先天性歯肉炎感受性ラットの血清中の免疫グロブリンおよび補体量

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Levels of Immunoglobulin and Complement in Serum of Rats with Congenital Susceptibility to Gingivitis

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ABSTRACT. Levels of serum immunoglobulins, complement and macrophage activity were compared between plaque-susceptible (SUS) and plaque-resistant (RES, control) rats. SUS rats with pregingivitis showed the significant lower levels in serum immunoglobulins than those in RES rats, especially at early months of age. C3 level and complement hemolytic activity in SUS rats were also lower than those of RES rats at the same age. IgA level and complement hemolytic activity in SUS rats remained at low levels throughout the observed period although the levels of the other immunoglobulins increased gradually thereafter. C3 levels in SUS rats aged 2 to 7 months were higher than that in RES control, while complement hemolytic activity was always lower than that in RES rats. Antibody response against sheep red blood cells (SRBC) was depressed in young SUS rats. The observations suggest that some abnormalities of immaturities in the humoral immune system are present in young SUS rats before occurrence of gingivitis. The susceptibility to periodontal lesions may be based on these abnormalities.—KEY WORDS: immunoglobulin , complement, periodontitis, plaque-susceptible rat.

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Periodontal disease has been considered as the inflammatory and destructive lesions in the periodontal tissues by bacterial infections [15]. Immunological aspects in this disease have been evaluated and support the concept that plaque-induced immunological effector mechanisms play a major role in the pathogenesis of periodontal disease [16, 18, 20]. The immunoglobulin and complement system are major humoral effectors for protective responses against foreign substances [2–5, 23]. Many studies have reported elevated levels of immunoglobulins against certain periodontitis-related bacteria, such as *Bacteroides gingivalis,* and C3 in the sera of patients with various forms of periodontal disease [14–15, 22]. In contrast, some reports have indicated that the levels of immunoglobulins are depressed in the patients [14] or that it is not different from healthy controls [4, 24]. In spite of many immunological approaches as above, there has been no precise explanation for the immunological conditions of host concerning the pathogenesis of periodontal disease. Majority of these observations has confirmed only the immunological conditions as a consequence of periodontitis, but has not clarified the immunological condition concerning susceptibility to periodontitis.

This study attempts to compare the immunological conditions between rats with congenital susceptibility to naturally occurring gingivitis [10–13] and rats with healthy periodontium by identifying the levels of humoral factors in serum. The results would provide basical information of the relationship between host immune conditions and the susceptibility to plaque formation and gingival inflammation.

MATERIALS AND METHODS

Animals: Plaque-susceptible (SUS) and plaque-resistant (RES) rats [10–13] were used in the present study. Both rats were fed on a commercial powder diet and examined at the age of 1, 2, 4, 7, 10 and 13 months. A total of 112 SUS and 93 RES rats was used, 60 SUS and 60 RES for determination of levels of Immunoglobulins, C3 and complement hemolytic activity, 47 SUS and 29 RES for determination of antibody producing activity, and 5 SUS and 4 RES for chemiluminescent assay of macrophages.

Collection of serum: Blood samples were obtained from rats at each experimental time. Serum was collected after clotting, and stored at −75°C.

Enzyme-linked-immuno-sorbent assay (ELISA): Serum IgM, IgG, IgA and C3 levels were determined by an ELISA, the details of which have been described elsewhere [6]. Briefly, anti-rat IgM, IgG, IgA (Serotec Co.) or C3 (Cappel Co.) antibodies were diluted to final concentration (10 μg/m*l*) and adsorbed to 96 well-microplate (100 μ*l*/well) at 4°C overnight. After washing the microplate (4 times with 0.05% Tween-PBS, pH7.2), 100 μ*l* of diluted sample (sera from the experimental rats, diluted to 1:10 to 1:100, optimal dilution was used), was added to the microplate and incubated at 37°C for 90 min. After washing, peroxidase-conjugated sheep anti-rat IgM, IgG or IgA antibodies (Serotec Co.) or goat anti C3 antibody (Cappel Co, diluted to 1:2,000) were added to the well and incubated at 37°C for 90 min. The wells were again rinsed and washed thoroughly, and the enzyme substrate, 5-aminosalycylic acid containing 0.001% H_2O_2 was added for color reaction. After incubation for 60 min, 0.2% NaN_3 (10 μ*l*) was added to each well. The levels of IgM, IgG, IgA and C3 were then determined by measuring the color changes in a 2 wave length microplate photometer (MTP-22, Corona Co.).

