Role of Activated Natural Killer Cells in Oral Diseases

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INTRODUCTION

The elderly population is currently rapidly increasing in industrialized countries. In Japan, the proportion of the population >65 years old was 7.1% in 1970 but it is predicted to be 29.6% by 2030 (1). Accordingly, the number of bedridden elderly requiring systemic care in residential and nursing homes is also increasing. Reports show that institutionalized elderly have poorer oral health than those who live independently at home (2,3). Moreover, changes in microflora related to poor oral health include an increase in the prevalence of bacteria and may also contribute to the development of pneumonia (4), as the aspiration of bacteria present in oropharyngeal flora moves them into the respiratory tract; therefore, their presence is a risk factor for the elderly and compromised hosts. Consequently, the oral cavity is a reservoir for pathogenic bacteria where re-colonization may infect systemic organs.

Dental caries and periodontal diseases are a major problem for the elderly and are significantly associated with tooth loss (5-8). Several species of bacteria including Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans are pathogens related to dental caries and periodontal disease in humans (9-12). However, definitive useful indicators or predictors for dental caries and periodontal disease have not been reported.

Natural killer (NK) cells are instrumental in the innate immune response for early production of gamma interferon (IFN-γ) and other cytokines necessary to control bacterial, parasitic and viral infections (13,14). Reports show that products prepared from broth extracts of Gram-positive bacteria, e.g., streptococci, staphylococci, and lactobacilli, activate human NK cells (15,16). Oral streptococci are the principal commensal bacteria that construct normal biofilms and play a role in the resistance to colonization by invading opportunistic pathogens in the oral cavity (17,18). Therefore, these Gram-positive bacteria interact with the human immune system and are competitors with the opportunistic pathogens on the mucosal tissues in the microbial ecosystem of the oral cavity. Individuals with either inherited or acquired immune deficiency are subject to increased risks for dental disease (19,20). However, it is not known if there is a relationship between the systemic immune response and oral microbial infection or oral disease.

Human blood NK cells responsible for antibody-dependent cell-mediated cytotoxicity constitutively express CD56 antigen and CD16. In addition, NK cells express C-type lectin receptors such as CD69 (which is an early activation marker) (21). CD69 is a type II integral membrane glycoprotein that is expressed on many activated cells of hematopoietic origin (22). The role of CD69 is to initiate cell activation. It presents a functional triggering molecule to activate NK and T cells where the cross-linking of CD69 induces cytotoxic activity and cytokine production (23). Therefore, CD69+NK cells are indicators for early activated NK cells.

Here we quantitatively measured activated NK cells in the bloodstream to determine if there is a relationship between oral bacterial infection and oral commensal bacteria such as streptococci and lactobacilli and opportunistic pathogens with comparisons to CD69+NK cells. We demonstrated that increased proportionate numbers of systemic activated NK cells correlate with oral disease, oral streptococci and opportunistic pathogen infections and are a possible indicator for oral infections.

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SUBJECTS AND METHODS

Elderly subjects: In 1998, a longitudinal interdisciplinary study of aging was initiated to evaluate the relationships between health status and dental status in Japan. Initially, questionnaires were sent to 4,542 residents aged 70 years old (born in 1927) in Niigata City. After the responders were divided by sex, 600 subjects were selected randomly with approximately the same numbers of each sex chosen for the baseline survey (24,25). The participants agreed to have medical and dental examinations, and they signed an informed consent form showing the protocol that was approved by the Ethics Committee of Niigata University Graduate School of Medical and Dental Science. The study was carried out according to the Declaration of Helsinki. Follow-up surveys have been carried out every year during June using the same methods as in the initial survey. Among the participants (n = 399) in the follow-up survey conducted in June 2005, 100 subjects (53 males, 47 females) were examined for the NK cell of their lymphocyte population. All subjects were 77 years old; the body height, body weight and number of teeth present were recorded, and the body mass index (BMI) was calculated for each subject. Some subjects had systemic diseases, including diabetes (9%), cerebral infarction (8%), cataract (6%), dyslipidemia (4%), osteoporosis (4%), anemia (4%), and smoking habit (9%), but did not require special care.

