Mini Review

Development of Useful Antibodies for Passive Immunotherapy against *Porphyromonas gingivalis* Infection

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Porphyromonas gingivalis 感染に対する有用な受動免疫用抗体の開発

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要旨：*Porphyromonas gingivalis* は歯周病の重要な原因菌であると考えられており，これまでの研究成果から本菌の病原因子を標的にした免疫療法は本疾患の予防に有用であると示唆されている。歯周組織に対する病原細菌の付着は定着，感染に不可欠であり本疾患の発症に重要であることから，本菌の付着因子に対する免疫療法は極めて重要である。歯肉縁下部位への歯周病原細菌の共凝集因子，赤血球凝集因子は重要な定着因子であることが明らかにされている。一般社会からは，歯周病が生命を奪う重要な疾患であることに対する認識は未だ深くないことから，免疫療法の実用化には完全な安全性を保証せねばならならない。その実現には安全性の高い受動免疫用抗体の作成が必要であろう。本稿では，歯周病の免疫療法の実現に向けてバイオテクノロジーを応用した安全性の高い受動免疫療法用抗体の作成戦略とその研究成果について紹介する。

キーワード：*Porhyromonas gingivalis*，単鎖可変部抗体，ヒト型モノクローナル抗体，鶏 IgY

Abstract：*Porphyromonas gingivalis* has been implicated as an important etiological agent associated with periodontal disease, and emerging evidence suggests that immunotherapy against virulence factors of this pathogen may provide disease protection. Bacterial adherence to periodontal tissues is a prerequisite for colonization, and one of the important steps in the disease process, thus immunotherapy against adherence molecules is considered to be vital. Bacterial coaggregation and hemagglutination are factors that likely play major roles in colonization in the subgingival area. Since the public at large may be skeptical about the seriousness of periodontal disease, any immunotherapy employed must be carried out with absolute safety. To achieve this goal, the development of safe antibodies for use in passive immunization is significant. Herein, we discuss recent salient advances in immunotherapy against periodontal disease and introduce some biotechnological approaches for developing safe passive antibodies. Nihon Shishubyo Gakkai Kaishi (J Jpn Soc Periodontol) 47：239—249, 2005.

Key words：*Porphyromonas gingivalis*, single chain variable fragment antibody, human type monoclonal antibody, chicken IgY

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Introduction

Active immunization strategies for periodontal disease have been developed, and several reports have described their effectiveness and potential in experimental animals[1]. However, it is unlikely that active systemic immunization against periodontal disease will be utilized in humans in the near future, as its safety for practical use has yet to be shown. Further, It is known that disadvantageous immune reactions may occur with certain vaccines that very rarely result in sudden death, even though they have been previously well tested and confirmed safe.

Passively transferred immunization involves use of a specific antibody for a particular antigen, so-called serotherapy, and may provide immediate protection. As for periodontal disease, since the primary pathological site is gingival tissue, intravenous injections of antibodies may not be necessary, as effective immunization might be achieved with a simple mouth rinse or local application of the specific antibody to the periodontal pocket.

However, development of passive immunization therapy for practical use against periodontal disease has not significantly progressed, because of difficulties associated with the establishment of useful animal models and systems to deliver antibodies to the subgingival area. Further, several clinically relevant questions regarding which bacterial species are important and the etiologic agents involved in periodontal diseases remain. When considering passive immunization against periodontal diseases, the pathological focus has been primarily on gingival tissues, and application of a specific antibody that neutralizes bacterial adhesion to gingival tissues could provide a practical and satisfactory treatment approach. Recently, such an approach was used against *P. gingivalis*. In the experiment, patients with periodontitis, who harbored *P. gingivalis* in their subgingival plaque, were treated with scaling, root planing and metronidazole to suppress any detectable *P. gingivalis,* then a monoclonal antibody (MAb) against *P. gingivalis* was applied to the periodontal pocket. That treatment significantly reduced the numbers of *P. gingivalis* in sites showing the most severe periodontitis for up to 9 months after application of the MAb[2].

Although passive immunization in the oral cavity is thought to be safe, side effects can arise with use of animal antibodies, which may stimulate unwanted immune reactions that induce inflammation with further periodontal tissue destruction. Thus, it is still necessary to develop safe neutralizing antibodies for practical use.

In this review, recent salient advances in immunotherapy against periodontal disease are discussed and biotechnological approaches for developing safe passive antibodies such as recombinant single chain variable fragment, human type monoclonal antibodies, and chicken IgY, are introduced.