Hemolytic assay: The hemolytic unit [8, 17] of rat complement, CH50, was defined using CH50 assay system (Denka Bio. Lab. Co.). A 50% unit of complement was defined as the amount of serum required to lyse 50% of sheep red blood cells (SRBC) sensitized with hemolysin. Total reaction volume was 3m*l* (serial dilution of rat serum in 2.6m*l* and 0.4m*l* of 5×10^8/m*l* SRBC sensitized with hemolysin), and then the reaction mixture was incubated at 37°C for 1 hr. Reaction was stopped in ice bath. After centrifugation (350×g, 10 min), supernatant was measured for an absorption at optimal density (OD 541). The data for titer determination was done and CH50 was determined.

Antibody responses against SRBC: Hemolytic plaque assay was done by the method of Cunningham *et al.* [3]. For this experiment, SUS rats (4–5 week-old) which showed neither plaque formation nor gingivitis were selected. RES rats which were the same age as SUS rats were used for control. Briefly, each rat was inoculated with 2×10^8 SRBC intravenously. At 3 or 7 days after inoculation, the spleen was removed from each rat. Spleen cell suspensions were prepared, and 100 μ*l* containing 1×10^6 or 1×10^7 spleen cells in Eagle's MEM with 10% FCS was incubated with complement (Guinea-pig serum, 10 μ*l*), SRBC (10 μ*l*, 4×10^9/m*l*) and MEM (with 10% FCS, 30 μ*l*) at 37°C for 1 hr (direct counts, IgM producing cells). Similarly, spleen cells were incubated with complement, SRBC and rabbit anti-rat-IgG anti-serum (1:100, 10 μ*l*) and MEM (with 10% FCS, 20 μ*l*), at 37°C for 1.5 hr (indirect counts, IgG producing cells). Plaque forming cells (PFCs) were counted on 3 different glass slides and the mean counts were recorded as PFCs in each rat.

Preparation of opsonized zymosan: A 250mg amount of zymosan (Sigma Co.) was boiled in Hank's balanced salt solution

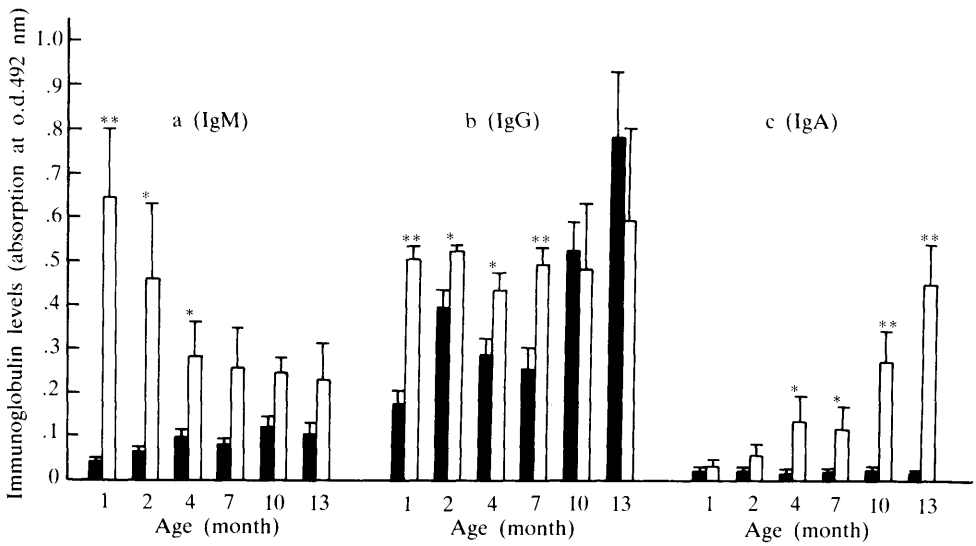

Fig. 1 Changes of mean immunoglobulin levels in serum of SUS (solid column) and RES (open column) rats. The results are expressed by absorption levels (o.d. 492 nm). Ten SUS and 10 RES rats were used at each temporal time. Standard errors are indicated by bars. Statistical differences between SUS and RES rats are shown by **(P<0.01) and *(0.01<P<0.05).