Antibodies: The following monoclonal antibodies (mAb) were purchased from BD Pharmingen (San Diego, Calif., USA): fluorescent isothiocyanate (FITC)-conjugated anti-human CD6 (clone 3G8) and phycoerythrin (PE)-conjugated anti-human CD69 (FN50). Phycoerythrin-cyanine 5 (PC5)-conjugated anti-human CD6 (N901), FITC, PE and PC5-conjugated mouse immunoglobulin G1 (IgG1)(clone 679.1Mc7) were purchased from Immunotech as isotype controls (Marseille, France).

Lymphocyte separation: Whole blood, 5-7 ml, was collected in sodium heparin tubes (10 ml VENOJECT; Terumo, Tokyo, Japan), diluted with an equal volume of Hanks’ Balanced Salt Solution (HBSS) (Gibco Laboratories, Life Technologies; Paisley, UK), layered on a Ficoll-Conray density gradient separation solution (Lymphosepar I; Immuno-Biological Laboratories, Gunma, Japan), and centrifuged at 1,800 rpm for 30 min at room temperature. The peripheral blood mononuclear cells (PBMC) layer was removed and washed twice in HBSS. The PBMC were stained using PC5-conjugated CD56 and FITC-conjugated CD16 mAb to detect NK cells and stained with PE-conjugated IgG1 isotype to identify NK cells and stained with PE-conjugated CD69 mAb and PC5- and PE-conjugated IgG1 isotype controls were used. PBMC (1 x 10⁶) were then inoculated into v-bottomed 96-well plates (Coster, Cambridge, Mass., USA), and each was incubated with 20 μl of 5% formalin/PBS solution; and stored at 4°C until flow cytometric analysis was performed. The proportions of the major subsets were determined using single and quadrant analysis. The percentage of FITC-, PC5- and PE-positive cells was measured using a FACSCalibur (Becton Dickinson, Franklin Lakes, N.J., USA) flow cytometer, and the data were analyzed using CellQuest Pro (Becton Dickinson) software.

Human saliva collection: Whole saliva samples were collected on swabs after saliva stimulation by the chewing of paraffin gum for 5 min, and the saliva was placed in transport fluid (0.4% agar, 0.15% thioglycolate/phosphate buffered saline). At the Bio Medical Laboratory (BML, Tokyo, Japan), the numbers of mutans streptococci, total streptococci, and lactobacillus microorganisms were determined.

Bacterial count: Cotton swabs containing the saliva samples from the elderly subjects were placed in transport fluid and taken to BML for analysis. Mutans streptococci, lactobacilli and total streptococci were noted to be typical cariogenic and commensal bacteria in the oral cavity. Each sample was plated onto Mitis-Salivarius agar (MS; Nippon Becton Dickinson Co. Ltd., Tokyo, Japan), modified Mitis-Salivarius agar containing 0.2 U/ml of bacitracin (MMSB) (27) or Rogosa SL agar (Nippon Becton Dickinson) using an EDDY JET spiral plating system (IUL, S.A., Barcelona, Spain) and incubated at 37°C under anaerobic conditions for 48 h. The total number (CFU) of total streptococci, mutans streptococci, and lactobacilli organisms was counted using MS, MMSB, and Rogosa SL agar. Colonies of mutans streptococci were identified by their characteristic appearance.

Tongue samples were collected from the tongue surface using a cotton swab (SeedsSwab No. 1) to determine if opportunistic pathogens were present. The sampling was performed by a physician from Niigata University trained in the mucosal surface sampling technique. The tongue bacterial samples were placed in transport fluid and transported to BML for analysis. Procedures were performed to detect qualitatively the following bacterial species (opportunistic pathogens): Acinetobacter spp., Citrobacter freundii, Enterobacter agglomerans, Enterobacter cloacae, Escherichia coli, Haemophilus parainfluenzae, Klebsiella oxytoca, K. pneumoniae, methicillin-resistant Staphylococcus aureus (MRSA), methicillin-sensitive S. aureus (MSSA), Pseudomonas aeruginosa, Proteus mirabilis, Serratia marcescens, Streptococcus agalactiae, and Stenotrophomonas maltophilia.

