Oral Microbiology Immunology 2003 18 220–225
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Oral Microbiology and Immunology
ISSN 0902 0055

Real-time interaction of oral streptococci with human salivary components

M Kawashima[1] N Hanada[2] T Hamada[1] J Tagami[1], H Senpuku[3]
[1]Department of Cariology and Operative Dentistry Tokyo Medical and Dental University Tokyo Japan [2]Department of Oral Health National Institute of Public Health Tokyo [3]Department of Bacteriology National Institute of Infectious Diseases Tokyo Japan

Kawashima M Hanada N Hamada T Tagami J Senpuku H Real time interaction of oral streptococci with human salivary components
Oral Microbiol Immunol 2003 18 220–225 © Blackwell Munksgaard 2003

Oral streptococci are present in large numbers in dental plaque and several types interact with the enamel salivary pellicle to form a biofilm on tooth surfaces The respective affinity of individual streptococci for salivary components has an influence on the etiologic properties of oral biofilm in the development of dental caries We studied real time biospecific interactions between oral streptococci and salivary components utilizing biosensor technology to analyze surface plasmon resonance *Streptococcus sanguis* and *Streptococcus mutans* showed significant responses for binding to salivary components in comparison with other bacteria Further the association rates ($4\,1 \times 10^{-11}$/bacterium) and dissociation rate ($5\,7 \pm 0\,9 \times 10^{-3}$ Second(s)$^{-1}$) were higher for *S sanguis* than for *S mutans* ($2\,4 \times 10^{-11}$ and $2\,9 \pm 0\,8 \times 10^{-3}$) and *Streptococcus mitis* ($1\,3 \times 10^{-11}$ and $3\,5 \pm 1\,3 \times 10^{-3}$) However the association equilibrium constants (8 2 S/bacterium) for *S mutans* was 2 times higher in than *S mitis* (3 8) and slightly higher than *S sanguis* (7 2) These findings may provide useful information regarding the mechanism of early biofilm formation by streptococci on the tooth surface

Key words *Streptococcus mutans Streptococcus sanguis Streptococcus mitis* BIA core salivary components

Hidenobu Senpuku Department of Bacteriology National Institute of Infectious Diseases 1 23 1 Toyama Shinjuku ku Tokyo 162 8640 Japan
E mail hsenpuku@nih go jp
Accepted for publication January 16 2003

The oral biofilm is produced by sequential attachment of a variety of bacteria which is dependent on both the species involved and the surface composition (1 8 19 22 24 25) Attached bacteria are able to grow in the biofilm that forms on tooth surfaces (8) There is a strong correlation between the attachment detachment and proliferation of these organisms in plaque on the tooth surface and the occurrence of dental caries in humans (13 19) Oral streptococci are present in large numbers in dental plaque and several types interact with the enamel salivary pellicle to form a biofilm on tooth surfaces Streptococci account for approximately 20% of the salivary bacteria (23) *Streptococcus salivarius* is the most abundant species and the density of *Streptococcus mutans* and *Streptococcus sanguis* in saliva is more than 1×10^5 cells/ml *S mutans* produces a surface protein antigen (PAc) that has a molecular mass of 190 kDa (28) which interacts with salivary components including lysozyme (31 33) amylase (31) proline rich proteins of 18 and 38 kDa (31) and agglutinin (16) It is also known as a late colonizer (27) and is considered to be one of the major causative agents of dental caries a disease related to the oral biofilm (5 10) In contrast *S sanguis* is among the first bacteria to adhere selectively to and colonize saliva coated teeth (10) and also interacts with the salivary film that is known to contain α amylase secretory IgA (12 17) mucin (36) and agglutinin (6) The two bacteria form a complex biofilm on the tooth surface by cell to surface and cell to cell interactions An understanding of these components is required to elucidate the mechanisms of biofilm formation *in vivo*

Gibbons *et al* suggested that biofilm formation consists of two processes that involve separate mechanisms (9 11) The first step is associated with adsorption of cells to the pellicle and requires specific adhesions on the cell surface while the second involves a build up of cells binding to each other The first step of formation has been investigated using saliva coated hydroxyapatite beads however physical interference of the interactions between cells and beads may occur in that model The kinetic rate of cell to saliva interaction is not yet entirely understood We studied biospecific interactions between oral streptococci and saliva components in real time utilizing biosensor technology that measures surface plasmon resonance (32) and then analyzed the kinetic rate constants of the interactions

Materials and methods

Bacterial strains and culture conditions

The strains used in this study were *S mutans* MT8148 ATCC 6229 and GS5

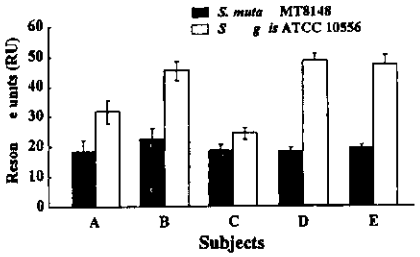

Fig 1 Binding of *S sanguis* ATCC 10556 (□) and *S mutans* MT8148 (■) to immobilized salivary components from five human subjects (A B C D and E) Forty microliters of each bacteria solution (OD = 0 5 550 nm) was applied to the sensor chip Results are expressed as mean ± SD for three independent assays Asterisks denote significant difference (*S mutans* vs *S sanguis* $P < 0\,01$)

S sanguis ATCC 10556 ATCC 15908 and ATCC 10558 *Streptococcus sobrinus* 6715 OMZ65 and MT8245 *S salivarius* ATCC 9759 and HT9R and *Streptococcus mutis* ATCC 903 and ATCC 6249 All bacteria were grown in an atmosphere of H_2 and CO_2 (GasPak^{TM} CO_2 System Becton Dickinson and Company Sparks MD) in Brain Heart Infusion broth (GHI Difco Laboratory Detroit MI)

Human saliva collection

Whole saliva from five subjects (25–55 years old females and males) was stimulated by chewing paraffin gum and collected into ice chilled sterile bottles over a period of 5 min and clarified by centrifugation at 10 000×*g* for 10 min at 4 C Fresh saliva samples from subject A (Fig 1) were used in every experiment while the other samples were stored until use

Monoclonal antibodies

To confirm whether the adherence of *S mutans* to the salivary components in BIAcore responses was dependent on interactions between the salivary components and the PAc anti PAc monoclonal antibodies (mAbs) (KH5 and SH2) obtained in our previous study (34) were used in inhibition BIAcore assays (BIAcore AB Uppsala Sweden) (35) The mAbs were collected from the culture fluid of the B cell clone and the culture fluid including the mAbs was pooled and stocked at −20 C for each experiment

Immobilizing the ligand

The binding of streptococci to salivary components was determined using a BIAcore^{TM} biosensor system (BIAcore AB) which has been shown to provide real time interactive analysis of two interacting macro molecules (33) This offers some advantages over conventional methods that use hydroxyapatite beads because the interaction is monitored directly in real time as it proceeds Comparisons of all bacteria interacting with salivary components in the BIAcore system were studied in detail in separated stages of association and dissociation This differs from conventional methods which analyze coupled stages of association and dissociation For the present study we used the pioneer sensor chip F1 (BIAcore AB) which has a shorter dextran matrix than other standard chips and is useful when working with large analyses such as those of cells and virus particles Whole salivary components were immobilized on the dextran surface of the sensor chip via carboxyl moieties and then activated by injecting 70 µl of 400 mM N ethyl N′ (3 diethylaminoprophyl) carbodiimide and 100 mM of N hydroxysuccinimide solution at a flow rate of 10 µl/min After activation 70 µl of 1 20 diluted whole saliva 500 µl of bovine serum albumin (BSA) and 500 µl of mouse immunogulobulin G (mIgG) in 10 mM of sodium acetate buffer at pH 5 0 were applied to the surface Residual N hydroxysuccinimide esters were then inactivated with 70 µl of 1 m ethanolamine hydrochloride The flow of PBS was maintained at 10 µl throughout the immobilization procedure

Determination of experimental conditions

To confirm the specific binding of *S mutans* its adherence to salivary components was compared with that of BSA and mIgG which are known to have no interaction with *S mutans* *S mutans* MT8148 was diluted with phosphate buffered saline (PBS pH 7 4) solution after centrifugation at 5000 × *g* for 10 min at 4 C and washing by PBS twice Ultrasonic processing (60 W 10 min) was carried out to disrupt the bacterial chain The bacterial concentration was adjusted using fresh PBS in a spectrophotometer (UV 2200A Shimazu Japan) to OD = 0 5 and 1 0 (550 nm) *S mutans* solutions were exposed to each ingredient (flow speed of 20 µl/min) and the dissociation phase was followed by injection of PBS at 20 µl/min All binding experiments were conducted at 25 C At the end of each binding cycle the surface of the sensor chip was regenerated by exposure to 50 mM of glycine NaOH (pH 9 5) for 60 s The detection system of the BIAcore^{TM} utilizes surface plasmon resonance (SPR) which is a quantum mechanical phenomenon that represents changes in optical properties at the surface of the sensor chip and eliminates the need to label the interactants The resonance angle depends on the refractive index in the vicinity of the surface which changes as the concentration of molecules on the surface is modified The signal was expressed in resonance units (RU) The SPR signal obtained in each binding cycle was recorded as a sensorgram which is a real time pattern with a sampling interval of 0 2–0 5 s plotted in RU versus time The amount of binding was represented as the increase of RU between the start and the end of each binding cycle A response of 1000 RU corresponds to a shift of 0 1 in the response angle which in turn represents a change in surface protein concentration of about 1 ng/mm^2 A 1 50 dilution of mAbs (KH5 and SH2) was pre incubated with 1 ml of *S mutans* (2×10^9/ml) by suspension in PBS at 4 C for 1 h After the incubation *S mutans* was washed with PBS and sonicated by ultrasonic dispersion (60 W power output) for 10 s and then 40 µl of mAb treated or untreated *S mutans* in PBS was injected onto the sensor at a flow rate of 20 µl/min

Interaction of oral streptococci with whole salivary components

S mutans MT8148 *S sanguis* ATCC 10556 *S sobrinus* 6715 *S salivarius* ATCC 9759 and *S mutis* ATCC 903 solutions were diluted to OD = 0 5 at 550 nm and then adjusted by the above mentioned methods and exposed to the saliva immobilized surface at a flow speed of 20 µl/min In addition *S mutans* MT8148 and *S sobrinus* ATCC 10556 were applied to the chips individually after being immobilized with saliva from five subjects To determine the association rates (*Kon*) of *S mutans* *S sanguis and S mutis* the organism solutions were adjusted from OD = 0 1 to 1 0 and then applied to the sensor chip that had been immobilized with salivary components Increases of RU were calculated 10 s after switching to PBS from the bacteria buffer for comparison with the baseline and to draw a kinetic graph showing dose dependency *Kon* was calculated by inclination and shown to be ΔRU per number and colony forming unit (CFU) of individual bacteria

CFUs were counted in BHI agar plates with various concentrations of bacteria suspensions The CFU to bacteria suspension ratio (OD = 1 0 550 nm) was then recalculated from a standard curve (Y axis CFU X axis OD) The dissociation rate (*Koff*) was determined using BIAevaluation 2 1 software (BIAcore)

Statistical analysis

Statistical analysis was performed using ANOVA P values of 0 05 or less were considered to indicate statistical significance