Molecular targets of immunotherapy

Adherence of bacteria to host tissues is a prerequisite for colonization and an important factor in bacterial pathogenesis. We have selected 2 colonization factors, coaggregation factor and hemagglutinin, as targets of passive immunotherapy[3],

Coaggregation factor, which plays a role in colonization through aggregation with other oral bacteria. *P. gingivalis* is able to adhere to the surface of several Gram-positive bacteria including *Actinomyces viscosus* and various streptococci[4], and interbacterial adherence appears to be an essential step in the process of colonization by *P. gingivalis*. It is well known that an accumulation of bacteria on tooth surfaces follows a sequence, beginning with Gram-positive facultative species and later shifting to Gram-negative facultative and anaerobic species. Another property of the coaggregation factor is its relationship to pathogenicity, since more abscesses have been shown to be formed by co-aggregates of 2 strains, for example, *A. viscosus* and *Streptococcus mitis,* than those caused by infection with a pure suspension of each microorganism and coaggregated cells were found to be more resistant to phagocytosis and killing by neutrophils *in vitro* and *in vivo*[5].

We previously cloned the gene for a 40-kDa outer membrane protein (OMP) from *P. gingivalis* 381 and produced large amounts of recombinant protein[6,7]. A rabbit antiserum against the purified

recombinant protein reacted with a polypeptide of a similar size in the outer membrane fraction and in vesicles of *P. gingivalis* 381. Previous studies have shown that monospecific rabbit polyclonal antibody purified using recombinant 40-kDa OMP (r40 kDa-OMP) affinity column significantly inhibited coaggregation of *A. viscosus* cells with *P. gingivalis* vesicles. These results indicated that the 40-kDa OMP functions as a coaggregation factor of *P. gingivalis*[8] and also found that the 40-kDa OMP is conserved among many strains of *P. gingivalis*[9]. Using the affinity-purified antibody against r40-kDa OMP, we also confirmed that the antibody contributed to the killing of *P. gingivalis* 381 which was mediated by the complement system through both classical and alternative pathways[10]. Further, we demonstrated that the antibody exhibited an opsonic activity on human neutrophil function leading to phagocytosis of *P. gingivalis*[11]. More recently, we found that 40-kDa OMP is one of hemin-binding protein[12]. These findings suggest that 40-kDa OMP is unique and useful target of immunotherapy.

Hemagglutinin is a second potential target for immunotherapy. Hemagglutinins mediate adsorption and penetration of bacteria into host cells[13]. *P. gingivalis* hemagglutinin domains are encoded in protease genes, which are able to degrade a broad range of host proteins[14]. The multivalent hemagglutinins are also encoded by different genes, and it is thought that the hemagglutinins and protease genes may share similar hemagglutinin domain sequences in a multigene family[15]. These findings support the notion that the hemagglutinins are the most important targets for passive immunization and that it may be of interest to develop safe antibodies capable of neutralizing the hemagglutinating activity of *P. gingivalis*.

In a previous study, we established several hybridoma clones using vesicles as the immunogen and produced a MAb, designated mAb-Pg-vc, that strongly inhibited activities of hemagglutination[16] and hemolysis[17], and also recognized the 43-and 49-kDa bands in a Western-blot analysis. This immunoblotting profile was the same as that of the monospecific antibody, anti-HA-Ag 2, which is known to be a hemagglutinating adhesin of *P. gingivalis*[13]. Our finding indicated that mAb-Pg-vc recognized the same epitope involved in the functional domain of hemagglutinin. Next, we constructed a *P. gingivalis* genomic library and successfully cloned a gene encoding a 130-kDa hemagglutinin protein (130 kDa-Hag) using mAb-Pg-vc. The complete nucleotide sequencing of the 4.6-kb cloned DNA fragment revealed 2 open reading frames (ORFs), with the upstream ORF shown to be the putative gene and responsible for 130 kDa-Hag, while the downstream ORF was found to specify the insertion sequence gene *IS1126*. Since an insertion sequence (IS) has the ability to move on a chromosome together with adjacent genes, it is likely that this IS may help spread the gene encoding the hemagglutinin domain on the *P. gingivalis* chromosome[18].

