than were counterpart measures (Environic Research Group Ltd., 2001). Therefore, visible oral symptoms of smokers likely afford potential with respect to prevention and cessation of smoking.

The present study was the first to demonstrate association of lip pigmentation with smoking and melanin pigmentation in the gingiva; thus, additional investigations involving a pathological approach and employing various variables as possible confounders of smoking are required. The striking relationship between exposure of smoking and the visible symptom of pigmentation in oral and perioral conditions could potentially influence not only smoking but also oral health behaviors due to increasing awareness of oral health.

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Table 1. Distribution of subjects with or without melanin pigmentation in lip and gingiva by smoking status

		Smoking status				
	Pigmentation	Never	Current	Subtotal	Former	Total
Lip	No pigmentation	39(67)	19(33)	58(100)	12	73
	Pigmentation	34(27)	93(73)	127(100)	16	150
Gingiva	No pigmentation	59(73)	22(27)	81(100)	14	95
	Pigmentation	14(13)	90(87)	104(100)	14	118
	Total	73(39)	112(61)	185(100)	28	223

Distributions of lip and gingival pigmentation were significantly associated with smoking status (P<0.0001). Former smokers were excluded.

Table 2. Comparisons in score, prevalence and odds ratio (OR) and 95% confidence interval (CI) of lip and gingival pigmentation by levels of exposure to smoking.

Levels of exposure (N)	Lip pigmentation			Gingival pigmentation		
	Score	Prevalence (%)	OR (95% CI)	Score	Prevalence (%)	OR (95% CI)
Smoking status						
Never (73)	1.1±1.3	47	1.0(reference)	0.5±1.2	19	1.0 (reference)
Former (28)	1.0±0.9*	57	1.4(0.6-3.5)	1.8±2.2*	50	4.5 (1.7-12.0)
Current (112)	2.1±1.3*	83	5.6(2.8-11.1)	4.6±3.0*	80	17.0 (8.1-36.0)
Daily consumption (pieces)						
1-19 (37)	1.8±1.4*	76	3.9(1.6-9.7)	4.5±3.3*	76	13.5 (5.2-35.3)
20 (58)	2.0±1.3*	85	6.0(2.5-14.0)	4.6±2.9*	83	20.4 (8.3-50.6)
>20 (17)	2.8±1.3*	94	16.4(1.3-132)	4.8±3.0*	82	20.5 (4.9-85.0)
Correlation	r=0.380 p<0.0001			r=0.594 p<0.0001		
Duration of smoking (years)						
1-9 (40)	1.6±1.4	70	3.6(1.4-9.1)	3.4±2.9*	70	9.5 (3.4-26.7)
10-19 (36)	2.2±1.3*	89	8.9(2.9-27.9)	5.3±2.9*	86	27.2 (8.9-84.6)
>19 (36)	2.4±1.2*	92	9.0(2.2-37.4)	5.2±2.9*	86	37.0 (8.5-160)
Correlation	r=0.377 p<0.0001			r=0.640 p<0.0001		
Lifetime exposure (piece-years)						
1-199 (46)	1.7±1.4	72	3.8(1.6-9.2)	3.7±3.1*	72	10.9 (4.1-28.7)
200-399 (34)	2.1±1.2*	88	8.0(2.5-25.2)	5.6±2.8*	88	33.3 (9.8-113)
>399 (32)	2.5±1.2*	94	13.3(2.6-66.8)	4.8±2.8*	84	33.5 (7.8-143)
Correlation	r=0.387 p<0.0001			r=0.632 p<0.0001		

N; Number of subjects, *Significantly higher than that of never smokers

Table 3. Contingency table by score of pigmentation between lip and gingiva for current and never smokers.

Lip	Gingiva								
	Current smokers (p<0.0001)					Never smokers (p=0.1728)			
	0	1-3	4-6	7,8	Total	0	1-3	4-6	Total
0	17	1	1	0	19	38	1	0	39
1,2	4	15	24	22	65	16	7	2	25
3-6	1	1	7	19	28	5	3	1	9
Total	22	17	32	41	112	59	11	3	73

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Detection of heat shock proteins but not superantigen by isolated oral bacteria from patients with Behcet's disease

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We isolated bacteria from periodontal sites and mixed saliva in eight patients with Behcet's disease and surveyed them to determine whether they produced heat shock proteins (HSPs) and superantigen. Cultivable bacterial compositions from periodontal sites and saliva were examined by anaerobic culture using blood agar plates. Gram-negative anaerobic rods such as *Prevotella intermedia, Fusobacterium nucleatum*, and *Capnocytophaga* species were predominant in the isolates from the subgingival plaque samples. The *Streptococcus mitis* group was the most common type isolated from the saliva samples. To detect the production of HSPs, Western blot analyses were performed using a polyclonal rabbit antibody to *Escherichia coli* DnaK and a monoclonal antibody to *Helicobacter pylori* Gro-EL. Sonic extracts of 27 of the strains (79.4%) reacted with the antibody against *E. coli* DnaK. Nine of these 34 strains (26.5%) were found to produce HSPs that reacted with antibody to *H. pylori* Gro-EL. A total of 54 isolates were examined for superantigen activity against human peripheral leukocytes. Twenty-five gram-negative clinical strains isolated from chronic periodontitis lesions and 20 ATCC strains of microorganisms were also examined. We could not detect any superantigen activity in 500× diluted supernatant of the strains isolated from the eight patients with Behcet's disease. The present study indicates that the anaerobic strains isolated from the oral cavity of these patients produce HSPs, the production being related to Bechet's disease.