Results

Establishment of experimental conditions

Salivary components were used as ligands for BIAcore analyses of the interactions between the ligands and *S mutans* To confirm the specific binding of *S mutans* to salivary components without non specific binding to the dextran chip coated BSA and mIgG were also used as experimental controls A sensorgram for immobilization of these ligands showed increases of 2100 RU (whole salivary components) 7250 RU (BSA) and 4600 RU (mIgG) which involved a covalent binding of approximately 2 1 ng 7 3 ng and 4 6 ng respectively to the activated carboxymethylated dextran on the biosensor surface *S mutans* MT8148 (OD = 1 0) was applied to the sensor chip immobilized with these ligands The increasing RU seen indicated the response level of *S mutans* to the ligands as shown in the sensorgrams in Fig 2(A) Thus approximately 102 6 RU were required for the binding of *S mutans* to the immobilized salivary components on the sensorchip surface (Fig 2A) The responses (72 2 RU and 45 1 RU) to BSA and mIgG were lower than whole salivary components but meaningful The response to BSA decreased to close to the RU level of mIgG within 80 s after switching to PBS from the *S mutans* solution A high concentration (OD = 1 0) of *S mutans* may induce non specific binding to the ligands because the binding of *S mutans* (OD = 0 5) was significant to the salivary components (Fig 3) but poor to mIgG [less than 10 pg (10 RU)] (data not shown) in three independent experiments Moreover the mAbs (KH5 and SH2) clearly inhibited the binding of *S mutans* (OD = 0 5) to the salivary components (data not shown) Therefore the bacteria concentration (OD = 0 5 or 0 6) was used in following experiments These results demonstrated that *S mutans* was able to bind specifically to whole salivary components in the BIAcore biosensor system

Interaction of oral streptococci with whole salivary components

S mutans MT8148 *S sanguis* ATCC 10556 *S sobrinus* 6715 *S salivarius* ATCC 9759 and *S mitis* ATCC 903 solutions (OD = 0 5) were injected onto the sensor chip that had been immobilized with whole saliva components SPR signals of *S sanguis* were drawn from the highest association and the highest decrease during the dissociation stage after changing the bacteria solutions to PBS (Fig 2B) SPR signals of *S mutans* were drawn from the highest levels of binding during the dissociation stage as compared to those of the other bacteria (Fig 2B) The association and dissociation of *S mutans* ATCC 6229 were also similar to those of *S mutans* MT8148 (data not shown) At 10 s after changing to PBS *S sanguis* ATCC 15909 ATCC 10556 and ATCC 10558 and *S mutans* MT8148 and ATCC 6229 showed significant levels of binding to the salivary components in comparison with the others (Fig 3) However the association level of *S mutans* GS5 was lower than the other *S mutans* strains (Fig 3) while the association level of *S sanguis* ATCC 10556 was higher than *S mutans* MT8148 in samples from every subject (Fig 1) As *S sanguis* ATCC 10556 *S mutans* MT8148 and *S mitis* ATCC 903 showed different binding activities in the above experiments we studied the dose dependent response kinetics of these three bacteria to salivary components A concentration dependent binding of each was observed and *Kon* was then calculated from the inclination of the graph (Fig 4 Table 1) Dissociation rates (*Koff*) were easily measured using the BIAevaluation 2 1 software (Fig 5)

Kon was 2 4 4 1 and $1\,3 \times 10^{-11}$/bacterium in *S mutans* *S sanguis* and *S mitis* respectively while *Koff* was $2\,9 \times 0\,8$ $5\,7 \times 0\,9$ and $3\,5 \times 1\,3 \times 10^{-3}$ Second(s)$^{-1}$ respectively (Table 1) *S mutans* and *S mitis* were dissociated at a significantly slower rate than *S sanguis* (Table 1) From these data the calculated association equilibrium constants ($Ka = Kon/Koff$) were 8 2 7 2 and 3 8 S/bacterium in *S mutans* *S sanguis* and *S mitis* respectively The *Kon* and *Koff* results were higher for *S sanguis* than for *S mutans* and *S mitis* however *Ka* for *S mutans* was two times

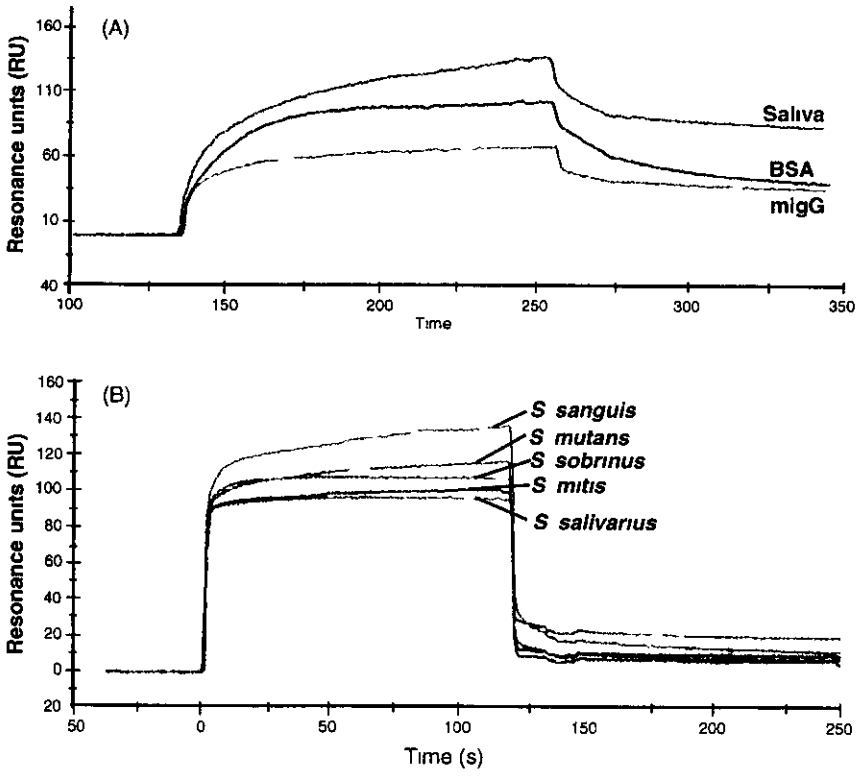

Fig 2 (A) Sensorgram illustrating the binding of *S mutans* MT8148 to immobilized salivary components (red) BSA (blue) and IgG (green) *S mutans* solutions with PBS (OD = 1 0 550 nm) were applied to the sensor chip Increases of 102 6 RU (to salivary components) 72 2 RU (to BSA) and 45 1 RU (to mIgG) were seen in the binding of *S mutans* to the immobilized ligands Data from three independent experiments with similar results obtained in each (B) Sensorgrams illustrating the binding of *S sanguis* ATCC 10556 (blue) *S mutans* MT8148 (red) *S sobrinus* 6715 (green) *S salivarius* ATCC 9759 (purple) and *S mitis* ATCC 903 (pink) to immobilized salivary components Bacteria solutions with PBS (OD = 0 5 550 nm) were applied to the sensor chips Various SPR signals were drawn during the association and dissociation stages for each streptococci Data from three independent experiments with similar results obtained in each

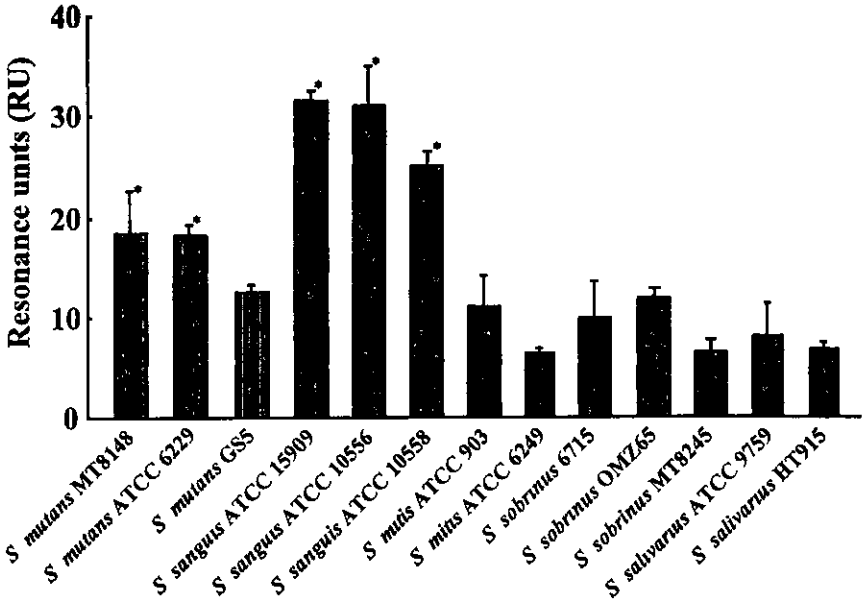

Fig 3 Binding of various streptococci to immobilized salivary components Forty microliters of bacteria solution (OD = 0 5 550 nm) was applied to the sensor chip Results are expressed as mean ± SD for three independent assays Asterisks denote significant difference (vs *S mitis* ATCC 903 $P < 0\,01$)

higher than *S mitis* and slightly higher than *S sanguis*

Discussion

Colonization of the oral cavity by strepto cocci is a crucial step in the sequence of events leading to infection and salivary proteins and glycoproteins can act as re ceptors for the binding of streptococci to enamel surfaces (10 16 36) The ability of oral streptococci to bind to immobilized salivary protein is of considerable etiologic significance (7 26 29) and *S mutans* and *S sanguis* are known to be primarily in volved when bacterial flora forms on the tooth surface *S sanguis* is an early colo nizer of the salivary pellicle whereas *S mutans* colonizes later The ability of both to bind to salivary proteins and gly coproteins is important in biofilm devel opment (3 15) The differences of affinity to the salivary pellicle between these two bacteria may be relevant in the etiology of biofilm conditions There is a lack of com parative research elucidating precisely the difference in affinity of both though their binding abilities to salivary components have been reported by various investiga tors (16 30) Recently a pioneer F1 chip that can be used to analyze bacteria whole cells was developed for the BIAcore sys tem (35) and with it we investigated the differences of real time affinity to salivary components by streptococci including *S mutans* and *S sanguis* The affinity of *S mutans* MT8148 to whole salivary com ponents was inhibited by mAbs (KH5 and SH2) that recognized PAc *S mutans* GS 5 which was PAc negative showed a lower affinity than *S mutans* MT8148 There fore affinity was dependent on PAc ex pression in the BIAcore system which was considered to be close to that of the pel licle

We first confirmed that *S mutans* ad hered only to whole salivary components and not to the surface of the sensor chip by covering the dextran surface entirely with ligands to shield it from *S mutans* (Fig 2A) The *Kon* value was determined as the increase of binding quantity (RU) per single bacterium using bacteria density instead of molarity since molarity could not be calculated in the bacteria solutions The *Koff* value was determined conven tionally using BIAevaluation because it does not require molarity From our re sults it was clear that *S sanguis* had a strong association with the saliva compo nents however it could also be easily dissociated in comparison with the other streptococci In contrast although *S mutans* showed a lower RU value than *S sanguis* for association (though higher than the other three bacteria tested) it could not be easily dissociated from the saliva components *S sobrinus S salivar ius* and *S mitis* showed low RU values and the ability of association with a simple substance was also considered to be low In the *Ka S mutans* was slightly higher than *S sanguis* which may support the presence of a mechanism in the biofilm itself The affinity of *S mitis* which is also an early colonizer (18) of tooth surfaces was lower than that of *S mutans* and *S sanguis* It has been reported that *S mitis* releases sub stances later recognized to be biosurfac tants that discourage the adhesion of *S mutans* (14) Therefore the present re sults may support a dominant role of *S mitis* in biofilm formation because of the