Each tongue sample was inoculated onto chocolate, OPA Staphylococcus and Drigalski agar plates (Nippon Becton Dickinson). The species numbers of opportunistic pathogens were counted in each sample. The plates were incubated under an atmosphere of 5% CO₂ in H₂ at 37°C for 24-48 h. Representative colonies from each plate were Gram stained, and isolates were made by the identification of characteristic appearance as well as by hemolysis, catalase and oxidase reactions (28). Isolates were suspended in 1 ml of 0.5% saline, gently shaken and tested using microbial identification kits to detect the above bacteria (ViTEK; BioMerieux Vitex Japan, Tokyo, Japan) (29).

Oral disease examinations: Dental examinations were conducted under artificial white light by four trained dentists. All subjects were examined at local community centers in Niigata City. All functioning teeth including third molars were assessed (except for partially erupted teeth). Using the World Health Organization (WHO) criteria (30), decayed teeth (DT), missing teeth (MT), and filled teeth (FT) (DMFT) scores were recorded along with findings of dental caries. The dentists assessed each patient’s periodontal condition using six measurement points (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, and disto-lingual) for each tooth. The number of sound teeth, crowns, bridges, and retained roots were counted. Intra- and inter-examiner reliability was confirmed using a kappa statistic (κ = 0.56 - 0.92 for attachment loss (AL)). To estimate periodontal status, rCA (the rate of sites with dental calculus), rAL4 (the rate of sites with greater than 4 mm of AL), rAL6 (the rate of sites with greater than 6
mm of AL, rPD4 (the rate of sites with greater than 4 mm of periodontal pocket (PD)), rPD6 (the rate of sites with greater than 6 mm of PD), AL [length (mm) of attachment loss] and BOP (the rate of sites with bleeding on probing) were measured at the same six points for each tooth (31). Mouth mirrors were used incorporating a light and pressure-sensitive plastic periodontal probe set to give a constant force of 20 g and graded at 1-mm intervals (VIVACARE TPS PROBE®). In cases where a restorative margin was apical to the cement-enamel junction (CEJ), AL was measured using the observed anatomical features of the teeth; and if present, the CEJ of the adjacent tooth/teeth. The BOP was measured at six sites per tooth. Before and during the survey, examination calibrations were conducted at an institution for the aged and at the Faculty Hospital of Dentistry, Niigata University. Intra-examiner agreement ranged from 86.6 to 95.9% and from 65.8 to 94.4% for PD and AL, respectively. The kappa values ranged from 0.79 to 0.93 and from 0.56 to 0.92 for PD and AL, respectively. Thereafter, the indicators were assessed and used to estimate the periodontal status of each subject.

Statistical analysis: All encrypted data were provided by the epidemiological research group of preventive dentistry at Niigata University and analyzed by the authors. All data were analyzed using the Statistical Package for SPSS for Windows (version 100; Chicago, Ill., USA). The student’s t test was used to compare the means of the parameters between males and females. Correlations between two variables were tested using the Pearson rank correlation. A P value of 0.05 or less was considered to indicate statistical significance.

RESULTS

Characteristics of elderly patients: The characteristics of elderly subjects and dental and periodontal disease status were compared between males and females. We recorded the height, weight, highest PD, highest AL, mean AL, % of sites with an AL >4 mm, and % sites with an AL >6 mm (Table 1). Males had a significantly higher AL at >6 mm than females. However, there were no other significant gender differences found in the other parameters.

Analysis of NK cells: For all patients, the proportionate number of NK (CD56+CD16+) cells in lymphocytes was 23.6 ± 13.9% and the proportion of CD69+ cells in the NK cell population was 31.2 ± 14.3%. There was a significant gender difference in the proportion of NK cells in lymphocytes (male 26.9 ± 13.1%, female 19.8 ± 13.9; P < 0.05). There was no significant gender difference in the proportion of CD69+ NK cells (male 30.7 ± 14.2%, female 31.8 ± 14.7). Even though some subjects had systemic diseases and a smoking habit, there were no significant differences in the proportion of NK and CD69+ NK cells shown in total systemic diseases, diabetes and cerebral infarction, and smoking habit (data not shown).