Key words: Behcet's disease; heat shock proteins; oral anaerobe; superantigen

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Behcet's disease is an inflammatory disorder of unknown etiology characterized by recurrent oral aphthous ulcers, genital ulcers, uveitis, and skin lesions (14, 27). Microbial infections have been implicated in its development (14, 18). Herpes simplex virus DNA and serum antibodies against the virus have been found in a higher proportion of patients with Behcet's disease than in controls (14). The *Streptococcus mitis* group has been suggested as a causative agent, because these bacteria and the antibodies against them are frequently found in the oral flora and serum, respectively, of patients with the disease (16, 22, 23). However, none of these infectious agents has been proven to cause Behcet's disease.

It is well known that microbial infections can moderate host responses. The production of superantigens and heat shock proteins (HSPs) by infected microorganisms is closely associated with such changing host responses (3, 6, 34, 35, 37, 38). A series of studies has led to the hypothesis that specific antigens including superantigen and HSPs of microorganisms may trigger cross-reactive immunopathologic responses in patients with Behcet's disease (5, 9, 10, 15, 26, 31, 32). In fact, several oral bacterial species including the *S. mitis* group (16, 22, 23), *Prevotella intermedia* (20), *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (7) have been shown to produce superantigens that stimulate T cells to

proliferate nonspecifically through interaction with class II major histocompatibility complex products. HSPs are highly conserved immunogenic proteins that are often immunodominant antigens produced in bacteria and mammalian cells by a variety of stresses (37, 38). Many research groups have indicated that HSPs have been implicated in the etiology of Behcet's disease (5, 9, 15, 19, 26).

In the present study, we investigated the bacterial composition of the subgingival plaque and saliva collected from patients with Behcet's disease and determined the levels of superantigen and HSP production by the oral bacteria isolated from these patients.

Materials and methods

Subjects

After their informed consent was obtained, eight patients (five men and three women) with Behcet's disease who consulted the Second Hospital of Tokyo Women's Medical University, School of Medicine for periodontal treatment were enrolled in the present study. The mean patient age was 44.4 years (32–61 years). These patients had not received any antibiotics within the previous 4 weeks.

Sampling of dental plaque and saliva

Sites for sampling subgingival dental plaque were isolated with sterilized cotton rolls. Subgingival plaque samples were collected with a sterilized scaler. Mixed saliva samples were also obtained. All samples were immediately transferred into 0.9 ml RTF transport medium (29), diluted in RTF from 10^{-1} to 10^{-7}. Then 100 µl aliquots of each dilution were incubated on Tryptic soy agar plates (BBL Microbiology Systems, Cockeysville, MD) with 10% horse blood, hemin (5.0 µg/ml), and menadione (0.5 µg/ml) and incubated in an anaerobic chamber containing 10% CO_2, 10% H_2, and 80% N_2 for 7 days.

Identification

Colonies from an appropriate number of plates were picked, purified by repeated transfer, and characterized. Colony morphology, gram-staining, cell morphology, motility, and aerobic growth were checked. Biochemical tests included indole production, esculin hydrolysis, nitrate reduction, gelatinase activity, catalase, and fermentation of glucose, lactose, sucrose, cellobiose, and mannitol and the BAPNA test for trypsin-like enzyme described by Loesche (21). The enzymatic activity of the examined strains was also evaluated by the API ZYM system (Bio Merieux S.A. Marcy-L'Etoile, France).

Assay for superantigen production

The cultured supernatants of the isolated strains were examined for the production of superantigen. Twenty ATCC strains of *Actinomyces naeslundii* 15987 and 12104, *Actinomyces israelii* 12102, *Propionibacterium acnes* 11827 and 11828, *A. actinomycetemcomitans* 43718, 33384 and 29523, *P. intermedia* 25611, *Prevotella nigrescens* 33563, *P. gingivalis* 33277 and 53977, *Fusobacterium nucleatum* 25586, *Campylobacter rectus* 33238, *Tannerella forsythia* 43037, *Eikenella corrodens* 23834, and *Treponema denticola* 33521 and 35405, *Mycoplasma penetrans* 15845, and *Mycoplasma buccae* 14851 were also included in this experiment. The Tryptic soy broth culture media for gram-positive and gram-negative bacteria, the TYGVS broth for *T. denticola* strains described in our previous paper (12) and *Mycoplasma* broth (BBL Microbiology Systems) for *Mycoplasma* species were used. Culture supernatants were filtered through 0.1 µm sterile Millex-VV filters (Millipore, Bedford, MA) and evaluated for superantigen activity. The assay for superantigen production was carried out as described by Uchiyama et al. (35). Staphylococcus enterotoxin A (SEA; Toxin Technology, Inc., Miami, FL) was used to stimulate lymphocyte culture as a positive control at a concentration of 400 ng/ml. For assay purposes, the appropriate concentration of SEA as a positive control or broth alone as a negative control was added to the cells. Heparinized venous blood (30–50 ml) from healthy volunteer donors was diluted with phosphate-buffered saline (PBS) and layered over Ficoll–Conray medium containing 100 g of Ficoll 400 medium (Amersham Biosciences, Piscataway, NJ) and 240 ml of Conray 400 medium (66.8% w/v, Daiichi Seiyaku, Tokyo, Japan) per liter, a mixture whose density had previously been adjusted to 1.077 µg/ml with saline. The tubes were centrifuged for 30 min at 500 × g at room temperature. Leukocytes were harvested from the Ficoll–Conray interface and washed twice to remove the Ficoll medium with Hanks' solution (Nissui, Tokyo, Japan) containing 2% fetal calf serum. Cells were collected by centrifugation at 200 × g for 10 min at 4°C, and then resuspended in the RPMI 1640 culture medium with the supplementation of 100 µg/ml streptomycin and 100 IU/ml penicillin. For the superantigen assay, cells were distributed at 1×10^5 viable cells per well in 96-well round-bottomed microplates (Iwaki, Chiba, Japan). After incubation at 37°C in a humidified environment containing 5% CO_2 for 2 days, the cultures were pulsed with methyl-^{3}H thymidine (1 µCi/well) for the last 16 h of incubation, and thymidine incorporation was determined in a liquid scintillation counter. The data are presented as averages of triplicate cultures.