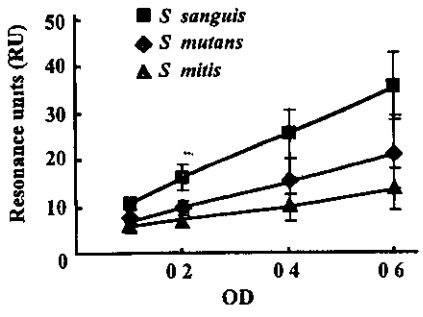

Fig 4 Dose dependent binding of *S sanguis* ATCC 10556 (■) *S mutans* MT8148 (◆) and *S mitis* ATCC 903 (▲) to the immobilized salivary components Forty microliters each of four concentrations (OD = 0 1 0 2 0 4 and 0 6 at 550 nm) was applied to the sensor chip Res ults are expressed as mean ± SD for three inde pendent assays Asterisks denote significant difference (*S sanguis* vs *S mutans* $P < 0\,05$ *S sanguis* vs *S mutans* $P < 0\,01$ *S sanguis* vs *S mutans* $P < 0\,01$) Association rates (*Kon*) were calculated using the inclination shown on this graph and CFU of individual bacteria

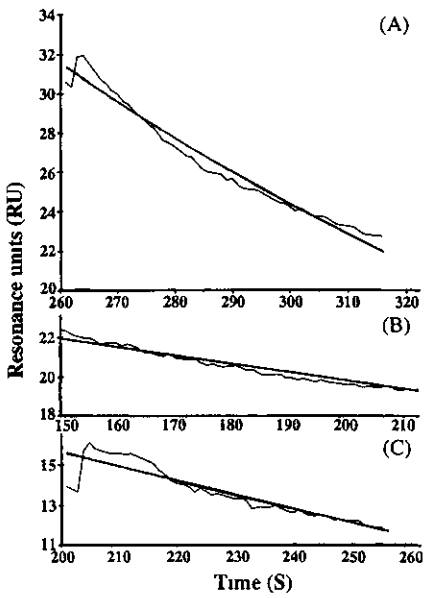

Fig 5 Sensorgrams illustrating the dissociation of *S sanguis* ATCC 10556 (A) *S mutans* MT8148 (B) and *S mitis* ATCC 903 (C) SPR signals were drawn 60 s after changing the bac teria solutions to PBS Dissociation rates (*Koff*) were calculated from the lines in graphs A B and C using BIA evaluation 2 1 software Data from 3 independent experiments with similar results obtained in each

Table 1 Kinetic rates of *S mutans S sanguis* and *S mitis* to salivary components

Bacteria	*Kon* ($\times 10^{-11}$)	*Koff* ($\times 10^{-3}$ S^{-1})	*Ka* = *Kon*/*Koff* ($\times 10^{-9}$ S)
S mutans	2 4	2 9 ± 0 8	8 2
S sanguis	4 1	5 7 ± 0 9	7 2
S mitis	1 3	3 5 ± 1 3	3 8

Kon results were calculated from lines in Fig 4 by the procedure described in Materials and methods
Koff results were calculated from lines in Fig 5 using BIA evaluation 2 1 software The *Koff* results are expressed as mean ± SD for three independent assays
Asterisks denote significant difference (vs *S sanguis* $P < 0\ 01$)

protection provided by the released sub stances rather than physical competition by cell adhesion against colonization of pellicle coated surfaces by *S mutans*

The A region in the PAc molecule is essential for the adhesion of *S mutans* to salivary film Further differences in the alanine rich sequence in *S sanguis* as compared to *S mutans* may have an effect on the affinity of *S sanguis* to interact with salivary receptors The A region is com posed of three long and two incomplete repeating sequences (28) In the region sequenced from *Streptococcus gordonii* M5 (previously designated *S sanguis*) the cell surface adhesin of SSP 5 showed an extensive homology [44% identity with SpaP (PAc)] (6) Further each repeating sequence contains sequences that are homozygous to the amino acid sequence ^{365}TYEAALKQYEADL^{377} PAc (365–377) which is an important region for the initial attachment of *S mutans* to the tooth surface (32 35) These analogs or variant peptides from *S gordonii* M5 to PAc (365–377) inhibited the binding of *S sanguis* ATCC 10556 to the salivary components in BIAcore competitive inhibi tion assays (unpublished data) Therefore the sequence may influence the affinity of *S sanguis* to salivary components

Colonization by both *S sanguis* and mu tans streptococci is also dependent upon the presence of teeth (2 4) Caufield et al reported that levels of *S sanguis* increase with age prior to the colonization of mu tans streptococci after mutans streptococ ci colonize the levels of *S sanguis* decrease (4) Several other investigators have also suggested that the *S mutans*/ *S sanguis* ratio may serve as an indicator of caries risk (20 21) These findings in dicate that the binding of *S sanguis* to salivary components may compete with the action of *S mutans* salivary component binding in the oral cavity *S sobrinus* also produces a surface protein antigen that shows a homology to *S mutans* of approxi mately 66% (37 38) However in the present study *S sobrinus* did not present a definite response to the salivary compo nents in the real time biosensor Three homologous PAg sequences (286–298 69 2% 368–380 61 5% and 450–462 38 5%) were found in the three long re peating sequences of PAg and varied by amino acid sequence ^{286}D—K A—K–^{2398} ^{368}N—K A—QK–^{280} and ^{450}D—LK SK QEE ^{462} respectively to PAc (365 377) These variants may have influenced the lower affinity of *S sobrinus* to salivary components

The differences in *Kon Koff* and *Ka* between *S sanguis S mutans* and *S mitis* in the present real time biosensor results may explain one of the mechanisms of bacterial flora formation on teeth seen in previous epidemiologic findings (10 18 27) Therefore it is suggested that bacterial interactions with salivary components are influenced by the respective etiologic abil ities of streptococci in the development of dental caries Our findings also demon strated the early colonization of *S sanguis* as well as later colonization of other mu tans streptococci in the real time fluid phase and may provide useful information about the mechanism of early biofilm for mation on the tooth surface by oral strep tococci

Acknowledgments

This work was supported in part by grants in aid for Development Scientific Re search (13671991 and 12557158) from the Ministry of Education Science and Culture of Japan and by a grant from the Japan Health Science Foundation to H S This study was also funded by a part of Ground Research for Space Utiliza tion promoted by NASDA and the Japan Space Forum

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Assessment of oral transmission using cell-free human immunodeficiency virus-1 in mice reconstituted with human peripheral blood leucocyte

RYOMA NAKAO NOBUHIRO HANADA † TOSHIHIKO ASANO ‡ TAKASHI HARA § MD ABDUS SALAM ¶ KHAIRUL MATIN † YOSHIHITO SHIMAZU ** TADASHI NAKASONE,§ SHIGEO HORIBATA § TAKAAKI AOBA ** MITSUO HONDA § TERUO AMAGASA & HIDENOBU SENPUKU¶ **Maxillofacial Surgery Department of Maxillofacial Reconstruction and Function Division of Maxillofacial and Neck Reconstruction Graduate School Tokyo Medical and Dental University Tokyo Japan †Department of Oral Health National Institute of Public Health Tokyo Japan ‡Division of Experimental Animal Research §AIDS Research Center ¶Department of Bacteriology National Institute of Infectious Diseases Tokyo Japan and **Department of Pathology The Nippon Dental University Tokyo Japan*

SUMMARY

Oral–genital contact is one of the risk factors for the transmission of human immunodeficiency virus (HIV) in adults. In recent reports oral exposure to simian immunodeficiency virus (SIV) was found to have important implications for the achievement of mucosal transmission of HIV in a rhesus monkey animal model. In the present study we aimed first to establish a small animal model which did not develop tonsils suitable for HIV oral mucosa transmission using non obese diabetic/severe combined immunodeficiency (NOD/SCID) mice and NOD/SCID B2m$^{-/-}$ mice grafted with human peripheral blood leucocytes (hu PBL) and stimulated with interleukin (IL) 4 and second to investigate whether oral exposure to cell free R5 and X4 HIV-1 could lead to oral transmission of HIV through intact or traumatized mucosal tissues in humanized mice. Both low and high concentrations of cell free R5 and X4 viruses failed to cause oral transmission with or without trauma in hu PBL NOD/SCID and NOD/SCID 2m$^{-/-}$ mice which presented a number of $CD4^+$ cells in gingival tissues and oral cavities with or without tissue injury. The present results show that IL-4 administrated NOD/SCID B2m$^{-/-}$ mice are useful as a small humanized model for the study of HIV oral infection which may help to define the window of opportunity for oral transmission by the HIV virus in animal model experiments.

INTRODUCTION

Mucosal exposure of infants to maternal infectious cervical secretions and/or blood during delivery is a great risk for human immunodeficiency virus (HIV) infection.[1] Further oral transmission of HIV-1 through breast milk occurs during the lactation period, while oral–genital contact has been shown to be one of the risk factors for transmission of HIV-1 in adults.[3,4] However, there is no risk of infection via saliva transfer from light kissing or the common use of dishes or toothbrushes.[3] In recent reports oral exposure of simian immunodeficiency virus (SIV) or simian HIV (SHIV) has been shown to be a risk factor for mucosal infection in rhesus monkey models.[5,6] Moreover, HIV-1 proviral DNA was detected in 74% of semen samples from HIV-1 infected patients,[7] while it has also been demonstrated that epithelial cells in the oral cavity can be productively infected by a cell borne HIV-1 virus.[8] These findings demonstrate that both oral and upper gastrointestinal mucosa have important roles in achievement of mucosal transmission of HIV-1.

A small animal model for the *in vivo* study of human peripheral blood leucocytes (hu PBL) would be valuable for the elucidation of human specific infectious agents such as HIV-1. Severe combined immunodeficiency (SCID) and non obese diabetic (NOD)/LtSz *scid* (NOD/SCID) mice reconstituted with hu PBL mice have been established[9,10] and their systemic infection with HIV-1 by intraperitoneal injection has been achieved.[11] The NOD strain is characterized by a functional deficit of natural killer (NK) cells,[13,14] absence of circulating complement[15] and defects in the differentiation and function of antigen presenting cells.[11,16,17] Recently NOD/SCID β2 microglobulin knock out (NOD/SCID 2m$^{-/-}$) mice which are major

Received 3 October 2002; revised 27 January 2003; accepted 6 March 2003.