Based on the results of our Western blot analysis of nested deletion clones, we performed binding assays of the phage-displayed peptide library using mAb-Pg-vc and competition experiments of hemagglutinating activity using synthetic peptides with purified r130 k-kDa-Hag, which identified PVQNLT as a functional stretch. Further analysis of the identified short motif using NCBI databases revealed 14 genes containing hemagglutinin associated domains. It is noteworthy that additional homology searches with the short motif indicated a similarity with the hemagglutinin domain HA 1 in the influenza virus. These findings suggest that *P. gingivalis* expresses multiple sequence related hemagglutinin-associated motifs, which encode a number of hemagglutinins and/or proteases. This gene structure seems ideal for growth of the bacterium in periodontal pockets, as it facilitates the availability of heme molecules as an iron source. Since bacterial cell attachment to erythrocytes is an important initial step for expressing hemolysis activity, we also examined the effect of mAb-Pg-vc on the hemolytic activity of *P. gingivalis* cells. The MAb significantly inhibited hemolytic activity, while the inhibition was reduced by the synthetic peptide corresponding to the 130-kDa HAG functional motif PVQNLT.

Together, these findings support the notion that hemagglutinins are the most important targets for passive immunization and confirm the importance

of developing safe antibodies that are capable of neutralizing the hemagglutinating activity of *P. gingivalis*.

Recombinant ScFv antibody

The IgG molecule consists of 2 heavy (H) and 2 light (L) chains, and the N-terminal end in both the H and L chains contains variable (V) regions. These V regions are known as VH and VL, and together they form the antigen-binding site of the V fragment (Fv) in Fab. The constant region Fc plays important roles in complement activation via binding of C 1 and in opsonization via binding to the Fc receptor. When considering the effectiveness and safety of antibodies for neutralizing virulence factors, Fv in Fab without Fc may avoid unwanted cellular immune responses and is a reasonable candidate for use as an antibody. The single chain variable fragments (ScFv) antibody, which consists of the Hand L chain variable fragment, is also an attractive molecule, because its small size allows faster delivery to tissues when compared to an ordinary MAb[19]. Further, an intact MAb may attach non-specifically to tissues via the constant regions. Thus, an ScFv antibody may be more advantageous due to its specific binding to pathogens.

To construct an ScFv antibody, we isolated mRNA from mAb-Pg-vc antibody-producing hybridomas, synthesized the cDNA corresponding to the VH and VL genes, amplified the genes by polymerase chain reaction (PCR) using immunoglobulin gene-specific primers or oligo dT primers, and then amplified the VH and VL genes that were interconnected by a short polypeptide linker, $(Gly_4 Ser)_3$. The short polypeptide linker ensured the functional expression of the VH and VL domains, and increased stability. Further, improvement of the vector with histidine hexamer tails attached to the ScFv fragment allowed for rapid purification using metal ion resin affinity chromatography. Our ScFv from hybridoma producing mAb-Pg-vc found to be secreted into the periplasmic space and then purified using an anti-E tag antibody affinity column from the periplasmic fraction. As expected, the ScFv significantly inhibited the hemagglutinating activity of *P. gingivalis* vesicles in a dose-dependent manner. Interestingly, a DNA sequence homology search with the ScFv gene showed that CDRs in the VH gene exhibited a high degree of homology with those in the VH gene of the MAb that recognized the antigenic site of the A/PR/8/34 influenza virus hemagglutinin. Compilation of these data may help clarify the stereochemistry between antibody CDRs and antigen epitopes, which could be critical for designing novel antibodies in the establishment of a passive immunization system[16](Fig. 1).

Although we succeeded in the construction of an ScFv capable of inhibiting hemagglutinating activity, the recovery rate and activity were not satisfactory. Eukaryotic proteins expressed in the *Escherichia coli* host often accumulate within the cells as insoluble protein aggregates or inclusion bodies. The recovery of structure and activity from inclusion bodies is a complex process, with efficient *in vivo* folding of functional proteins a major bottleneck of high-level production in the *Escherichia coli* host, and simple optimization protocols are not available. To accomplish the mass production of functional ScFv antibodies, we attempted to express the ScFv gene in a *Bacillus brevis* protein-producing system[20]. A chimera gene was constructed from the secretions of the leader peptide of the *B. subtilis* α-amylase gene and our ScFv gene, using a shuttle vector between *E. coli* and *B. brevis,* which was then transformed in the *B. brevis* host. As expected, the novel *B. brevis* transformant produced a large amount of ScFv protein that was secreted extracellularly, and the that protein, which was purified from conditioned culture fluid, significantly inhibited *P. gingivalis* hemagglutinating activity[21](Fig. 2).

Our ScFv protein may be capable of inhibiting *P. gingivalis* colonization onto gingival tissues as well as Fe ion intake, and might also have a potential to prevent periodontal diseases.