Heat shock protein production of isolates from Behcet's patients

To detect the production of HSPs, Western blot analyses were performed using polyclonal rabbit antibody to *Escherichia coli* DnaK (Hsp 70, Upstate Biotechnology Inc., Waltham, MA) and a monoclonal antibody to *Helicobacter pylori* Gro-EL (Hsp 60, Wako Pure Chemical Industries, Ltd, Osaka, Japan). Cells grown in each medium were harvested by centrifugation and washed twice with PBS (pH 7.2). The cell suspension was homogenized with a sonicator (Branson, Danbury, CT) at 100 W for 5 min on ice, and the supernatants were used in the experiment. These sonic extracts of bacterial strains were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Towbin et al. (33). Separated proteins by SDS-PAGE were transferred to PVDF membranes, and the membranes were then washed twice and incubated with rabbit anti *E. coli* DnaK antibody or mouse anti-*H. pylori* Gro-EL antibody. After washing, the membranes were reacted with peroxidase conjugated goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody. The peroxidase reaction was initiated with Tris buffered saline containing 4 methoxy-1-naphthol and 0.02% H_2O_2.

Results

Bacterial flora of dental plaque and saliva

The bacterial composition of samples obtained from eight patients with Behcet's disease were examined by culture on blood agar plates in an anaerobic chamber. The cultivable compositions of the subgingival plaque and mixed saliva are summarized in Tables 1 and 2, respectively.

The predominant strains from the subgingival plaque samples were groups of *S. mitis* and *Streptococcus salivarius*. Isolated strains of black-pigmented colonies on blood agar in subgingival plaque

Table 1. Comparison (%) of anaerobically cultivable bacteria in samples of subgingival dental plaque obtained from patients with Behcet's disease

	Patients							
Nos.	1	2	3	4	5	6	7	8
Age	37	42	40	51	41	51	32	61
Sex	F	M	M	M	F	M	M	M
Bacterial species in dental plaque	Detection rate (%)							
Streptococcus mitis group	20.2	21.4	9.1	20.1	1.9	–	26.1	17.5
Streptococcus salivarius group	19.0	8.6	12.0	–	–	–	4.3	10.0
Streptococcus mutans group	–	–	–	–	–	–	4.3	–
Actinomyces naeslundii	3.7	10.0	6.0	6.7	–	23.0	13.0	5.0
Actinomyces israelii	–	–	–	–	4.5	–	–	–
Porphyromonas gingivalis	–	–	4.0	20.8	–	–	–	–
Prevotella intermedia	5.1	4.3	6.8	5.4	1.0	–	–	–
Prevotella melaninogenica	–	–	0.9	10.7	22.2	–	8.7	7.5
Prevotella denticola	–	–	–	–	–	3.8	–	–
Prevotella species	–	7.1	6.4	–	–	11.5	–	12.5
Fusobacterium species	8.9	–	1.6	–	–	15.4	–	–
Fusobacterium nucleatum	–	2.8	2.0	7.3	–	7.7	4.3	–
Capnocytophaga species	7.6	7.1	1.5	–	11.2	–	4.3	5.0
Leptotrichia buccalis	–	2.9	2.9	1.0	2.9	7.7	–	–
Eikenella corrodens	–	2.9	–	2.6	–	–	–	10.0
Selenomonas sputigena	–	–	5.0	–	–	–	4.3	–
Veillonella species	1.3	2.9	4.0	7.9	4.0	–	–	–
Unidentified	34.2	30.0	37.8	17.5	52.3	30.9	30.7	32.5

Table 2. Comparison (%) of anaerobically cultivable bacteria in samples of mixed saliva obtained from patients with Behcet's disease

		Patients							
Nos.		1	2	3	4	5	6	7	8
Age		37	42	40	51	41	51	32	61
Sex		F	M	M	M	F	M	M	M
Bacterial species in dental plaque	Detection rate (%)								
Streptococcus mitis group		30.3	25.0	4.7	14.3	10.6	–	77.5	70.0
Streptococcus salivarius group		22.2	21.7	68.1	7.1	9.1	8.8	–	10.0
Veillonella species	–	–	–	7.1	–	–	–	–	
Actinomyces naeslundii		–	–	–	14.3	18.2	–	–	–
Fusobacterium nucleatum		3.0	–	–	–	–	–	–	10.0
Capnocytophaga species		2.0	–	–	7.1	–	–	–	–
Prevotella intermedia	–	–	–	–	–	2.9	–	–	
Prevotella species	–	–	4.7	–	–	23.5	3.8	–	
Selenomonas sputigena		–	–	–	–	–	–	3.8	–
Unidentified	42.5	53.3	22.5	50.1	62.1	64.8	14.9	10.0	