Correspondence: Dr H. Senpuku, Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. E-mail: hsenpuku@nih.go.jp

histocompatibility complex (MHC) class I deficient B and T cell deficient C 5 deficient and have a low number of NK cells were established [18 0] These support up to six to sevenfold higher levels of hu cell engraftment than NOD/SCID mice which is associated with increased levels of hu $CD4^+$ T cells [1]

A long term culture of hu lymphocytes in the NOD/SCID mice system induces cytotoxic T cells and up regulates infiltration of $CD8^+$ cells into various tissues Interleukin (IL)-4 is a pleiotropic cytokine produced by activated T helper 2 (Th2) cells mast cells and basophils [3,4] and has been identified as an important regulator for $CD4^+$ Th2 subset development [5] It is also well known that a change in Th1/Th2 balance is the result of various immunoreactions [6] Therefore in the present study IL-4 was used as the effective agent for grafting of hu $CD4^+$ cells in the NOD/SCID mouse system

Studies with newborn and older macaques have shown that a non traumatic inoculation of SIV into the oral cavity can lead to productive viral infection and acquired immune deficiency syndrome (AIDS) [5] which is possibly initiated in the tonsils by M cell uptake and transported by rapid infection of local lymphoid tissue [7] In the oral infection of SIV Robert Guroff noted that palatine and sublingual tonsils contained deep crypts rich in M cells and suggested them as another potential route of HIV infection [8] These studies have focused on non traumatic infection using SIV or SHIV *in vivo* experiments however the traumatic infection by HIV 1 on oral mucosa have not been widely reported These sites are not a well established entry of HIV 1 though the question of whether periodontal mucosa condition is a risk factor for HIV infection is of obvious importance Because mice do not develop palatine sublingual or pharyngeal tonsils we investigated first whether IL 4 administrated NOD/SCID B2m [11] mice could be useful as a small humanized model for the study of cell free HIV oral infection and second whether oral mucosa with or without trauma in a model have important implications for the achievement of mucosal transmission of HIV 1 by using oral exposure of cell free HIV 1 in the present study mouse systems

MATERIALS AND METHODS

Mice

NOD/SCID and NOD/SCID B2m [11] mice were purchased from The Jackson Laboratory (Bar Harbor ME) and maintained at the National Institute of Infectious Diseases (NIID) The present study used female mice at the age of 6–9 weeks All experiments were performed in accordance with our institutional guidelines

Human leucocyte transplantation

Transplantation of hu PBL into both types of NOD/SCID mice was performed using procedures and conditions described previously hu PBL cells were separated from hu peripheral blood by Ficoll Conrey (Immuno Biological Laboratories Cunma Japan) density gradient centrifugation then washed three times in Hanks balanced salt solution (HBSS) (Gibco Laboratories Life Technologies Paisley UK) and adjusted to a $4.0–8.0 \times 10^7$/ml concentration of cells in HBSS A hu PBL suspension was then administered intraperitoneally at 0.5 ml per mouse Mice were irradiated (2.2 Gy gamma irradiation) from a ^{137}Cs source (Gammacell 40 Atomic Energy of Canada Ltd Kanata Canada) 0–1 days before human cell transfer On days 1 7 and 14 after hu PBL transplantation mice were administrated intraperitoneally with either 62.5 ng or 250 ng of hu IL 4 (204-IL R & D system Inc Minneapolis MN) in 300 µl of phosphate buffered saline (PBS) pH 7.4 Control mice were injected intraperitoneally with 300 µl of PBS alone On days 21 and 28 mice were administrated with various HIV 1 strains by intraperitoneal or oral inoculation On day 35 all mice were killed and analysed

Virus inoculation

We used the following cell free HIV 1 strains HIV 1_{NDK} and HIV_{MN} as X4 viruses and HIV $1_{TH\ PF6}$ HIV $1_{JR\ CSF}$ and HIV $1_{JR\ FL}$ as R5 viruses HIV 1_{NDK} HIV 1_{MN} HIV $1_{Th\ PF6}$ and HIV $1_{JR\text{-}CSF}$ were obtained from the NIH AIDS Research and Reference Reagent Program and $HIV_{JR\ FL}$ was kindly provided by Dr Yoshio Koyanagi (Department of Virology Tohoku University Graduate School of Medicine) Fifty percent tissue culture infection doses ($TCID_{50}$) were determined by limiting the dilution of virus stocks with phytohemagglutinin and IL 2 activated hu PBL The cell free supernatants were stored at −130 until used as a virus source All procedures for infection and maintenance of hu PBL NOD/SCID mice were performed in biosafety level 3 facilities at the National Institute of Infectious Diseases Tokyo Japan under standard caging conditions Grafted mice were intraperitonrally injected with $1 \times 10^{-1} \times 10^{4}$ $TCID_{50}$ of various strains of HIV 1 as positive controls The strains were transmitted into the oral cavities by two different means One method was to inoculate the virus into the oral cavity without trauma as a model of an intact oral cavity while the other was to inoculate HIV 1 into the oral cavity after trauma which was induced in the periodontal mucosa by injury with scissors under anaesthesia as a model of an oral disease such as periodontal disease A 100 µl cell free virus sample was pipetted for 5 min onto non injured and injured periodontal mucosa in the mice oral cavities The mice were not allowed to drink or eat overnight after HIV 1 exposure Seven days after the last inoculation of HIV 1 the mice were killed and analysed

Tissue preparation

On day 35 after transplantation of hu PBL into mice the upper and lower jaws spleen cervical lymph nodes lung liver salivary gland and serum were extracted for the following experiments Some mice were anaesthetized with sodium pentobarbital (0.008 ml/g mouse) and killed and fixed by exsanguinations then underwent perfusion with 4 paraformaldehyde (PFA) in a 0.1 M cacodylic acid buffer administered through the left ventricle of the heart Subsequently oral tissues including the jaw and surrounding soft tissue were removed and placed in 4 % PFA for 12 hr After fixation the specimens were washed then decalcified in 10 neutral buffered ethylendiaminetetraacetic acid (EDTA) using a magnetic stirrer at 4 for 3 weeks

Flow cytometry

Single cell suspensions from the removed spleens were prepared Contaminated red blood cells were lysed in ammonium chloride potassium (ACK) buffer and rinsed twice in HBSS and

the number of viable cells was determined by exclusion of trypan blue (Sigma Aldrich St Louis MO) using a haemocyt ometer Single cell suspensions were stained with the following antibodies fluorescein isothiocyanate (FITC) conjugated anti mouse CD45 (30 F11) anti mouse CD4 (H129 19) anti hu CD45 (H130) anti hu CD4 (RPA T4) and phycoerythrin (PE) conjugated anti hu CD8 (RPA T8) Each was purchased from BD PharMingen (San Diego CA)

The expressions of galactocylceramide (GalCer) CCR5 and CXCR4 in mouse oral mucosa were also examined by flow cytometry Surgically resected mouse gingival and palatal mucosa were minced and then dispersed with 0 25 / trypsin and 0 02% EDTA by continuous stirring for 90 min at 37C Prepared single cell suspensions were incubated with the fol lowing antibodies rabbit antiserum to GalCer (CovalAb Oul lins Cedex France) FITC conjugated goat anti rabbit immunoglobin specific polyclonal antibody (BD PharMingen) PE conjugated anti mouse CD45 (30 F11 BD PharMingen) FITC conjugated anti hu CCR5 (2D7/CCR5 BD PharMingen) and PE conjugated anti hu CXCR4 (12G5 BD PharMingen) Normal rabbit immunoglobulin (DAKO Glostrup Denmark) was used as a control isotype Staining was evaluated for a minimum of 10^4 cells with a FACSCalibur (Becton Dickinson Franklin Lakes NJ) flow cytometer and data were analysed with Cellquest (Becton Dickinson) software

Immunohistochemistry

The decalcified oral tissues including the jaw were snap frozen and embedded in OCT (Tissue Tek Sakura, Torrance CA) Serial 10 μm cryosections were placed on matsunami adhesive silane (MAS) coated glass microscope slides (Matsunami Glass IND Osaka Japan) and dried then rinsed three times in PBS for 5 min each time Endogenous peroxidase activity was quenched with 3 % H O in PBS (v/v) for 20 min at room temperature then the sections were washed again three times in PBS for 5 min each time and the slides were incubated with 2% blocking horse serum (Vector Laboratories Burlingame CA) for 1 hr at room temperature in a humidified chamber Excess serum was drained and biotinylated antibodies (murine mono clonal anti hu CD45 clone HI30 anti hu CD4 clone RPA T4 and anti hu CD8 clone RPA T 8 (BD PharMingen)) in 1 / bovine serum albumin (BSA)–PBS were directly added to the sections and incubated overnight at 4 in a humidified chamber For a negative control non specific mouse immunoglobulin G1 (I_gG1) was used at the same concentration Sections were rinsed and then the antigen–antibody complex was detected using an avidin–biotin peroxidase kit (Vector Laboratories) 3 3 diaminobenzidine tetrahydrochloridedihydrate (Vector Laboratories) was used as the chromogen and sections were counterstained with methyl green (Mutoh Igaku Tokyo Japan) then mounted with ENTELLANneu (Merck Darmstadt Ger many) These periodontal sections were also stained with hae matoxylin and eosin (H&E) for conventional histological assessment

Nested polymerase chain reaction (PCR) and DNA sequence analyses

DNA from the spleen cervical lymph nodes lung liver salivary gland and periodontal tissues was extracted using a DNeasy tissue Kit (QIAGEN GmbH Hilden Germany) according to the manufacturer s instructions and tested for proviral DNA by nested PCR with primers derived from conserved HIV 1 *env* and *gag* sequences The primers (first OA and OD and second SB and SC) used for the *env* PCR assay were OA 5′ TGTACA CATGGAATTAGGC CAGTAG 3 (HIV genome location 6962–6986) and OD 5 AAATTCCCCTCCACAATTAAAA CT 3 (7345–7369) for the primary reaction and SB 5 TCAACTCAACTGCTGTTAAAT 3 (6989–7009) and SC 5 AATTTCTGGGTCCCCTCCTCAGG 3 (7314–7336) for the secondary reaction Primers (first JA152 and JA155 and second JA153 and JA154) for the *gag* PCR assay were JA152 5 ATCTCTAGCAGTGCCGCCCGAACAG 3 [6 4–648] and JA155 5 CTGATAATGCTGAAAACATGGGTAT 3 (1276–1300) for the primary reaction and JA153 5 CTCTCGACGCAGGACTCGGCTTGCT 3 (679–703) and JA154 5 CCCATGCATTCAAAGTTCTAGGTGA 3 (1213–1237) for the secondary reaction These primers have been reported previously [9 30] The primary reaction mix contained 10–100 μg of DNA 12 5 μl of Premix Taq Takara Ex Taq™ Version (Takara Bio Inc Shiga Japan) and 1 μM of each primer in a total volume of 25 μl The samples were first preheated for 9 min at 94 then cycled 34 times at 95 for 1 min annealed at 55 for 2 min extended at 72 for 1 min and finally incubated at 72 for 5 min The secondary reaction used 2 5 μl from the primary PCR product under the same conditions as in the primary reaction To confirm whether the PCR products were recovered from the HIV 1 strains used nested PCR products were purified using PCR Kleen Spin Columns (Quantum Prep® Bio Rad Laboratories Hercules CA) and sequenced using a sequencing kit with fluorescent dye terminators (Perkin Elmer Foster City CA) according to the manufacturer s instructions These PCR primers and amplification conditions were also subjected to sequencing The sequence data were collected with an ABI PRISM® 377 DNA Sequencer (Applied Biosystems Foster City CA)

Quantitative PCR assay

Serum virus RNA copy numbers were determined using a quantitative PCR assay (Amplicor Roche Molecular Systems Somerville NJ) One hundred μl of mice serum was taken on day 35 after hu PBL transplantation and subsequently assayed according to the instructions provided by the manufacturer The detection limit for the RNA copy numbers was 800 in this experiment

Enzyme linked immunosorbent assay (ELISA) of secretory leukocyte protease inhibitor (SLPI) from saliva