Human type monoclonal antibody

One of the major obstacles to the development of therapeutic applications using MAbs derived from animals for humans is their intrinsic immuno-

genicity. The full benefits of antibody therapy can only be properly assessed with a fully human MAb that can be administered repeatedly. To develop a safe regimen for passive immunization therapy, several methods have been developed for the production of human monoclonal antibodies (hMAbs), including Epstein-Barr virus (EBV) immortalization of antibody producing cells and hybridoma production using human immunoglobulin genes from transgenic mice.

EBV infects human B-lymphocytes via the membrane antigen CD 21 and immortalizes antibody producing cells[22]. However, B-lymphocytes that produce efficient hMAbs are difficult to obtain, since immunization of a human population for that purpose is not practical. In order to overcome this problem, we have used severe combined immunodeficiency (SCID) mice[23]. Most implanted human peripheral blood lymphocytes (hPBLs) are depleted by an immune response involving cytotoxic cells, mainly NK cells, in SCID mice. We administered anti-asialo GM 1 to SCID mice to reduce NK cell activity and then transferred hPBLs (Fig. 3).

We obtained hPBLs from a donor with a high serum level of antibodies against r40 kDa-OMP, and B cell clones were established using SCID mice and an EBV immortalization system. EBV-B cells that produced anti-r40 kDa-OMP antibodies were collected by panning using r40 kDa-OMP. IgG isotype clones, representing the IgG 1 and IgG 2 subclasses, were successfully isolated, and the clone showing the highest inhibitory activity against coaggregation was part of the IgG 2 subclass and named HAb-omp 1. The hMAb significantly inhibited the coaggregation activity of *P. gingivalis* vesicles to *A. naeslundii* cells. Next, the equilibrium binding constant of the interaction between HAb-omp 1 and r40 kDa-OMP was examined using a Fluorescence Polarization System, and the Kd value of the binding was determined from the fitted curve to be 4.4 nM, which is similar to the value for mouse MAbs[24].

We highly purified recombinant 130-k HMGD using an electro-osmotic medium pump system for preparative disc gel electrophoresis[25]. Human lymphocytes were isolated from a donor who had a high antibody titer against purified recombinant 130-k HMGD and immortalized with EBV, after

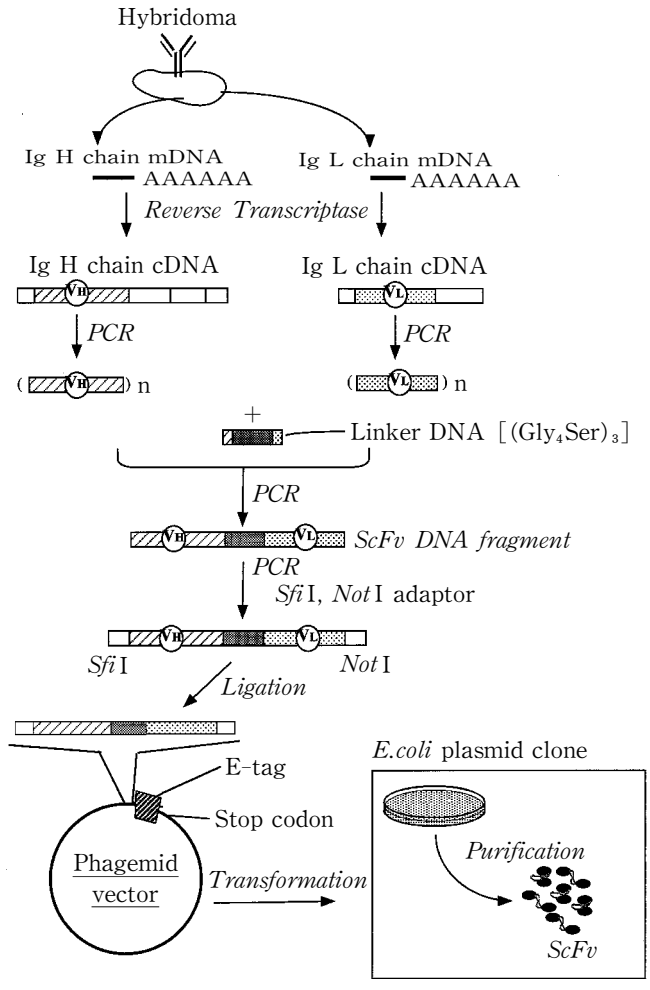

Fig. 1 Strategy for molecular cloning of VH and VL genes and construction of ScFv

which specific antibody producing B cells were established by panning using purified r130-k HMGD. The constructed hMAb-HMGD 1, which was of the IgG subclass, recognized r130 k HMDG as well as the 43-and 49-kDa major bands in *P. gingivalis* cells and vesicles, and significantly inhibited the hemagglutinating activity of *P. gingivalis* vesicles in a dose dependent manner. Further, the HuMAb-HMGD 1 recognized the synthetic peptide, EGSNEFAPVQNLTGSSVG, which contained the functional domain of 130-k HMDG[26].