samples from Behcet's patients were identified as *P. intermedia*, *Prevotella melaninogenica* and *P. gingivalis*. However, many of the isolated strains of gram-negative short rods could not be identified by the biochemical characteristics surveyed in this study. More than 10% of the total colony forming units (CFUs) in subgingival samples from patient Nos. 2, 3, 4, 5, 6, and 8 were *Prevotella* species. In addition, we found more than 20% of *P. gingivalis* in the samples obtained from patient No. 5. Genus *Fusobacterium*, including *F. nucleatum* and *Capnocytophaga* species, were also isolated from almost every patient at a moderate detection rate. Most of the isolates in the saliva samples were *Streptococcus* species, mainly groups of *S. mitis* and *S. salivarius*. We could not identify all of the strains isolated from each sample.

Superantigen production

We examined 54 strains isolated from the oral cavities of eight patients with Behcet's disease for their ability to produce superantigen using healthy volunteers' lymphocytes. These 54 strains included 8 strains of the *S. mitis* group, 6 strains of the *S. salivarius* group and 3 strains of the *Streptococcus mutans* group, 4 strains of unidentified *Streptococcus* species, 2 strains of *Veillonella* species, 6 strains of *A. naeslundii*, one strain of *A. israelii*, 2 strains of *P. gingivalis*, 4 strains of *P. intermedia*, 2 strains of *Prevotella denticola* and an unidentified *Prevotella* species, 6 strains of *F. nucleatum*, 4 strains of unidentified *Capnocytophaga* species, 2 strains of *Leptotrichia buccalis*, and one strain each of *E. corrodens* and *Selenomonas sputigena*. The superantigen production was examined using 50× and 500× dilution of the culture supernatant from each culture. The 50× diluted culture supernatant sample from some strains of *Streptococcus* species induced weak thymidine incorporation. However, repeated examinations using 500× diluted culture supernatant sample failed to find superantigen activity in strains isolated from the oral cavities of the eight Behcet's patients.

We also examined the production of superantigen by 25 gram-negative clinical strains isolated from chronic periodontitis lesions and 20 ATCC strains. No superantigen production was found in strains of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *C. rectus*, *E. corrodens*, *F. nucleatum*, *P. intermedia*, *T. denticola*, *T. forsythia*, *M. buccae* and *M. penetrans*.

Production of HSPs by isolated bacterial strains

The HSPs produced that reacted with the polyclonal antibody against *E. coli* DnaK (HSP 70) and with the monoclonal antibody against *H. pylori* Gro-EL (HSP 60) are summarized in Table 3. The strains examined were 4 strains of *P. intermedia*, 3 strains of *F. nucleatum*, 2 strains each of *S. sputigena* and *L. buccalis, and* one strain each of *P. denticola, P. gingivalis*, and *E. corrodens*. Ten unidentified strains of *Prevotella* species, 5 strains of unidentified *Streptococcus* species, 3 of *Capnocytophaga* species, 1 of *Veillonella* species, and 1 unidentified gram-negative rod were also examined. Twenty-seven strains (79.4%) reacting with anti-*E. coli* DnaK and 9 strains (26.5%) reacting with anti-*H. pylori* Gro-EL were found among 34 strains in the present study. We confirmed these results with repeated Western blot analysis.

Discussion

Oral ulceration is usually an initial symptom and is seen in all patients at some time in the reported clinical course of Behcet's disease (25). To clarify the relationship between the oral ulceration and oral bacterial interactions, including the immunopathologic factors, we attempted to isolate

Table 3. Reactivity of bacterial strains isolated from samples of subgingival plaque and mixed saliva from patients with Behcet's disease against HSPs of *E. coli* DnaK and *H. pylori* Gro-EL

	Number of positive strains/Number of examined strains	
Bacterial species	anti-*E. coli* DnaK	anti-*H. pylori* Gro-EL
Prevotella intermedia	3/4	1/4
Prevotella denticola	0/1	0/1
Unidentified *Prevotella* species	10/10	4/10
Porphyromonas gingivalis	0/1	0/1
Eikenella corrodens	1/1	0/1
Fusobacterium nucleatum	2/3	0/3
Unidentified *Capnocytophaga* species	2/3	1/3
Leptotrichia buccalis	2/2	0/2
Selenomonas sputigena	1/2	2/2
Unidentified *Veillonella* species	1/1	0/1
Unidentified gram-negative rod	1/1	0/1
Unidentified *Streptococcus* species	4/5	1/5
Total	27/34	9/34

and detect superantigen-producing anaerobic bacteria in subgingival dental plaque and saliva from patients with Behcet's disease. We isolated 620 strains from eight patients. Based on gram-staining, cell morphology, biochemical properties, and the results of the anaerobic API enzymatic kit, we identified most of the isolated strains at mainly the genus level. The anaerobic bacterial compositions of the subgingival plaque and saliva are listed in Tables 1 and 2, respectively. We obtained the subgingival plaque from inflammatory gingival sites but for ethical reasons did not record the periodontal status with probes or X-ray photographs. The predominant cultivable bacteria in the subgingival plaque samples were gram-negative short rods. A comparison of the present culture study with cultivable findings of subgingival plaque reported previously by Sutter et al. (30) and Umeda et al. (36) revealed no significant differences. The predominant bacterial composition of mixed saliva in the present study comprised *Streptococcus* species. The present findings are similar to those in a previous cultivable study (24). No specific species or inherent bacterial composition was found in the present study.