To obtain whole saliva from NOD/SCID and NOD/SCID 2m [ll] mice they were intraperitoneally injected (after anaesthesia) with a cocktail of isoproterenol (0 20 mg/100 g body wt) and pilocarpine (0 05 mg/100 g body wt Sigma) in PBS as secretagogue Subsequently saliva was collected from the mouth by a micropipette for 15 min and stored in 1 5 ml microfuge tubes Whole human saliva from three healthy individuals and whole mouse saliva from three mice of each strain were collected and centrifuged at 3000 × *g* for 15 min Each supernatant was sterilized using a 0 22 μm Millex GV filter (Millipore Products Bedford MA) Human SLPI was determined using a

sandwich ELISA technique as follows Ninety six well micro titre H plates (Sumitomo Beklite Tokyo Japan) were coated overnight at 4 with 100 μl of 1 1000 dilution goat anti hu SLPI polyclonal antibody (Cosmo Bio Co Ltd Tokyo Japan) in 50 mM of carbonate buffer (pH 9 6) The plates were washed with PBS containing 0 05% Tween 20 (PBST) and blocked with 1 % (wt/vol) skimmed milk in PBST This was followed by the addition of 100 μl of a twofold serial dilution of saliva to the wells and incubation for 1 hr at 37 The wells were then washed five times with PBST and further incubated for 1 hr at 37 with 100 μl of anti hu SLPI monoclonal antibody (clone 31 HyCult Biotechnology PB Uden Netherlands) diluted 1 1000 in PBST The wells were then washed five times with PBST and further incubated for 1 hr at 37 with 100 μl of alkaline phosphatase conjugated goat anti mouse IgG (γ) antibody (Zymed Laboratory South San Francisco CA) diluted 1 1000 in PBST After five washes with PBST bound SLPI were detected after the addition of 100 μl of 1 mg/ml paranitrophenyl phosphate (Sigma) as a substrate and incubation for 1 h at 37 SLPI differences between human saliva and mouse saliva were determined by colorimetric end points read at a wavelength of 405 nm in a microplate reader (Multiscan Bichromatic Bichromatic Lobsystems Helsinki Finland)

Statistical analysis

Statistical analysis was performed by Mann–Whitney s *U* test *P* values of 0 05 or less were considered to indicate statistical significance

RESULTS

Effects of hu IL 4 on grafting of hu CD45 CD4 and CD8 cells in NOD/SCID and NOD/SCID B2m ^{null} mice

To establish a small animal model for HIV infection we examined the effects of IL-4 on hu CD45$^+$ CD4$^+$ and CD8$^+$ cells in NOD/SCID and NOD/SCID B2m ^{null} mice In NOD/SCID mice the administration of low doses of IL-4 (62 5 ng/mouse) induced slight increases in [CD45$^+$ (86 3% ± 11 2 (represented by average ± SD) $n = 7$] CD4$^+$ CD8$^-$ (33 9% ± 10 9 $n = 7$) and CD4$^-$ CD8$^+$ (51 4% ± 6 3 $n = 7$) cells in comparison with the controls which were injected with PBS alone (CD45$^+$ 73 7% ± 31 6 CD4$^+$ CD8$^-$ 27 9 % ± 24 0 and CD4$^-$ CD8$^+$ 40 3% ± 11 5) though the differences were not significant Moreover the administration of high doses of IL-4 (250 ng/mouse) did not induce significant changes of CD45$^+$ (70 8% ± 16 7–69 4% ± 35 5 P =0 8273 $n = 6$)

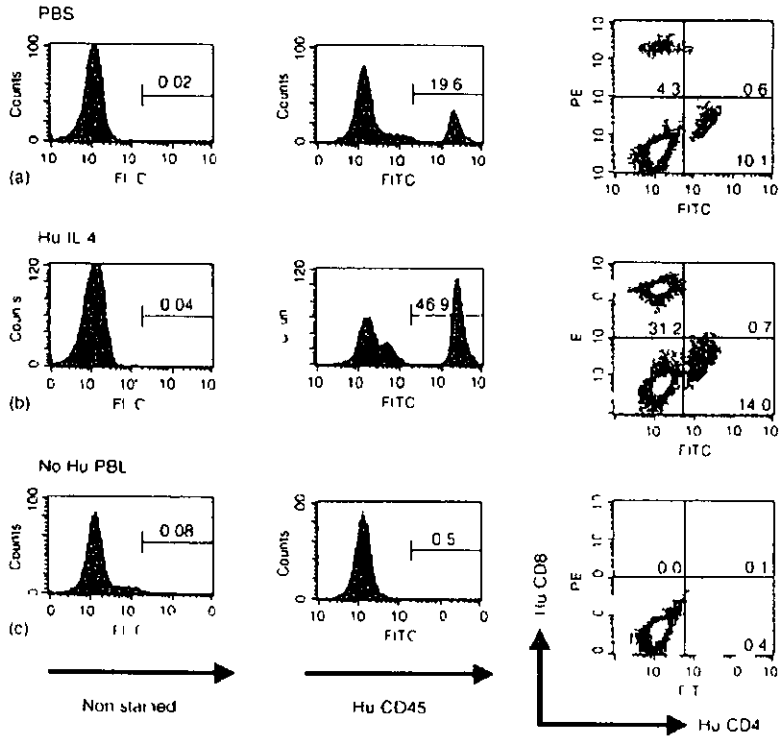


Figure 1 Flow cytometric analyses of hu CD45 CD8 and CD4 T cell populations present in the spleens of grafted NOD/SCID B2m ^{null} mice treated with hu IL 4 Hu PBL in spleen cells were analysed 3 weeks after the last administration using hu CD45 single and CD4 /CD8$^+$ double staining The analyses were performed on gated lymphocytes with forward/side scatter characteristics lymphocytes can be distinguished in the quadrant analysis The numbers in the upper left and lower right quadrants are the percentages of CD8 CD4 T cells and CD4 CD8 T cells respectively Determinations of the proportion of non stimulated cells hu CD45 cells hu CD4 CD8 hu CD4 CD8 T cells in the spleens of grafted mice given PBS (a) or IL 4 (250 ng/mouse) (b) as well as non grafted mice (c) are shown Data shown are representative of 7 (PBS) 14 (IL 4) and 3 (no PBL) in 3 independent experiments No Hu PBL spleens from NOD/SCID B2m ^{null} mice

$CD4^+$ $CD8^-$ (33 6% ± 23 9–30 9% ± 27 3 $P = 0\ 8293$ $n = 6$) or $CD4^-$ $CD8^+$ (32 2% ± 5 3–38 4 % ± 13 5 $P = 0\ 5127$ $n = 6$) cells in proportion to the control

In NOD/SCID B2m ^{II} mice the administration of high doses of IL-4 (250 ng/mouse) induced significant increases of hu $CD45^+$ $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ cells in comparison with the control (Figs 1 and 2c) Further the proportion of each type of hu cell in spleens from PBS and IL 4 treated mice was different from that of fresh hu PBL (Fig 2a) As for the hu CD4/CD8 relative ratio there were no significant differences between the PBS and IL 4 groups in either mouse strain (Figs 2b c) However the ratio in spleens from both strains after administration with IL 4 was half (approximately 1) of the ratio of fresh hu PBL before transplantation

Identification of hu leukocytes in periodontal tissues

To study circulating hu leucocytes treated with IL 4 in oral tissues hu $CD45^+$ $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ cells were stained for immunohistochemistry assay in hu PBL NOD/SCID and NOD/SCID B2m ^{II} mice Many hu $CD45^+$ cells were seen in tooth pulp samples from NOD/SCID B2m ^{II} mice at 5 weeks after grafting (Fig 3b c) However hu $CD45^+$ cells were not found in the upper area of epithelium in oral mucosa though they were found accumulated at the junction of the epithelium and lamina propria (Fig 3e f) Similar results to hu PBL NOD/SCID B2m ^{III} mice were also found in the hu PBL NOD/SCID mice (data not shown) A greater number of hu $CD45^+$ cells was induced by IL-4 than PBS administration in periodontal tissues from hu PBL-NOD/SCID mice (Fig 4) However in

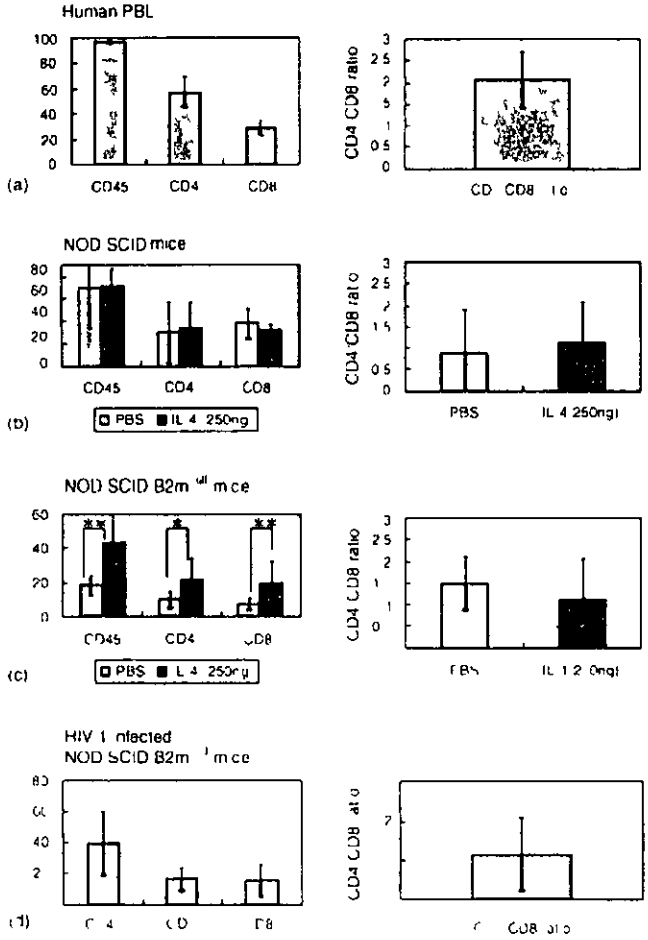


Figure 2 Effects of hu IL 4 alone on T cell proportions in grafted mice infected or not infected with HIV 1 Determinations of the proportion of hu CD45 cells in hu CD4 CD8 and hu CD4 CD8 T cells in the spleens of grafted mice (a) PBS or IL 4 (250 ng/mouse) or solvent in hu PBMC (a) grafted NOD/SCID mice (b) not infected NOD/SCID B2m ^{II} mice (c) and NOD/SCID B2m ^{II} infected with HIV 1 (d) The CD4/CD8 (CD4 CD8 T cells/CD4 CD8 T cells) ratio is shown in the right panel Hu lymphocytes in spleen cells were analysed 3 weeks after the first administration Analyses were performed on gated lymphocytes with forward scatter/side scatter characteristics Results are expressed as means ± SD for representative independent assays Statistical analysis was performed by Mann–Whitney *U* test Asterisks denote significantly different (versus PBS * $P < 0.05$ ** $P < 0.01$)