One of the most appealing approaches for constructing hMAbs is establishment of a mouse line engineered with human immunoglobulin genes capable of producing a large repertoire of human antibodies in the absence of mouse antibodies. This

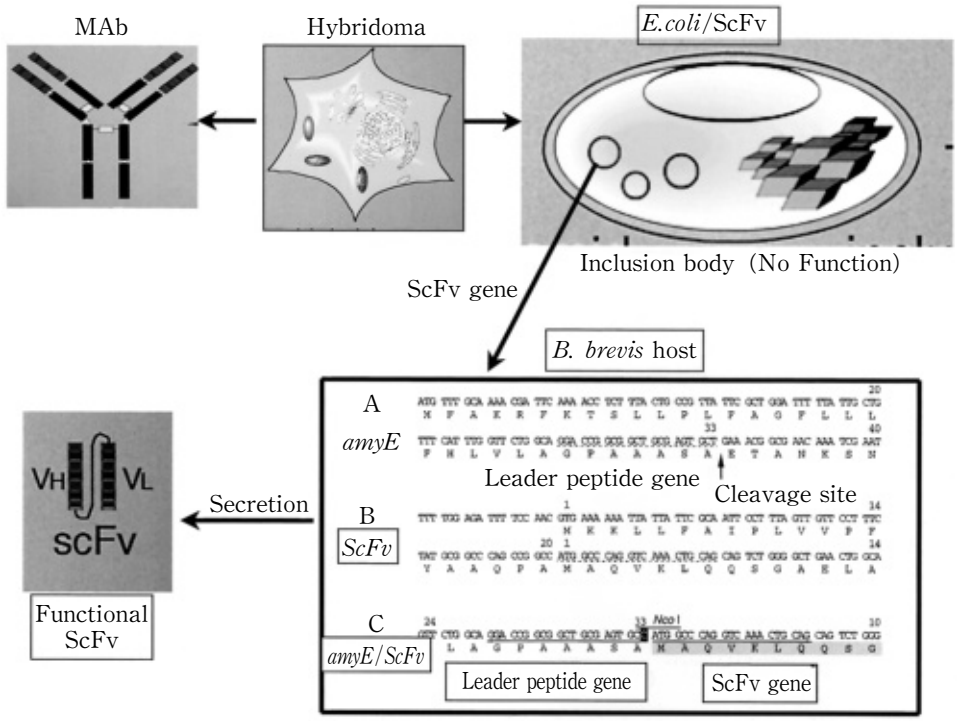

Fig. 2 ScFv gene cloning into B. brevis host. The leader peptide gene of *B. subtilis* α-amylase was joined with ScFv gene under adjusted triplet flame codon, and inserted into a shuttle vector between *E. coli* and B. brevis, which was then transformed in the *B. brevis* host

novel strategy involves inactivation of mouse immunoglobulin genes, and then introduction of human H and J chain loci into the mouse germline. Large fragments of human H and kappa L chain loci cloned on yeast artificial chromosomes have been introduced into mouse germlines via fusion of yeast spheroplasts with mouse embryonic stem (ES) cells. These fragments, which contain structural genes for the variable, joining, diversity, and constant domains as well as critical regulatory elements, proved to be sufficient for the production of a broad repertoire of human immunoglobulins and expressed significant levels of human antibodies in mice named Xenomice[27] (Fig. 4).

We also produced hMAbs capable of neutralizing the hemagglutinin activity of *P. gingivalis* by using Xenomice. Interestingly, our successfully constructed hMAb for IgG 1 showed inhibitory activity against the hemagglutinating activity of *P. gingivalis* vesicles[28].

Another transgenic line harboring a human Ig gene cluster is TranChromo mice, which have 2 transmittable human chromosome fragments, one containing the Ig heavy chain locus and the other the kappa light chain locus. These 2 human chromosome fragments were introduced into a mouse strain whose endogenous IgH and Igkappa loci were inactivated. In the resultant mice, a substantial proportion of the somatic cells retained both human chromosome fragments, and recovery from the defect in Ig production was shown by high levels of expression of human Ig heavy and kappa chains in the absence of mouse heavy and kappa chains[29] (Fig. 5).