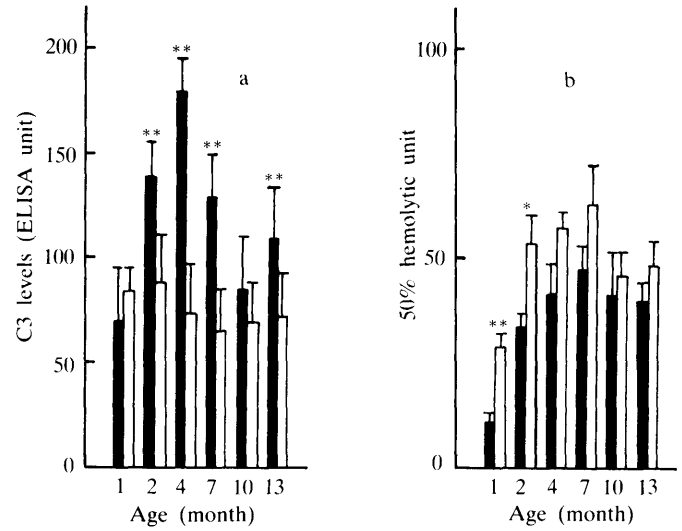

Fig. 2 Changes of mean C3 levels and complement hemolytic activity in serum of SUS (solid column) and RES (open column) rats. C3 levels are expressed by ELISA unit and complement hemolytic activities are expressed by 50% hemolytic unit (CH50 unit). Standard errors are indicated by bars. Statistical differences between SUS and RES rats are shown by **(P<0.01) and *(0.01<P<0.05).

(HBSS, 25 m*l*), washed 3 times with HBSS and centrifugation (350 ×g) and suspended in 5 m*l* of HBSS, and then a normal rat serum (15 m*l*) was added. This suspension was incubated in a 37°C water bath for 60 min, centrifuged and resuspended in 20 m*l* of HBSS. The suspension of opsonized zymosan was stored in 0.5 m*l* aliquots at −75°C.

Chemiluminescent response (CLR) of macrophages to opsonized zymosan: SUS and RES rats at 5 weeks of age were used for the sorce of peritoneal macrophages which were elicited by injecting 5% starch in 5% proteose peptone intraperitoneally. Peritoneal exudate cells were washed 3

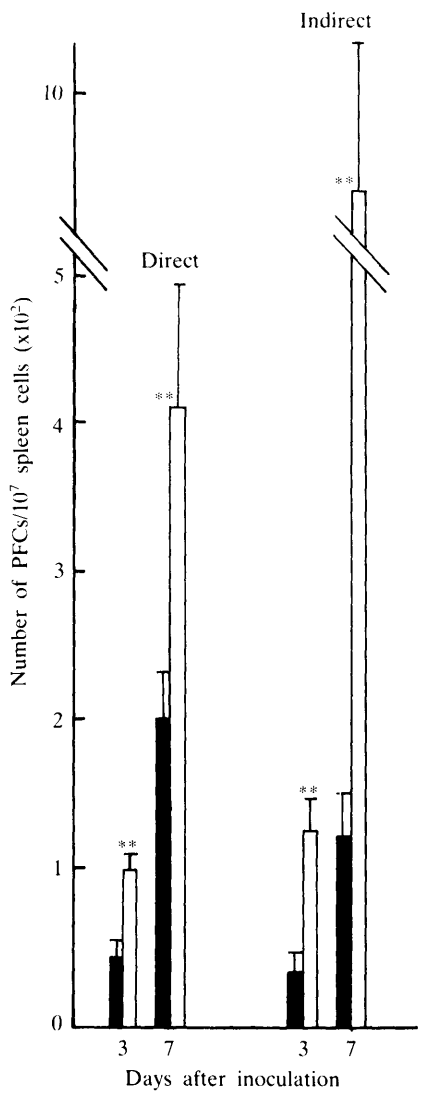

Fig. 3 Immune responses to SRBC in SUS (solid column) and RES (open column) rats. "Direct" and "Indirect" show the number of IgM and IgG antibody forming cells to SRBC, respectively. Standard errors are indicated by bars. Numbers of PFCs at preimmunization are 3.7±1.8 (SUS) and 3.5±1.7 (RES) in direct method and 0.5±0.3 (SUS) and 0.7±0.6 (RES) in indirect method, respectively. Statistical differences between SUS and RES rats are shown by **(P<0.01).