Correlation of oral bacteria numbers to activated NK cells: To analyze the relationships between activated NK cells and the number of oral streptococci and lactobacilli, the bacterial numbers were measured in the saliva and compared with the proportionate numbers of NK or CD69+ NK cells in the peripheral blood. In addition, infection by opportunistic pathogens on the tongue was also compared with the proportion of NK or CD69+ NK cells. A relatively increased proportion of CD69+ NK cells showed a positive correlation with the isolation numbers of total streptococci (r = 0.409, P < 0.01) and a statistically negative correlation with the species numbers of opportunistic pathogens (r = –0.318, P < 0.01) (Table 2). An increased proportion of CD69+ NK cells showed, in males, a positive correlation with the isolation numbers of total streptococci (r = 0.408, P < 0.01) and a negative correlation with the species numbers of opportunistic pathogens (r = 0.389, P < 0.05). There were no significant correlations between NK cells and any bacterial level nor between CD69+ NK cells and lactobacilli or mutants streptococci for either male or

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### Table 1. Characteristic of the subjects

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>155.8 ± 7.5</td>
<td>161.3 ± 5.1</td>
<td>149.6 ± 4.2</td>
<td>&lt;0.001**</td>
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<tr>
<td>Weight (kg)</td>
<td>54.9 ± 9.8</td>
<td>58.6 ± 9.9</td>
<td>50.8 ± 8.0</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.5 ± 3.2</td>
<td>22.4 ± 3.1</td>
<td>22.6 ± 3.3</td>
<td>0.762</td>
</tr>
<tr>
<td>Dental condition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of present teeth</td>
<td>15.9 ± 9.8</td>
<td>16.3 ± 9.8</td>
<td>15.4 ± 9.9</td>
<td>0.629</td>
</tr>
<tr>
<td>Sound teeth</td>
<td>7.4 ± 6.8</td>
<td>8.6 ± 7.0</td>
<td>6.0 ± 6.6</td>
<td>0.062</td>
</tr>
<tr>
<td>Decayed teeth</td>
<td>0.1 ± 0.6</td>
<td>0.2 ± 0.8</td>
<td>0.1 ± 0.2</td>
<td>0.263</td>
</tr>
<tr>
<td>Filled teeth</td>
<td>8.4 ± 5.5</td>
<td>7.6 ± 4.8</td>
<td>9.3 ± 6.2</td>
<td>0.127</td>
</tr>
<tr>
<td>Missing teeth</td>
<td>12.4 ± 9.3</td>
<td>12.1 ± 9.3</td>
<td>12.8 ± 9.5</td>
<td>0.698</td>
</tr>
<tr>
<td>Highest PD</td>
<td>5.5 ± 1.9</td>
<td>6.0 ± 1.8</td>
<td>5.0 ± 1.8</td>
<td>0.011*</td>
</tr>
<tr>
<td>Mean PD</td>
<td>2.3 ± 0.6</td>
<td>2.4 ± 0.6</td>
<td>2.2 ± 0.5</td>
<td>0.068</td>
</tr>
<tr>
<td>% site with PD ≥4 mm</td>
<td>12.8 ± 13.7</td>
<td>14.9 ± 13.6</td>
<td>10.3 ± 13.5</td>
<td>0.109</td>
</tr>
<tr>
<td>% site with PD ≥6 mm</td>
<td>2.5 ± 5.1</td>
<td>3.3 ± 6.0</td>
<td>1.5 ± 3.6</td>
<td>0.084</td>
</tr>
<tr>
<td>Highest AL</td>
<td>7.3 ± 2.3</td>
<td>8.0 ± 2.3</td>
<td>6.4 ± 2.0</td>
<td>0.001**</td>
</tr>
<tr>
<td>Mean AL</td>
<td>3.6 ± 1.2</td>
<td>3.9 ± 1.2</td>
<td>3.3 ± 1.0</td>
<td>0.021*</td>
</tr>
<tr>
<td>% site with AL ≥4 mm</td>
<td>43.6 ± 30.8</td>
<td>49.5 ± 29.4</td>
<td>36.7 ± 31.2</td>
<td>0.049*</td>
</tr>
<tr>
<td>% site with AL ≥6 mm</td>
<td>12.0 ± 19.1</td>
<td>15.8 ± 21.3</td>
<td>7.5 ± 15.2</td>
<td>0.040*</td>
</tr>
<tr>
<td>% site with BOP</td>
<td>11.3 ± 13.4</td>
<td>11.1 ± 13.0</td>
<td>11.5 ± 14.1</td>
<td>0.893</td>
</tr>
</tbody>
</table>

*P values evaluate gender differences.