We immunized human Ig-producing TransChromo mice with r 40 kDa-OMP and their spleen cells were fused with a mouse myeloma cell line. From that, we successfully constructed an hMAb that promoted phagocytosis of *P. gingivalis* by neutrophils[30]. Such novel hMAbs may be useful to develop a passive immunotherapy against periodontal disease through opsonic clearance of *P.*

gingivalis infection.

Chicken IgY antibody

Following immunization of hens with an antigen, specific antibodies have been induced and transported to the egg yolk, from which antibodies (IgY) can be purified in large quantities. Recently, the production of a specific antibody in chickens and extraction of IgY from egg yolks are attracting interest from the viewpoint of passive immunotherapy[31]. Chicken eggs are normal dietary components, thus there are no toxic side effects, and IgY Fc has no ability to activate the complement and opsonization systems. As compared to IgG, a chicken IgY production system is advantageous, because of the higher amount of IgY produced by a single hen in 1 year, which ranges from 20 to 40 g more than animal IgG production. Previous studies have shown that antibodies are actively transported to the egg yolk of an immunized chicken, thus the use of IgY for passive immunization avoids the need to bleed animals for antibody preparation. Since the amount of IgY obtained from an egg yolk is sufficient, hyper-immunized hens may be used as a convenient and economic method for passive immunization strategies (Fig. 6).

We immunized a highly purified r130-k HMGD to chickens, and isolated IgY recognized r130 k HMDG as well as the 43-and 49-kDa major bands in *P. gingivalis*, and significantly inhibited the hemagglutinating activity. Further, the IgY recognized the synthetic peptide, EGSNEFAPVQNLTGSSVG, which contained the functional domain of 130-k HMDG[32].

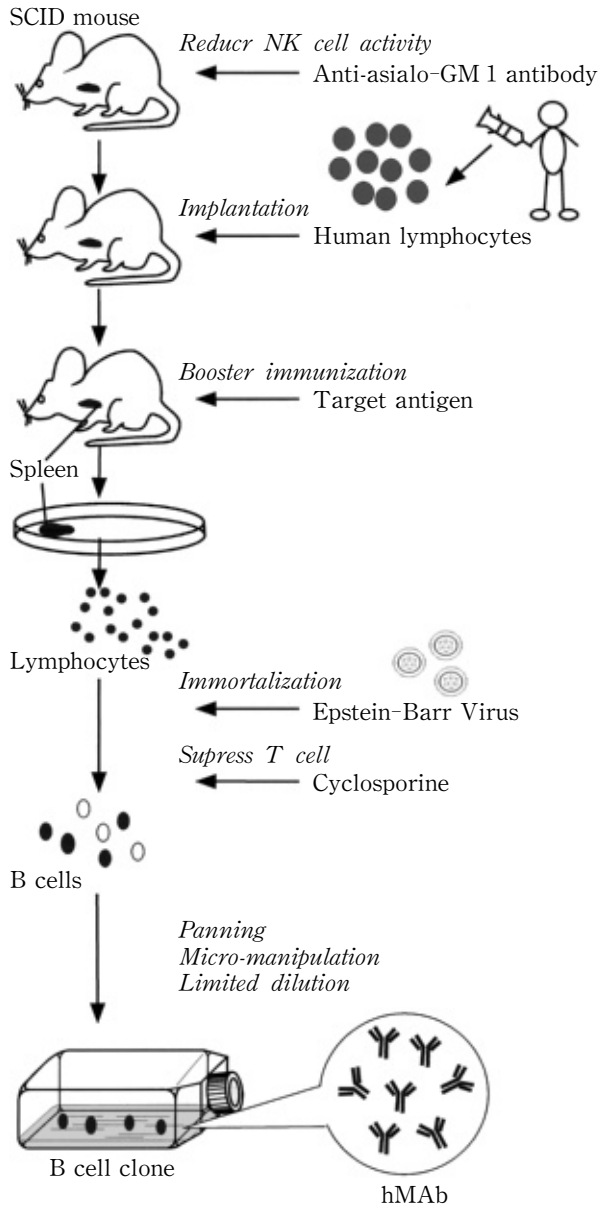

Fig. 3 An outline of the strategy to generate Hu-mAb using SCID mouse and EBV immortalization

Conclusions

Recently, the term periodontal medicine has come to mean the study of the contribution of periodontal infections toward systemic diseases such as atherosclerosis, myocardial infarction, stroke, diabetes, low birth weight, and osteoporosis. Thus, it is considered that prevention of periodontitis is relevant for both oral and systemic health. From now, dental researchers and practitioners must take a greater responsibility to enlighten general society regarding the development of new treatment methods, as well as effective strategies to prevent periodontal disease.