Immunomodulatory effects, especially lymphocyte stimulation, are thought to be implicated in the development of Behcet's disease (9, 18, 19, 27, 28). It has been found that *S. mitis* produces superantigen, and an association of extracellular products of oral *Streptococcus* with the pathogenesis of oral mucosal diseases has also been suggested (16, 22, 23). Periodontal disease-associated bacteria such as *P. gingivalis* produce substances with some of the characteristics of superantigens (7), and certain *P. intermedia* have been suggested to activate Vβ-specific T cells in a manner similar to that of other known microbial superantigens (20). We examined the culture supernatants of 54 isolated bacterial strains from eight patients with Behcet's disease, 25 gram-negative strains from chronic periodontitis lesions and 20 ATCC oral bacterial strains in order to detect superantigen, but none of the strains we examined produced any superantigen in the culture supernatant, although 50× diluted sample from some strains of *Streptococcus* species induced weak thymidine incorporation. Further study using concentrated culture supernatant is required to detect the superantigen activity from these strains.

We have previously reported that oral *Mycoplasma* species isolated from HIV-seropositive patients did not produce superantigen (2). It is possible that some culture conditions or stages affect superantigen production. Recent studies have indicated that dental plaque microorganisms form a biofilm with a complex bacterial community that can modulate pathogenic factors (4, 17). In this study, we examined planktonic bacterial cells grown in broth culture. It is possible that subgingival plaque bacteria form biofilms that then produce immunomodulating factors such as superantigen *in vivo*. Further analysis is required to examine the superantigen production of both intact dental plaque and its isolates obtained from patients with Behcet's disease in advancing stages of the disease under various culture conditions.

HSPs have been called 'common antigens' and have been implicated in immunomodulatory actions such as immunosuppression and the induction of autoimmune diseases (37, 38). One immunohistochemical study of HSPs revealed that they are expressed differently in experimental cells of patients with systemic lupus erythematous and atopic dermatitis (8). There are two kinds of responses to HSPs: the response to the infectious pathogen and the recognition of the conserved epitope, which is thought to play some role in autoimmune disease and has therefore been called the 'common antigen'. In addition, T cells with γδTCRs, which are abundant in mucosal membranes, exhibit unusually high reactivity with some HSPs. Oral streptococcus antigens such as HSPs are involved in the etiology of Behcet's syndrome (18, 19, 26, 28). The present study showed that many oral bacterial species isolated from patients with Behcet's disease produce HSP70. We have previously reported that strains isolated from patients with chronic periodontitis with pustulosis palmaris et plantaris (PPP), and HIV-seropositivity, produce HSPs (1, 2, 11). We have also examined the relationships between the onset of PPP, periodontitis and HSP levels of IgG against HSPs, including *A. actinomycetemcomitans* DnaJ, in sera (11). In that study, we found that periodontal therapy and extraction of teeth with periapical infection resulted in remission of PPP and a statistically significant reduction in the levels of IgG against HSPs.

In the present study, the mean ratios of HSP-producing bacterial strains that reacted with both antibodies were significantly lower than those in the strains isolated from patients with Behcet's disease we examined previously. We previously found that *C. rectus* strains isolated from adult periodontitis lesions possessed cross-reacting antigens including HSP cross-reactive with *H. pylori* strains (13). As infectious disease progress, it is possible that immune responses to HSPs may be initiated, and it is also possible that the differences we note may be related to immune suppression. The results of the present study do not provide sufficient data to allow discussion of the relationship between bacterial infections in the oral cavity and the progression of Behcet's disease. However, it is still possible that HSPs produced by microorganisms in the oral cavity may lead to the acceleration of oral membrane ulceration in patients with Behcet's disease.

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Salivary bisphenol-A levels detected by ELISA after restoration with composite resin

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Bisphenol-A diglycidylether methacrylate (Bis-GMA), which is synthesized from bisphenol-A (BPA), a compound with exogenous endocrine disrupter action, is widely used as a dental material. During clinical filling with sealants and composite resins, the compounds are solidified by polymerization and then used. However, it has been noted that unpolymerized monomers may become dissolved in saliva. In this study using a competitive ELISA system, we investigated the changes in the BPA concentration in saliva after restoration with composite resins. Commercial composite resins from nine companies were tested. Mixed saliva was collected from 21 subjects. Based on the dynamics of salivary BPA detected by this ELISA system, we concluded that several tens to 100 ng/ml of BPA were contained in saliva after filling teeth with composite resin but that sufficient gargling can remove it from the oral cavity. Our data suggest that sufficient gargling after treatment is important for risk management.

1. Introduction

Bisphenol-A (BPA) is a major component of epoxy resin and polycarbonate resin, and it has been suspected in recent years of having endocrine disrupter action as an environmental hormone. It has been reported that 2.3–3.6 ng/ml of BPA is generated in the air [1] and that its influence on the water environment and ecosystem is of concern [2, 3]. Regarding the effects of BPA on animals, vom Saal *et al.* [4] reported that administration of 1/25 of the acceptable daily intake, 2 μg/kg body weight, to pregnant mice induced hypertrophy of the prostate in newborn males, and Takai *et al.* [5] reported that growth of early mouse embryos was promoted.