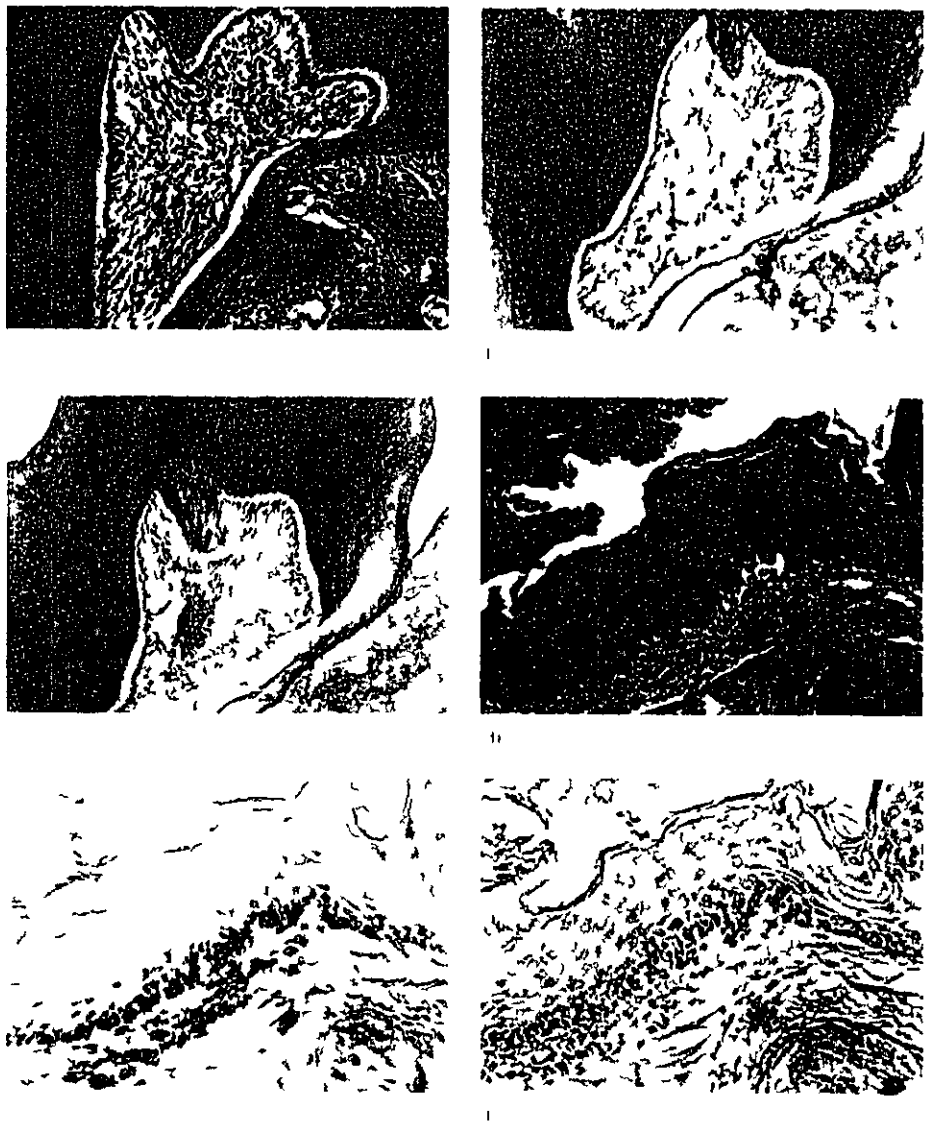


Figure 3 Photomicrographs of pulp and junction of epithelium and lamina propria specimens from hu PBL NOD/SCID B2m $^{-/-}$ mice given PBS (× 400) H&E staining of pulp (a) and junction of epithelium and lamina propria (d) are shown in the left column Immunohistochemical staining of CD45 cells and non staining are shown in the pulp (b and c respectively) and junction of epithelium and lamina propria (e and f respectively) Data shown are representative of four independent experiments

these positive cells hu $CD8^+$ cells were found in much greater numbers than $CD4^+$ cells (Fig 4) In contrast in NOD/SCID B2m $^{-/-}$ mice IL-4 and PBS induced not only $CD8^+$ cells but also $CD4^+$ cells (Fig 5) These results indicate that hu PBL NOD/SCIDB2m $^{-/-}$ mice treated with IL-4 may be useful as a small animal model for oral transmission of HIV

Oral transmission of HIV in hu PBL NOD/SCID B2m $^{-/-}$ mice

To evaluate oral transmission of HIV with or without trauma various HIV-1 strains were given either intraperitoneally or orally to hu PBL NOD/SCID and NOD/SCID B2m $^{-/-}$ mice Hu PBL NOD/SCID mice inoculated with HIV 1_{NIN} intraperitoneally at a dose of 100 $TCID_{50}$ showed *env* and *gag* PCR products in all spleen cells and one of three lung tissues as well as HIV-1 RNA copies in serum (Fig 6) Similar results were also obtained for hu PBL NOD/SCID B2m $^{-/-}$ mice as well as those inoculated intraperitoneally with other strains of HIV-1 (data not shown) Mice infected with HIV-1 did not show a significant reduction of hu $CD4^+$ cells among spleen cells after intraperitoneal inoculation (Fig 2d) Further PCR products were not found in cervical lymph node liver salivary gland or heart specimens (data not shown) Mice inoculated with 100

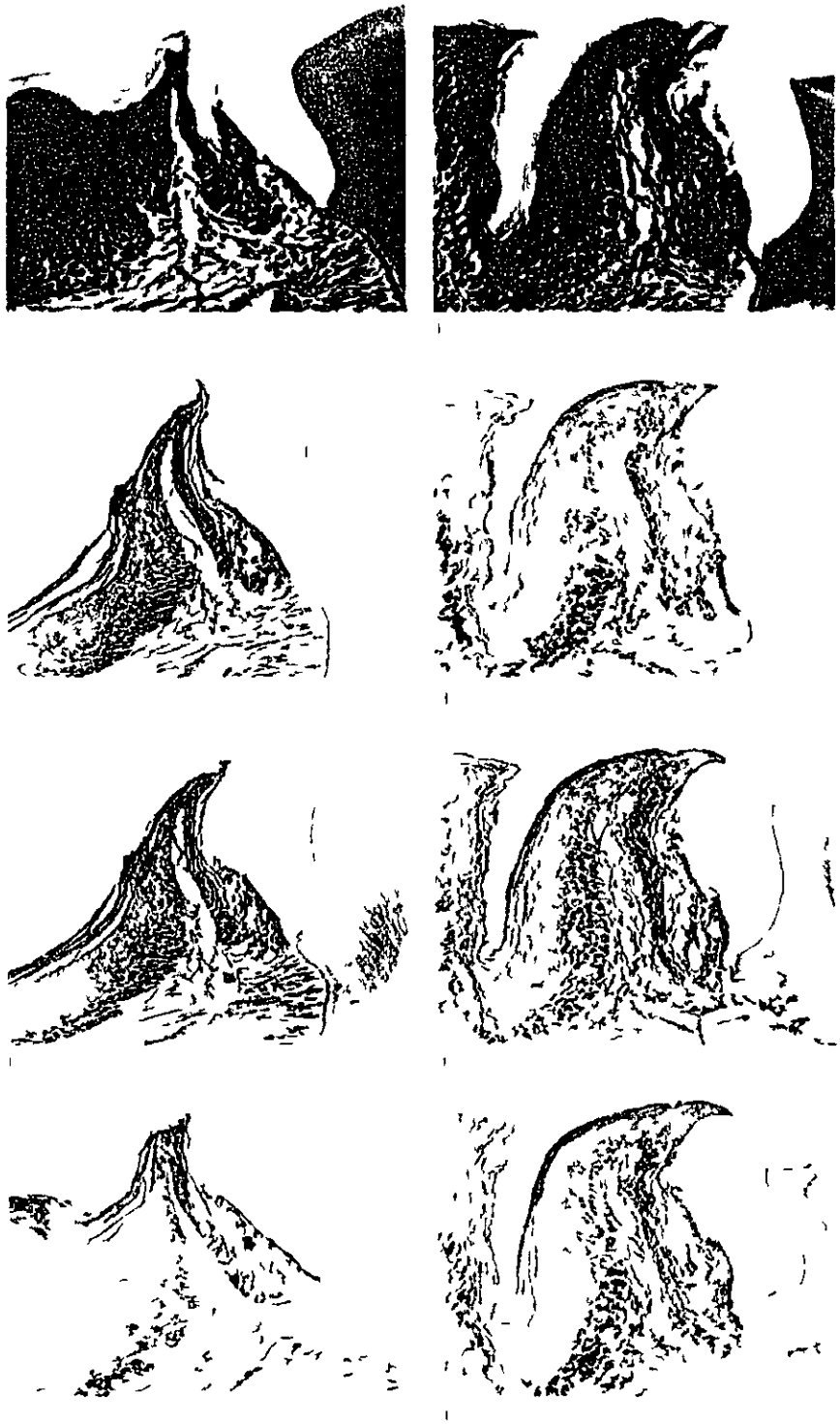


Figure 4 Photomicrographs of periodontal tissue specimens from hu PBL-NOD/SCID mice (× 200) H&E staining (a and b) immunohistochemical staining of hu CD45+ cells (c and d) and hu CD4+ (e and f) and hu CD8+ T cells (g and h) in periodontal tissues from hu PBL-NOD/SCID mice given PBS and IL-4 are shown. Left and right panels show periodontal sections from mice given PBS and IL-4 respectively and analysed 7 and 14 Photos show serial sections in all figures. From upper to lower Data show are representative of 3 independent experiments

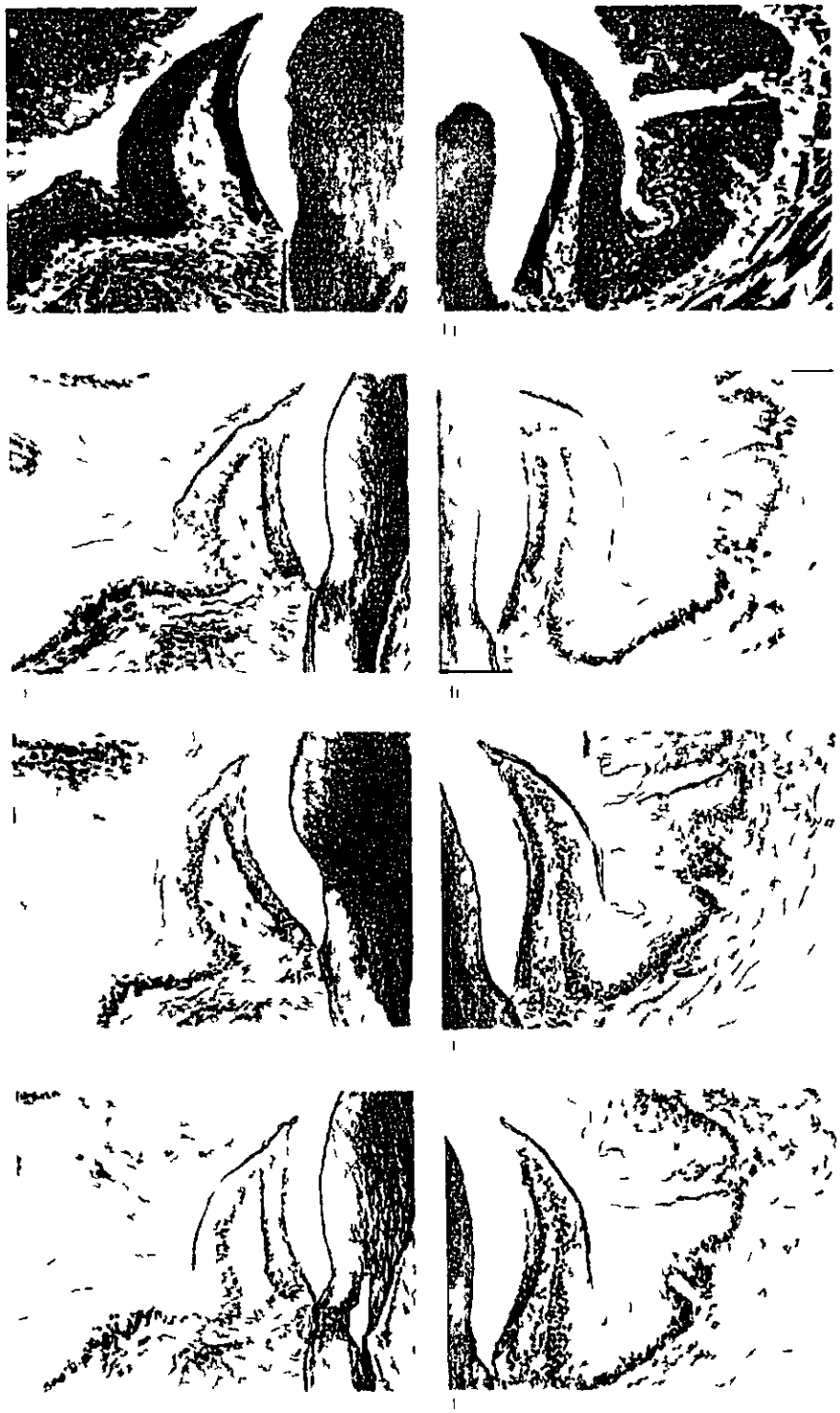
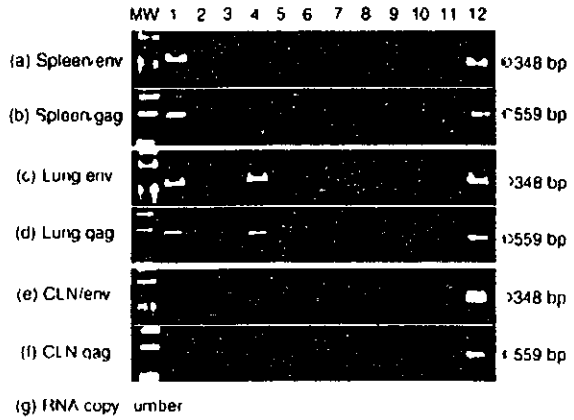