Herein, we have introduced some passive immunotherapy strategies that utilize the development of useful antibodies against *P. gingivalis*. Development of a recombinant antibody, ScFv, now makes it possible to mass-produce ScFv by its expression

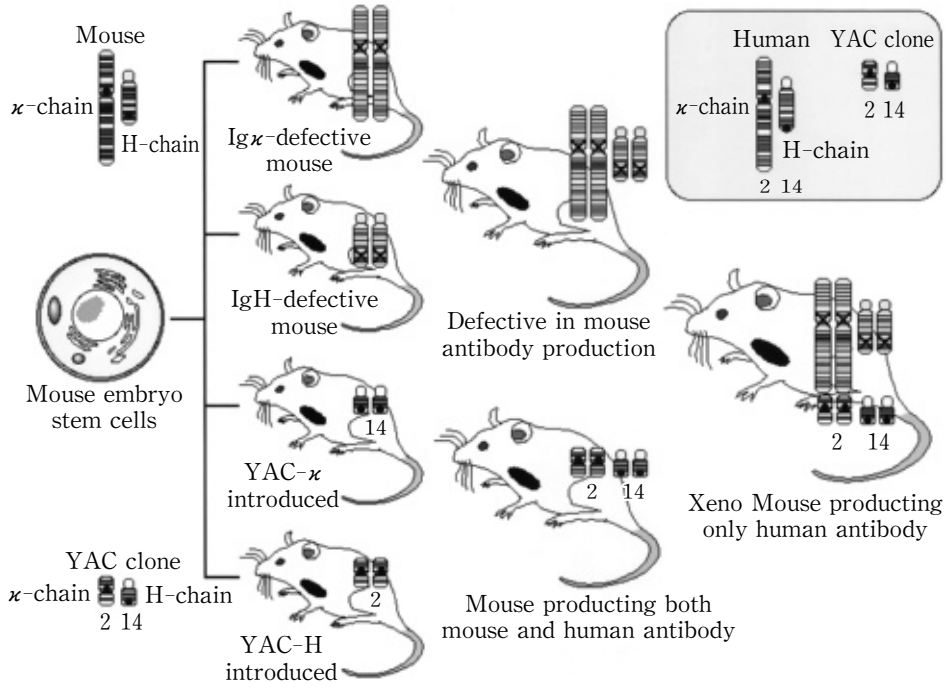

Fig. 4 The strategy for construction of human IgG gene transgenic mice, Xenomice

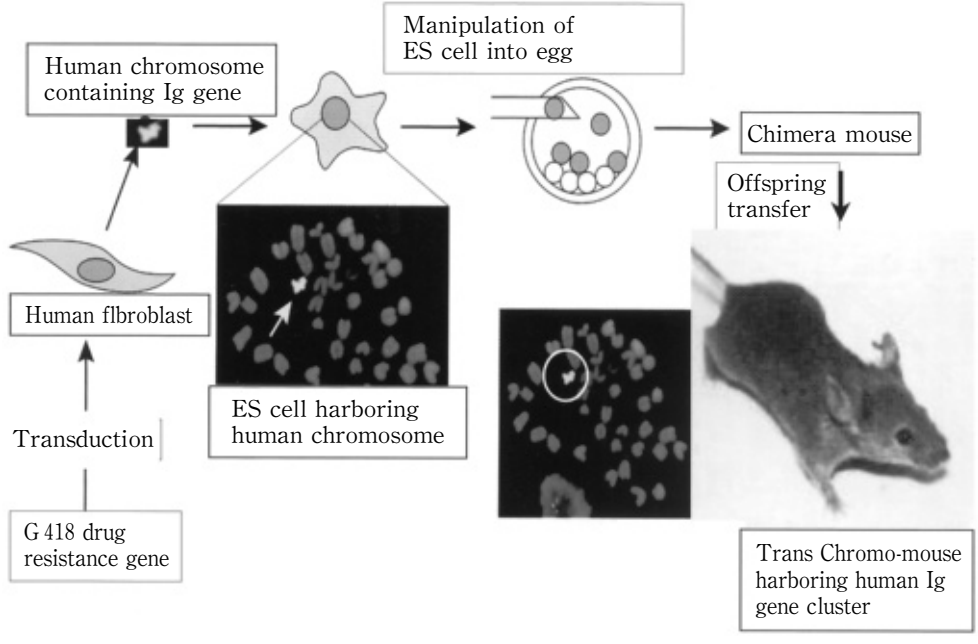

Fig. 5 The strategy for construction of human entire Ig gene transgenic mice, Trans-Chromo-mouse