BPA is widely used as a starting material for sealants and composite resins worldwide. In clinical filling with sealants and composite resins, the compounds are solidified by polymerization and then used. However, it has been noted that unpolymerized monomers may be dissolved in saliva and thus the patient may be exposed to the monomer [6–11]. Recent improvements of instrumental performance have allowed more precise analytical results than those obtained by the previous analytical methods. However, such analyses were performed using liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS), and UV [12], requiring complex pretreatments, and these methods are not suitable for the treatment of a considerable number of samples. Using an enzyme-linked immunosorbent assay (ELISA) system that readily measures serum and plasma BPA without pretreatment by extraction, BPA in saliva samples was measured before and after restoration with various composite resins.

2. Materials

Nine commercially available products were used in this study: A: Z 100 (3 M, St. Paul, MN); B: Progress (Kanebo, Ltd., Tokyo, Japan); C: Palfique Toughwell (Tokuyama Corp., Tokyo, Japan); D: Matafil Flo (Sun Medical Co., Ltd., Shiga, Japan); E: Unifil S (GC Corp., Aichi, Japan); F: Beautifil (Shofu Inc., Kyoto, Japan); G: Xeno CFII (Sankin Kogyo, Tochigi, Japan); H: Prodigy (Kerr Corp., Orange, CA); and I: Cleafil ST (Kuraray Co., Ltd., Okayama, Japan) (Table).

3. Methods

3.1. Method of restoration with composite resin

After informed consent was obtained, 21 patients underwent cavity preparation. All of the method in this

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study followed "The Guidelines for Human Studies". Almost the same size of cavity was used in each treatment. The bonding agent provided by each maker was applied to a prepared cavity, which was then irradiated with visible radiation for 30 s. according to the manufacturer's recommended procedure. Each cavity was then filled with 0.1 g of composite resin, irradiated with visible radiation for one min, and ground with a silicon point for polishing.

3.2. Collection of saliva samples

Saliva was collected for five minutes while the subject bit a paraffin pellet used in saliva sampling for oral bacterial testing. Each specimen was centrifuged at 3,000 rpm for 10 min, and the supernatant was used. Samples were collected before filling, immediately after filling with composite resin, and after gargling with tepid water at about 37 °C for 30 s. All samples were analyzed after incubation at 4 °C for 24 h in glass tubes. Aloka RIA Program ARCAS (Aloka Co., Ltd., Tokyo, Japan) was used for data management. In one patient treated with composite resin-A (A-4), BPA in saliva was measured over 120 h after gargling as described above.

3.3. Measurement of BPA

BPA was measured using the BPA ELISA 'EIKEN' Kit (Eiken Chemical Co. Ltd., Tokyo, Japan). This method is a competitive enzyme linked immunosorbent assay (ELISA) developed by Ohkuma *et al.* [13] that measures BPA in biological specimens such as serum and plasma. To the secondary antibody-coated microplates, 20 μl of the standard BPA or sample, 50 μl of enzyme-labeled antigen [horseradish peroxidase labeled BPA-4-carboxyphenolether (CPhE)], and 50 μl of anti-BPA serum were added and reacted at room temperature for one hour. After the reaction solution was removed, 300 μl of washing solution was added to each well and removed. After this procedure was repeated three times, 100 μl of o-phenylenediamine (OPD) solution was added. After the enzyme reaction proceeded at room temperature for 30 min, the reaction was stopped by adding 100 μl of 2 N sulfuric acid, and the optical density (OD) was measured at 492 nm.

4. Results

The composite resins used in this study are summarized in the Table I. The composite resins in A, C, F, G, H, and I are Bis-GMA monomer-based composite resins and bonding agents, and those in B, D, and E are urethane dimethacrylate (UDMA) monomer-based composite resins and bonding agents. In the four patients treated with composite resin-A, the salivary BPA levels before and after restoration and after gargling were 0.3–2.0 ng/ml (mean ± standard deviation: 0.87 ± 0.69 ng/ml), 21.0–60.1 ng/ml (32.1 ± 16.27 ng/ml), and 1.6–4.7 ng/ml (3.1 ± 1.47 ng/ml), respectively (Fig. 1). In one (A-4) of the above patients, saliva was collected for five days after gargling by the same procedure, and BPA was measured. The BPA level varied within the range from that before restoration to that after gargling within a half day; then it converged to the level before restoration (Fig. 2). When we tested C, F, G, H and I, which are made of the same Bis-GMA as A, the BPA level was low even immediately after restoration in those patients treated with G and I. In contrast, in three patients treated with F, the BPA level tended to be high, and a higher level was also present even after gargling (Fig. 1). In patients treated with the non-Bis-GMA materials, the BPA concentration was 40 ng/ml or lower, even in the patient with the highest level immediately after restoration (D-1); these data indicate that the level was generally low (Fig. 3).

TABLE I Composite resins and bonding agents used in this study

	Restoratives (Manufacturers)	Source monomer	Bonding agent
A	Z 100 (3 M)	Bis-GMA/TEGDMA	Bis-GMA
B	Progress (Kanebo)	UDMA/TEGDMA	UDMA/TEGDMA
C	Palfique Toughwell (Tokuyama)	Bis-GMA	Bis-GMA
D	Metafil Flo (Sun Medical)	UDMA/TEGDMA	UDMA
E	Unifil S (GC)	UDMA	UDMA
F	Beautifil (Shofu)	Bis-GMA/TEGDMA	Bis-GMA
G	Xeno CFII (Sankin Kogyo)	Bis-GMA	Bis-GMA
H	Prodigy (Kerr)	Bis-GMA/TEGDMA	Bis-GMA
I	Cleafil ST (Kuraray)	Bis-GMA/TEGDMA	Bis-GMA

Bis-GMA: Bisphenol-A diglycidylether methacrylate.
TEGDMA: Triethylen glycol dimethacrylate.
UDMA: Urethane dimethacrylate.