Figure 5 Photomicrographs of periodontal tissue specimens from hu PBL NOD/SCID B?m [illegible] nuc (× 00) H E staining (a an l b) Immunohistochemical staining of hu CD45 [illegible] lls (c and d) and hu CD4 (e and f) an l hu CD8 T cells (g and h) in periodontal tissues from hu PBL NOD/SCID mice given PBS and IL-4 respectively Left and right panels show periodontal sections from mice administered PBS and IL-4 respectively on days 1 7 and 14 Photos show serial sections in all figures from upper to lower Data shown are representative of three independent experiments



Lane	1	2	3	4	5	6	7	8	9	10	11	12
Dose of HIV (X4 virus)	1001CID$_{50}$			500TCID$_{50}$			1000TCID$_{50}$					
Route of inoculation	ip	inj	po	ip	inj	po	inj	po	no cell	no HIV		
RNA copy number	6842	ND	ND	2205	ND	ND	ND	ND	ND	ND	no tem	pc

Figure 6 PCR products for *env* and *gag* genes in spleen (a and b) lung (c and d) and CLN (e and f) tissues from NOD/SCID mice and NOD/SCID B2m$^{-/-}$ mice respectively infected with HIV 1 RNA copy numbers are shown in g Hu PBL NOD/SCID mice were inoculated with HIV 1_{MN} via different routes the peritoneal cavity (lane 1) and oral cavity with (lane 2) and without (lane 3) injured tissues NOD/SCID B2m$^{-/-}$ mice were inoculated with HIV 1_{MN} via different routes peritoneal cavity (lane 4) and oral cavity with (lanes 5 and 7) and without (lanes 6 and 8) injured tissues Lane MW Molecular weight standards (*Hae*IIIdigest of ΦX174) As shown in the table (g) the detection limit of RNA copy numbers was 800 in this experiment Data shown are representative of 3 independent experiments ND not detected ip intraperitoneal cavity inj oral cavity with injured tissues po oral cavity no cell tissues from NOD/SCID B2m$^{-/-}$ mice inoculated intraperitoneally with HIV 1 no HIV tissues from hu PBL NOD/SCID B2m$^{-/-}$ mice no tem negative control for PCR assay (no template DNA) pc positive control DNA (HIV 1_{MN})

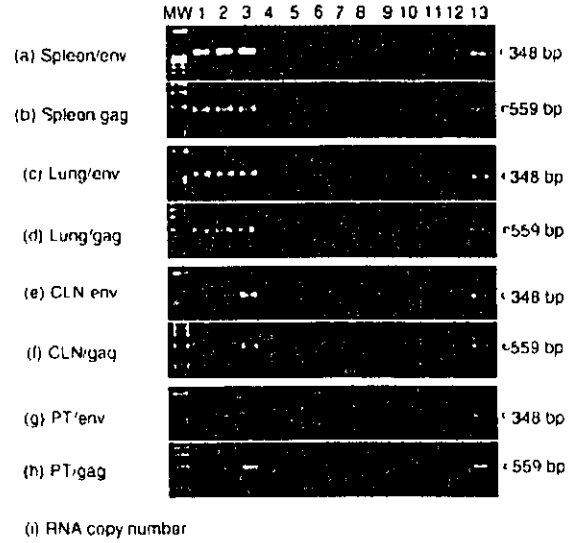
500 and 1000 TCID$_{50}$ HIV 1_{MN} as X4 viruses into the oral cavity with and without trauma did not show PCR products of *gag* and *env* in any tissues or HIV 1 RNA copies in serum (Fig 6) HIV 1_{NDK} at a high dose (100 TCID$_{50}$) also failed to cause oral infection in the mouse system (data not shown) Further the R5 viruses HIV $1_{TH\ PF7}$ and HIV $1_{JR\ FL}$ did not infect the oral cavity of mice with or without trauma at doses of 100 and 1000 TCID$_{50}$ (Fig 7 and 500 TCID$_{50}$ data not shown) HIV $1_{JR\ CSF}$ at a high dose (1000 and 10000 TCID$_{50}$) also failed to cause oral infection in the mouse system (data not shown)

Expression of coreceptor to HIV 1 in mouse periodontal tissues

To evaluate the expression of the coreceptor to HIV 1 in mouse periodontal tissues samples were extracted and homogenized in HBSS and stained with various FITC or PE conjugated anti coreceptor antibodies for fluorescence activated cell sorting analysis CXCR4 was expressed on 13 7 % of the CCR5 cells and 1 3 % of the CCR5^{+} cells in mucosal tissues from NOD/SCID 2m$^{-/-}$ mice (Fig 8) Moreover CXCR was expressed in 1 1 % of the CD45^{-} cells (epithelial cells) but not in CD45^{+} cells (leucocytes Fig 8)

DISCUSSION

In the present study we first examined the effect of hu IL 4 in an attempt to establish an animal model for HIV oral transmission using NOD/SCID mice and NOD/SCID B2m$^{-/-}$ mice which were considered to be useful recipients for the migration and grafting of hu lymphocytes It is known that IL-4 preferably activates Th2 subset T lymphocytes[31] and causes an increase in immunoglobulin production[3] As for the effect of hu IL 4 on hu lymphocytes in a human mouse chimera construction hu IL 4 causes an escape from activation of hu cytotoxic T cells and natural killer cells in NOD/SCID mice[33] Further cytotoxic hu cells induced in the SCID mouse system are accompanied by an increased sensitivity to apoptosis which is known to regulate proliferation and maintain lymphocyte homeostasis[34,35] The appearance of apoptotic hu T cells is dependent on cytotoxicity against mouse tissues and is up regulated during infiltration in a long term NOD/SCID culture The infiltration of a large number of CD8^{+} cells has also been confirmed in several types of NOD/SCID tissues from th kidney liver periodontal tissues and bone marrow (?? Fig 4) In the present study hu IL 4 significantly stimulated migration of hu CD4^{+} and CD8^{+} cells in spleen and periodontal tissues from NOD/SCID B2m$^{-/-}$ mice Furthermore th migration or circulation of hu CD4^{+} cells without apoptosis to peripheral tissues such as periodontal tissues was higher in NOD/SCID B2m$^{-/-}$ mice than NOD/SCID mi Thes results indicate that hu IL 4-administrated hu PBL NOD/SCID B2m$^{-/-}$ mice may be a useful model for *in vivo* studies of hu lymphocyte response in the oral cavity as the method is less artificial and easy to perform b cause it is not necessary to preculture hu lymphocytes



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
Dose of HIV (R5 virus)	$100TCID_{50}$							$100TCID_{50}$					
Route of inoculation	ip 1	ip 2	ip 3	inj 1	inj 2	po 1	po 2	inj	po	no cell	no HIV		
RNA copy number	15928	16728	17494	ND	ND	ND	ND	ND	ND	ND	ND	no tem	pc

Figure 7 PCR products for *env* and *gag* genes in spleen (a and b) lung (c and d) CLN (e and f) and PT (g and h) tissues from NOD/SCID mice and NOD/SCID $B2m^{null}$ mice respectively infected with HIV-1 RNA copy numbers are shown in i Hu PBL NOD/SCID mice were inoculated with HIV $1_{TH22PF7}$ via different routes the peritoneal cavity (lanes 1 and 2) and oral cavity with (lane 4) and without (lane 6) tissue injury Hu PBL NOD/SCID $B2m^{null}$ mice were inoculated with HIV $1_{JR\ FL}$ via different routes the peritoneal cavity (lane 3) and oral cavity with (lanes 5 and 8) and without (lanes 7 and 9) tissue injury Lane MW Molecular weight standards (*Hae*III digest of ΦX174) As shown in the table (i) the detection limit of RNA copy numbers was 800 in this experiment Data shown are representative of one or two independent experiments ND not detected ip intraperitoneal cavity inj oral cavity with injured tissues po oral cavity no cell tissues from NOD/SCID $B2m^{null}$ mice inoculated intraperitoneally with HIV-1 no HIV tissues from hu PBL NOD/SCID $B2m^{null}$ mice no tem negative control for PCR assay (no template DNA) pc positive control DNA (HIV 1_{MN})

Primary isolates from HIV-1-infected patients obtained in the early stages of infection have been shown to be primarily R5 viruses [36] which are very efficient in their infection of epithelial cells through monocytes [37] However both low and high concentrations of cell-free R5 viruses failed to infect hu PBL-NOD/SCID and NOD/SCID $B2m^{null}$ mice by oral transmission with and without oral trauma Recently it was suggested that a potential mechanism of HIV-1 entry into epithelial cells was direct transfer between productively infected cells and epithelial cells [38] Moreover Meng *et al* noted that intestinal epithelial cells that expressed glycosphingolipid GalCer might selectively transfer R5 HIV-1 to CCR positive cells such as macrophages or T lymphocytes via the lamina propria in a cell contact dependent manner [39] We confirmed that epithelial cells from the present experimental mice slightly expressed GalCer and CCR5 the chemokine receptor that serves as a coreceptor for R5 viruses Therefore a cell free R5 virus may not be able to invade the mouse mucosal barrier without tonsil even if hu $CD4^+$ cells are present in gingival tissues or injured tissues of the oral cavity As a result it is supposed that cell–cell contact by infected cells leads to transmission and infection in mice

The HIV 1_{MN} strain one of the most prevalent HIV-1 strains in North America and Europe [40,41] (classified as subtype B and known as an X4 virus) was also inoculated orally in the present mice A X4 virus requires interaction of the envelope glycoprotein with CD4 and a second receptor a chemokine receptor CXCR4 In the present study cell free X4 viruses did not transmit to or infect injured gingival mucosa infiltrated by a large number of hu $CD4^+$ cells in hu PBL NOD/SCID $B2m^{null}$ mice stimulated by IL-4 as well as the R5 virus Further direct contact of the cell-free X4 virus to hu $CD4^+$ $CXCR4^+$ cells in the oral cavity with injured tissues did not result in HIV-1 infection in the mice It is known that the human saliva contains resistants to HIV-1 infection as anti HIV-1 agents such as human-SLPI [42,43] and thrombospondin [44] that aggregate viruses (by blocking virus–CD4 interactions during virus entry) and the hypotonicity of saliva contribute to inhibit cell associated viral replication [45] We measured SLPI as a representative of the inhibitors however it was undetected in the mouse saliva samples