and secretion in a *B. brevis* system. It is possible that human MAbs can be produced using a large quantity of ScFv with the recombinant technology introduced here. However, chicken IgY also has the potential for mass production for human use.

All antibodies developed in the future must be useful and safe, as well as show a potential for practical use in passive immunotherapy against

periodontal disease by neutralizing *P. gingivalis* colonization. Although efforts have been made to develop effective immunotherapies against periodontal disease, it is a matter of great importance to ensure that safety is provided along with effective protection. Further, as a result of the increased commercial demand for safe therapeutic antibodies, an efficient and low cost production process is essential. It is doubtful that an active systemic immunization method against periodontal disease can be put into practical use in humans in the near future, because of safety concerns. However, investigations must continue and the goal of achieving a practical active immunization strategy against periodontal diseases maintained.

It is thought that treatment of the local immune system using mucosal immunotherapy is safer than active systemic immunization. The mucosal immune system functions to protect mucous membranes against colonization and invasion by pathogens, thus preventing the harmful immune responses to those antigens, if they reach the body interior. However, despite the many attractive features of mucosal vaccination, it is often difficult to stimulate strong secretory IgA immune responses and protection. As for specific adjuvants, the best-studied and most potent mucosal adjuvants in experimental systems are cholera toxins.

We assessed the efficacy of the 40-k OMP of *P. gingivalis* in a nasal vaccine. Mice were nasally immunized with the 40-k OMP using cholera toxin as an adjuvant and significant levels of 40-k OMP specific serum IgG 1, IgG 2 b, and IgA as well as mucosal IgA in saliva and nasal secretions were observed. Further, the 40-k OMP-specific IgG significantly inhibited the coaggregation activity of *P. gingivalis*[33]. Transcutaneous immunization, a topical vaccine method, combines the advantages of needle-free delivery with targeting of the immunologically rich milieu present in skin. In animal studies, this simple technique has been shown to induce robust systemic and mucosal antibodies against vaccine antigens[34]. The development of a transcutaneous 40-k OMP vaccine for human use may be a significant milestone in the quest for an effective vaccine. In assessing the efficacy of transcutaneously administered 40-k OMP, the 40-k OMP alone induced significant 40-k OMP-specific IgG responses in both serum and saliva samples, while the OMP plus cholera toxin used as an adjuvant further increased the levels of IgG responses and induced 40-k OMP-specific serum IgA. Those 40-k OMP-specific IgG induced by the transcutaneous administered vaccine also significantly diminished coaggregation activity of *P. gingivalis*[35]. These findings indicate that mucosal and transcutaneous vaccines using appropriate target antigens from *P. gingivalis* may be feasible for human immunization, and effective for the prevention of periodontal diseases.

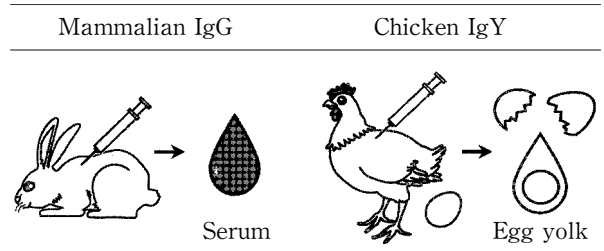

Fig. 6 Comparison of mammalian IgG and chicken IgY

In 1991, the National Institute of Dental Research sponsored a workshop titled "Genetically Engineered Vaccines : Prospect for Oral Disease Prevention," which focused on important topics such as, oral diseases and host immune response, vaccines and the mucosal immune system, optimizing mucosal and systemic immune responses, delivery systems, targeted antigen selection, and vaccine development. Although 15 years have passed since this meeting, immunotherapy against periodontal disease remains a goal to be achieved. It is a matter of great importance to ensure safety along with effective protection. Further, as a result of increased commercial demand for safe therapeutic antibodies, there is a need for an efficient and low cost production process. The successful development of immunotherapy against periodontal disease requires a concerted effort by industry, government, and dental society.

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