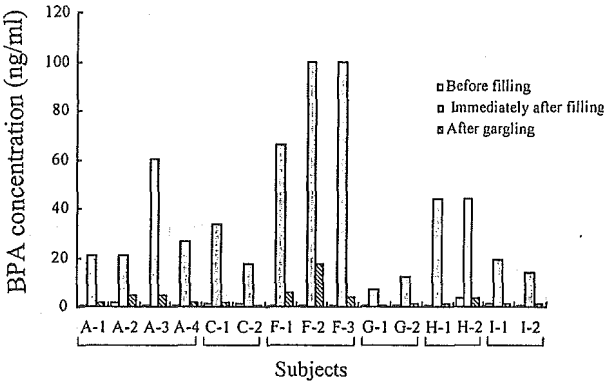

Figure 1 Salivary BPA concentrations before and after restoration with Bis-GMA- and TEGDMA-based composite resins.

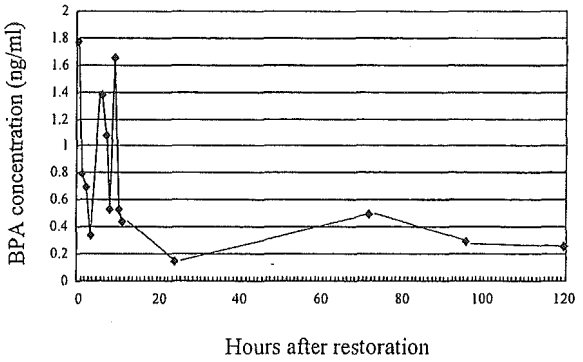

Figure 2 Time course of BPA concentration after restoration with composite resin-A, which is mainly composed of Bis-GMA.

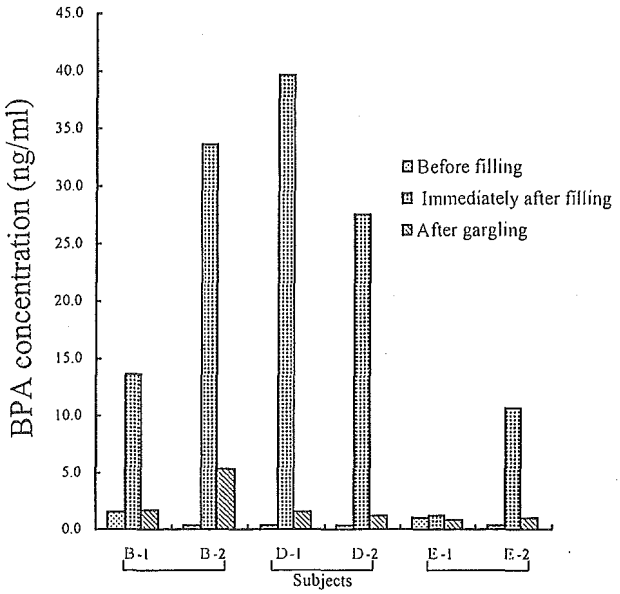

Figure 3 Salivary BPA concentrations after restoration with urethane monomer-based composite resin

5. Discussion

Olea *et al.* [6] initially reported the problem of dissolution of BPA. The amount of BPA dissolution from one resin-based sealant used in their study was abnormally high, and this product contained Bis-DMA. Many researchers have performed additional studies [7, 8, 14–17]. Recently, Tarumi *et al.* [18] analyzed the BPA content in three types of sealant and five types of bonding material by HPLC equipped with a UV detector and evaluated the estrogen activity in these materials by a receptor gene assay using Hela cells. They tested three types of sealant but did not detect BPA in any of the products. However, estrogen activity was detected in two products containing Bis-DMA, and they concluded that this activity was due to Bis-DMA, not due to BPA. Ohsaki and Imai [19] performed tissue analysis of commercial Bis-GMA, and confirmed that in addition to Bis-GMA and its structural isomer, iso-Bis-GMA, 2,2 [4-(2-hydroxy-3-metacryloiroxy-1-propoxy)-4-(2,3-dihydroxy-1-propoxy)] diphenylpropane (Bis-GMA-H), which possesses a structure in which one metacrylate ester bond of Bis-GMA is hydrolyzed, was present. They pointed that this Bis-GMA-H may appear as a peak overlapping the BPA peak under certain analytical HPLC conditions. The high BPA content reported by Olea *et al.* [6] might be due to inappropriate separation conditions.

Because BPA may dissolve from the inner coating materials of canned food, the subjects were instructed to avoid drinking canned beverages from one week before their dental treatment in our study. As in the study reported by Noda *et al.* [12], dissolution of BPA from non-Bis-GMA composite resins was detected in this study. Dissolution of BPA from non-Bis-GMA suggests slight contamination during the synthesis process. However, the level after gargling was very low. The amount of BPA in saliva collected before treatment was equal to that in the umbilical cord, and this detection in saliva suggested contamination *via* pathways other than dental treatment.