In a recent prospective clinical trial 16.2% of the cases of vertical transmission of HIV could be ascribed to breast feeding [2] Oral transmission of HIV-1 among adults is controversial In a study of 122 people with primary HIV-1 infection Dillon *et al* estimated that 6.6% of the infections resulted from oral sex [46] In the presence of severe periodontal disease very low

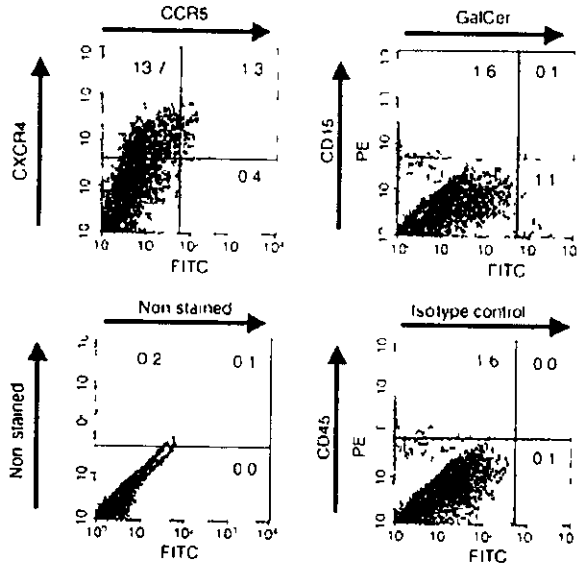


Figure 8 Flow cytometric analysis of hu CXCR4$^+$ and CCR5$^+$ cells and GalCer$^+$ epithelial cell populations in mucosal tissues from the oral cavities of treated NOD/SCID B2m^{null} mice The analyses were performed on gated cells larger than lymphocytes with forward scatter/side scatter characteristics Mucosal tissue cells can be distinguished in the quadrant analysis The proportions of CXCR4$^+$ and CCR5$^+$ cells were compared to non stained cells as a negative control The proportion of GalCer$^+$ cells among CD45$^-$ cells was compared to cells stained with the control isotype antibody Data shown are representative of three independent experiments

concentrations of infectious virus were present in 21% of the saliva samples from HIV-seropositive patients at all stages of HIV infection [47] However mucosal and gingival lesions such as gingivitis and candidiasis can cause bleeding thereby releasing the virus and infected cells into saliva and increasing the potential risk of oral transmission [48] A research agenda that clarifies in detail the risk from oral-genital contact in adults is important for rational counselling and prevention From results of the present study we supposed two hypotheses First human mucosa which likely has a pathogenic role in bleeding diseases such as periodontal diseases relocates the infected cells to other lymphoid tissues through the receptor-mediated homing properties of the mucosal system Second tonsils may be an essential gate for oral-transmission or infection of HIV-1 over contact of cell free virus to cells with receptors in animal models Therefore a definitive conclusion regarding the difference between oral mucosa with and without tonsils in the role of the oral transmission of HIV 1 requires further investigation Our findings demonstrated useful small humanized model for the study of HIV oral transmission or infection and may help to define the window of opportunity for initiation by the HIV virus in experiments using animal models of oral transmission

ACKNOWLEDGMENTS

This work was supported in part by grants in aid for Development Scientific Research (13671991 and 12557158) from the Ministry of Education Science and Culture of Japan and by a grant from the Japan Health Science Foundation to H S This study was also funded by a part of Ground Research for Space Utilization promoted by NASDA and Japan Space Forum

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Bone Regeneration by Recombinant Human Bone Morphogenetic Protein-2 Around Immediate Implants: A Pilot Study in Rats

Khairul Matin, BDS, PhD[1]/Hidenobu Senpuku, DDS, PhD[2]/Nobuhiro Hanada, DDS, PhD[3]/
Hidehiro Ozawa, DDS, PhD[4]/Sadakazu Ejiri, DDS, PhD[5]

Purpose *Difficulties relating to bone regeneration that complicate immediate implant placement include buccal and/or lingual fenestrations, primary anchorage of the implants, and the need for protection from functional loading during the osseointegration period. The objective of this pilot study was to evaluate bone regeneration by recombinant human bone morphogenetic protein-2 (rhBMP-2) around immediate implants placed in maxillary sockets in rats.* ***Materials and Methods*** *A total of 16 cylindric 0.8×1.8 mm commercially pure, solid titanium implants were placed immediately after gentle extraction of the maxillary first molar teeth of 8 male Wistar rats. The sockets were randomly divided into 3 groups: group 1 (n = 6) received rhBMP-2 with polylactic acid/polyglycolic acid copolymer–coated gelatin sponge carrier, group 2 (n = 5) received only the carrier, and group 3 (n = 5) received no grafting materials following placement. The rats were euthanized at 90 days postsurgery for microscopic analysis.* ***Results*** *In group 1, the implant body remained submerged completely, including the coronal part, which was fully covered by a significant amount (30% of total height) of regenerated cortical bone, even though the implant could easily be pulled out by a tweezer at the time of placement. Close approximation between the implant surface and regenerated bone could also be detected, indicating good bone to implant contact. In contrast, only peri-implant bone regeneration occurred in group 2, and an approximate 0.3 mm coronal part of the implant remained exposed. When no grafting materials were used (group 3), almost one third of the total length of the implant was exfoliated out of the socket when no grafting materials were used.* ***Discussion and Conclusions*** *Based on previous study and data from 16 sockets of the present study, it could be concluded that rhBMP-2 facilitated the regeneration of bone around immediate implants. In particular, the bone covering the coronal part could have been regenerated shortly after surgery, which helped to maintain the implant body inside the socket during the integration period in rats.* (Int J Oral Maxillofac Implants 2003;18:211–217)

Key words *bone morphogenetic proteins, dental implantation, dental implants, gelatin sponge, immediate implant placement*

[1]Postdoctoral Fellow (Japan Society for the Promotion of Science), Department of Oral Health, National Institute of Public Health, Tokyo, Japan. Assistant Professor, Department of Prosthetics, Dental Unit, Bangladesh Medical Studies and Research Institute, Dhaka, Bangladesh.
[2]Head, Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan.
[3]Director, Department of Oral Health, National Institute of Public Health, Tokyo, Japan.
[4]Professor, Institute for Dental Science and Department of Oral Anatomy, Matsumoto Dental University, Nagano, Japan.
[5]Associate Professor, Division of Anatomy and Cell Biology of the Hard Tissue, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Science, Niigata, Japan.

Placement of implants immediately after tooth extraction is a treatment modality used increasingly commonly in implant-supported oral rehabilitation.[1] To date, there have been several studies documenting this immediate implant placement technique.[1–3] Major difficulties[4,5] relating to bone

Reprint requests Dr Sadakazu Ejiri, Division of Anatomy and Cell Biology of the Hard Tissue, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Science, 5274-2 Bancho Gakkocho-dori, Niigata 951-8514, Japan. Fax: +81-25-227-0804. E-mail: ejiri@dent.niigata-u.ac.jp

regeneration that complicate immediate implant procedures include buccal and/or lingual fenestrations primary anchorage of the implants and the need for protection from functional loading during the osseointegration period In addition stability of the implant body during the osseointegration period and osseointegration at the coronal part of the implant may be of concern There is always a risk of trauma to the implant bone interface which can compromise implant success or increase crestal bone loss To overcome these difficulties various techniques are being investigated and applied and special efforts have been devoted to improving the bone implant interface by regenerating enough bone of sufficient quality around implants Recent reports have demonstrated bone regeneration around nonsubmerged implants placed immediately in extraction sites and supported by grafting materials or bone augmentation materials [4–7] Some of these studies involved the use of bioabsorbable materials which did not significantly enhance peri implant bone regeneration in immediate implantation

Recombinant human bone morphogenetic protein 2 (rhBMP 2) is the most actively studied of the recombinant proteins produced by recombinant technology for human bone morphogenetic proteins [8,9] The most recent report on successful oral application of rhBMP 2 in humans is very encouraging and it suggests that further studies of various oral applications of rhBMP 2 would be worthwhile [10] In previous work the present investigators were able to demonstrate that rhBMP 2 accelerated socket healing so as to preserve the cortical bone volume in rat maxillary root sockets [11] Bone regeneration around immediate implants supported by rhBMP 2 has not previously been evaluated in animal models Therefore the purpose of the present study was to evaluate the bone regenerative efficacy of rhBMP 2 around immediately placed implants in the maxillary root sockets of rats

MATERIALS AND METHODS

The protocol for this animal experiment was approved by the Niigata University School of Dentistry's Committee on the Guidelines for Animal Experimentation Six week old male Wistar rats (170 to 190 g Charles River Laboratory Yokohama Japan) were housed under similar conditions (22°C room temperature 40% humidity and a 12 hour daylight cycle) fed commercial rat food (MF Oriental Yeast Tokyo Japan) and given access to tap water ad libitum

In this experiment a polylactic acid/polyglycolic acid copolymer (PLGA) –coated gelatin sponge (GS) was used as the rhBMP 2 carrier (PLGA/GS) [11] The molar ratio of the PLGA polymers was 1 1 and the weight ratio of PLGA to GS was 4 1 with porosity of approximately 90% [17]

A total of 16 maxillary first molar teeth were gently extracted from 8 Wistar rats under anesthesia, and the rats were divided into 3 groups Group 1 (n = 6) received rhBMP 2 and the carrier in their sockets group 2 (n = 5) received only the carrier and group 3 (n = 5) received no grafting materials with the implants The socket walls were delicately trimmed with a spiral type low speed (500 rpm) engine bur for less than 5 seconds under sterile saline cooling then debrided and cleaned with sterile saline so that each implant could reach the base of the socket and fit tightly A commercially available commercially pure titanium implant bur was used to prepare the sites for machined solid cylinder implants (diameter = 0 8 mm length = 1 8 mm rounded apices) These were placed in each of the 16 sockets keeping the coronal part approximately 0 1 mm out of the socket The implants showed no side to side movement on probing but could be pulled out easily by a tweezer Placement of the implant was followed by placement of the grafting materials (groups 1 and 2 only) and suturing of the gingival mucosa The implant remained diagonally in the socket because of the diagonal anatomy of the anterior root and its socket of otherwise vertical rat maxillary first molar teeth

The rats were provided with soft food and monitored every day for the first 2 weeks after the operation Monitoring was continued at regular intervals over a 90 day period Rats were sacrificed by perfusion fixation under general anesthesia as described in a previous report [11] and block biopsies were harvested The status of each implant was verified using scanning electron microscopy (SEM) contact microradiography (CMR) and confocal laser microscopy (CLM) For examination using the SEM biopsy specimens were chemically treated to remove soft tissue and then dehydrated and gold coated before examination Conventional methods were used to embed the block biopsies in methyl methacrylate resin after fixation in 70% ethanol and Villanueva staining Using a cutting grinding technique 250 µm thick sagittal sections were obtained these were then examined by CMR and CLM

Regenerated bone height around the implants and the position of the implants were evaluated by a similar procedure as described previously [11] In brief 3 vertical lines were drawn on an imaginary