times with RPMI 1640 medium and suspended (10^7 cells/m*l*) in RPMI 1640 supplemented by 10% heat inactivated FCS, L-glutamine (2mM), sodium pyruvate (1mM), penicillin (50 U/m*l*), streptomycin (50 μg/m*l*) and Hepes buffer (20 mM). The suspension was seeded in 6 cm plastic dish (Falcon). After incubation for 60 min at 37°C in 5% CO_2, non adherent cells were removed. Adherent cells were harvested and resuspended in HBSS with 1mM $CaCl_2$ (5×10^5 cells/m*l*). CLR of macrophages was determined by the method of Allen and Loose [1]. One hundred μl of macrophage suspension and 100 μl of luminol solution (2×10^{-4}M) were mixed. After 2 min later, 100 μl of opsonized zymosan (12.5 mg/m*l*) was added and CLR was measured by luminescence analyzer (Biolumat LB9500T, Berthold Co.).

RESULTS

The gingival inflammation was observed in SUS rats at 2 months of age and thereafter. SUS rats showed significantly lower IgM levels than RES rats at 1 month of age (Fig. 1a). Similarly, lower IgM levels were recognized in SUS rats at 2 and 4 months of age. Similar results were observed in serum IgG levels. SUS rats showed lower IgG level than RES rats up to 7 months of age (Fig. 1b). Thereafter IgG level in SUS rats increased with aging and reached to almost similar level of that in RES controls at 10 months of age. SUS rats showed significantly lower IgA levels than that in RES rats at and after 4 months of age (Fig. 1c).

C3 level in SUS rats was not different from that in RES rats at 1 month of age. C3 level in SUS rats increased thereafter and was significantly higher than that in RES rats (Fig. 2a). Complement hemolytic activity in SUS rats was lower than that in RES rats at the age of 1 and 2 months (Fig. 2b).

The potency of antibody production in SUS and RES rats was estimated by determing the specific antibody to SRBC at 1 month of age. At the 3rd day post-inoculation, the mean PFCs per 10^7 spleen

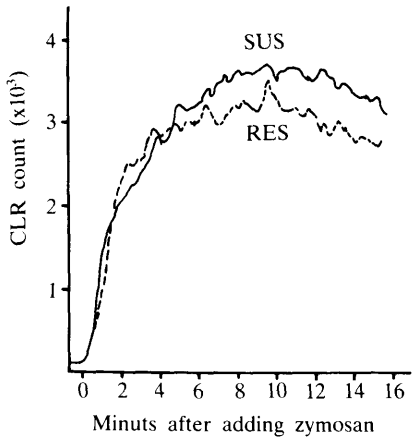

Fig. 4 Typical chemiluminescent response of macrophages from SUS and RES rats. One hundred μl of the peritoneal exudate adherent cells (5×10^5 cell/m*l*) and 100 μl of luminol solution (2×10^{-4} M) were mixed. After 2 min later, 100 μl of opsonized zymosan (12.5 mg/m*l*) was added.

cells were 45.6 in SUS and 103.3 in RES rats (IgM producing cells) and 36.7 in SUS and 130.6 in RES rats (IgG producing cells), respectively (Fig. 3). At the 7th day post-inoculation (Fig. 3), the mean PFCs per 10^7 spleen cells were 203.5 in SUS and 414.0 in RES rats (IgM), and 128.2 in SUS and 950.0 in RES rats (IgG), respectively.

The macrophages from both strains showed CLR by stimulation with opsonized zymosan. The typical CLR pattern was shown in Fig. 4. The mean peak counts of CLR in the macrophages from SUS and RES rats were 3416±460 and 2320±598 counts respectively. No significant difference was observed between these CLR counts.