**P < 0.05.

***P < 0.01.

BMI, body mass index; PD, probing depth; AL, attachment level; BOP, bleeding on probing.
Correlation of dental disease status to activated NK cells: To analyze the relationships between activated NK cells and oral disease status, decayed teeth and teeth cured by bridges and crowns were measured and compared with the proportion of CD69+NK cells. A relatively increased proportion of CD69+NK cells showed significant correlations with bridges (r = 0.219, P < 0.05) and decayed teeth (r = –0.223, P < 0.05) (Table 3). An increased CD69+NK cell population showed significant correlations with crowns (r = 0.362, P < 0.001) but was not significant in other oral disease status observations (Table 3). There were no other significant correlations between parameters of periodontal diseases and NK cell or CD69+NK cell proportions in male and female subjects (Table 4). The numbers of subjects having partial dentures and full dentures were 33 and 17, respectively. There was no significant difference between subjects with partial or full dentures and subjects without in terms of the proportions of NK cells and CD69+NK cells.

DISCUSSION

The oral carriage of bacteria causing pneumonia such as *K. pneumoniae*, *Pseudomonas* sp. and *Staphylococcus* sp. is low in healthy subjects and higher in immunodeficient, myelosuppressed and elderly subjects requiring care (7,32-34). Smith et al. report that coagulase-negative staphylococci sp. and *Candida* spp. levels were also higher in the oral cavities of critically ill patients and in those with oral Crohn’s disease (35). *Pneumococcus* sp. emerged in many debilitated elderly patients and in myelosuppressed and elderly subjects requiring care (7,32-34).

Correlation of oral bacterial numbers and NK cells: Table 2 shows the correlation between oral bacterial numbers and NK cells. The oral carriage of bacteria causing pneumonia such as *K. pneumoniae*, *Pseudomonas* sp. and *Staphylococcus* sp. is low in healthy subjects and higher in immunodeficient, myelosuppressed and elderly subjects requiring care (7,32-34). Smith et al. report that coagulase-negative staphylococci sp. and *Candida* spp. levels were also higher in the oral cavities of critically ill patients and in those with oral Crohn’s disease (35). *Pneumococcus* sp. emerged in many debilitated elderly patients and in myelosuppressed and elderly subjects requiring care (7,32-34).
the change in systemic conditions involving immune activity may interact with and restrict oral microbial ecology. In this study, we found the activated NK cells to be a responsible immune indicator for the incidence of oral disease and oral bacterial infection in elderly subjects.

Products prepared from Gram-positive bacteria including streptococci, staphylococci, and lactobacilli activate human NK cells (15,16) and induce CD14-independent pathways to stimulate human monocytes (23,37). The proportionate increase of CD69 NK cells correlates with infection by Gram-positive bacteria such as oral streptococci and lactobacilli. However, we show a correlation to the number of total streptococci but not to the numbers of lactobacilli in the saliva. Moreover, the proportion of CD69 NK cells did not correlate with periodontal disease status. This suggests that systemic activated NK cells are susceptible to oral total streptococci, the primary organisms in the oral cavity, but not to lactobacilli, mutants streptococci or the pathogens that cause periodontal disease in the elderly.

The correlation between activated NK cells and microbial infection and dental caries was greater in males than females. The reason for this is not understood. However, Willemse et al. reported that mental and cold stress increased NK cells and secretory immunoglobulin A (sIgA) in males but not in females (39). This may partially explain the gender differences in the correlation with activated NK cells.

We show that lower proportionate numbers of CD69 NK cells suggest that the oral streptococcal level may decrease, the opportunistic pathogen level may increase, and dental caries and pneumonia may develop in the future. Therefore, our data suggest that the proportion of active CD69 NK cells may be a useful indicator of oral infection in elderly subjects. However, it is not clear how CD69 NK cells are associated with the number of total streptococci and with dental status. Further studies are required to clarify these mechanisms. A limitation of our study is the bias introduced by the level of participation. The participants were 100 subjects (25.1%) out of a target population of 399 subjects; therefore, the data may be somewhat biased, as the present elderly subjects were generally in good health and might have been more eager and/ or able to participate in this survey. Thus, our findings may indicate an association in generally healthy elderly subjects. Therefore, additional follow-up studies with these elderly subjects are required to provide clear findings of interest.

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