DISCUSSION

In this study, the levels of almost all humoral factors analyzed, except for C3, were depressed in SUS in comparison with those in RES control rats, and the antibody response and the complement hemolytic activity in SUS rats were lower than those in RES rats. These results have something in common with the previous findings that the serum proteins of SUS rats differed from those of RES control in the number of components and in the concentrations of protein [9].

The low immunoglobulin levels in SUS rats at early age may be due to an immaturity of immunoglobulin-producing ability. Immune systems are known to be constructed by complicated interactions between the cells including macrophages and various subsets of lymphocytes. There are some interpretations for causes of immunodeficiencies, such as abnormalities of macrophages, B and T lymphocyte series and their interactions. Abnormality of macrophages was not observed in CLR assay in SUS although CLR is only one of the parameters in many macrophage functions. Mitogenic responses of the spleen cells from SUS rat have been reported that these responses are depressed to PHA and Con A at 1 and 1.5 months of age but not to LPS [12]. It may be possible that the low Ig levels in SUS rats are caused by the depressed functions of T lymphocytes which are responsive to these mitogens. However, further studies will need to clarify the macrophage-lymphocyte and lymphocyte-lymphocyte interactions in SUS rat.

The levels of these humoral factors except for IgA were found to be increase with aging in SUS rats, and there are two possible explanations for this. One is that the increase in immunoglobulin production is due to the maturation of immunological potency. This explanation is supported by the data in the present study that the levels of immunoglobulins and specific antibody production in SUS were significantly lower than that in RES control at 1 month of age. Furthermore, the previous study has indicated that the responses to mitogens recover to the normal level at 2 months and thereafter [12]. The subsequent elevations of immunoglobulin levels in SUS may be recog-

nized as the delayed maturation of immunological potency. The other is that the antigenic effects of accumulated plaque stimulated the immunoglobulin production in SUS rats. It is supported by the previous findings that the antibody against some of the bacterial strains forming the plaque in periodontitis has been demonstrated in the serum of patients [7] and that the plaque accumulation has been associated with an increase in immunoglobulin levels in serum and saliva [21–22]. Perhaps. the elevations might be caused by both maturational and reactive effects. Namely, immune abnormalities in SUS rats allow the bacterial growth in the gingiva at early age and the elevations of the levels of humoral factors in SUS rats are induced by the combined effects of antigenic stimulation with plaque and the immunological maturation process.

The IgA level in SUS rats remained at low levels throughout the period, and it is, therefore, suggested that some relationships exist between the depression of IgA production and occurrence of gingival plaque formation and inflammation in SUS rats.

Complement hemolytic activity in SUS rats was always lower than that in RES control while higher amounts of C3 levels were found in SUS rats than RES controls. This may suggest that SUS rats lack in one or more complement components (except for C3) or have some unbalanced regulation in the complement system. The complement system is a major humoral mechanism that contributes to protective responses against foreign substances and to damage to host tissue in periodontal disease [7, 19], and therefore the incompletion of this system may be associated with the onset of gingivitis.

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要　約

先天性歯肉炎感受性ラットの血清中の免疫グロブリンおよび補体量：磯貝　浩・磯貝恵美子・脇坂仁美・井藤信義・首藤文栄・石井　冬・高野一雄（東日本学園大学歯学部口腔解剖学教室・[1]口腔衛生学教室、[2]北海道大学獣医学部生化学教室）——歯肉炎自然発症（SUS）ラットおよび歯肉炎を起こさない対照（RES）ラットの血清中の免疫グロブリン，補体量およびマクロファージの活性を比較した。前歯肉炎段階の若齢SUSラットはRESラットに比べて，免疫グロブリン量が著明に低く，C3量および補体の溶血活性も低かった。SUSラットにおけるIgA量および補体の溶血活性は観察期間中低いままであったが，他の免疫グロブリン量は次第に増加した。2～7か月齢のSUSラットにおけるC3量はRESラットに比べて高くなったが，補体の溶血活性は常に低かった。羊赤血球に対する抗体応答はSUSラットにおいて抑制されていた。