Not only Bis-GMA but also many BPA analogues are used as monomers in sealants, composite resins, and bonding agents for dental treatment [19]. Based on the published range, these dental materials are not composed of a single compound, and removal of simple unpolymerized compounds, byproducts, and impurities is difficult at the time of polymerization from a practical standpoint. This fact makes any discussion of safety confirmation of materials concerned in endocrine disrupter action complex. Is BPA released? What level is the detection level? It is undeniable that separation analysis by HPLC and GC/MS is too complex for frequent analysis near clinical practice [12, 14, 15, 17, 20, 21]. Although the values measured using this ELISA system may include a large amount of crossed compounds among impurities and polymerization byproducts contained in dental materials composed of multiple ingredients, because the cross-reactivity among Bis-DMA, TEGDMA, and HEMA contained in monomers is low, only salivary BPA may have been detected [13]. Based on the dynamics of salivary BPA detected by this ELISA system, we conclude that several tens to 100 ng/ml BPA were present in saliva after filling cavities with composite resin but that sufficient gargling can remove this compound from the oral cavity. After removal by gargling, the BPA concentration converged to a constant level after half a day. Depending on the restorative material, the concentration can be reduced to a level lower than 10 ng/ml even immediately after restoration. Therefore, for dental treatment of pregnant women and children, who are readily affected by endocrine disrupters, it is important to insist upon sufficient gargling after treatment and/or to select materials with consideration of risk management.

It has been reported that BPA is degraded slowly by gram-negative aerobic rods [22], and possible conversion to stilbene during the bacterial degradation process is being clarified. The questions of how oral bacteria degrade BPA dissolved in saliva, how they convert BPA to stilbene, and which species of bacteria are involved in this process remain for future clarification.

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RESEARCH ARTICLE

Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV

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Immunization involving a DNA vaccine prime followed by an adenovirus type 5 (Ad5) boost elicited a protective immune response against SHIV challenge in monkeys. However, the hepatocellular tropism of Ad5 limits the safety of this viral vector. This study examines the safety and immunogenicity of a replication-defective chimeric Ad5 vector with the Ad35 fiber (Ad5/35) in BALB/c mice and rhesus monkeys. This novel Ad5/35 vector showed minimal hepatotoxicity after intramuscular administration with the novel Ad5/35 vector. In addition, an Ad5/35 vector expressing HIV Env gp160 protein (Ad5/35-HIV) generated strong HIV-specific immune responses in both animal models. Priming with a DNA vaccine followed by Ad5/35-HIV boosting yielded protection against a gp160-expressing vaccinia virus challenge in BALB/c mice. The Ad5/35-HIV vector was significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector. These findings indicate that an Ad5/35 vector-based HIV vaccine may be of considerable value for clinical use.
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Keywords: *Ad5/35 vector; HIV; animal model; vaccine; immune response*

Introduction

A vaccine capable of preventing HIV infection is needed to control the global AIDS pandemic. In the past decade, multiple strategies to produce an immunogenic HIV vaccine have been explored. This included production of HIV subunit peptide vaccines,[1] DNA vaccines,[2] recombinant virus-vector vaccines (including modified vaccinia virus,[3] adenovirus (Ad),[4,5] rabies virus,[6] flavivirus,[7] sendai virus,[8] Venezuelan equine encephalitis virus,[9] and adeno-associated virus[10,11]), and bacterial vector-vaccines (bacille Calmette–Guerin,[12,13] and *Lactococcus lactis*[14]). Each of these strategies showed some promising results in animal models, either alone or in combination.

Among these vectors, the replication-defective human Ad type 5 (Ad5) recombinants (with the deletion of a replication-essential gene, E1) and the replication-defective modified vaccinia Ankara (MVA) elicited the most potent $CD8^+$ T-cell responses and provided the highest degree of protection in non-human primates.[3,4,15,16] A major limitation for the clinical application of the Ad5 and MVA vectors is the pre-existing immunity against these viruses in humans, since most of the human population has been infected with Ad5[17] and vaccinia virus on being administered the smallpox vaccine. The pre-existing antiviral immunity may strongly influence the efficacy of the HIV vaccine using Ad5 and MVA vectors.

Human Ads are classified into six subgroups from A–F.[18] Most of Ad serotypes belonging to subgroups A, C, D, E, and F use the coxsackievirus and adenovirus receptor (CAR) as a cellular receptor.[19] The Ad5 (subgroup C) has well-defined biological properties and has been widely used as a vector for gene therapy and vaccine. The replication-defective Ad5 vector can easily be produced in high titers and is highly effective in boosting HIV-specific immunity.[4,15] However, this virus uses CAR as its primary attachment receptor, which confers tropism for liver parenchymal cells.[19–22] This raises important safety concerns,[22] particularly because the administration of an Ad5-based vector for gene therapy resulted in the death of a patient.[23] In response to these shortcomings, our laboratory has examined the immunogenicity and safety of a replication-defective chimeric Ad5 vector with Ad type 35 fiber (Ad5/35) (Ad35 virus was classified as subgroup B). The Ad35 fiber showed 25% amino-acid homology with the Ad5 fiber.[24] Cell entry of Ad35 is CAR independent and may involve CD46 receptor, which expresses on most human cells.[25] Ad35 can be transducted to liver nonparenchymal cells on a level 4–5 log orders lower than Ad5, but not to

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liver parenchymal cells.[20] In the present study, we found that the Ad5/35 recombinants not only induced strong antigen-specific humoral and cellular immune responses and exhibited minimal hepatotoxicity in both mice and non-human primates, but were also significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector.

Results

Biodistribution of Ad in mice

In the initial experiments, mice were injected intramuscularly (i.m.) with 10^{11} viral particles (vp) of a luciferase-expressing Ad5 (Ad5-Luc) or Ad5/35 vector (Ad5/35-Luc). Luciferase expression was monitored using an *in vivo* imaging system (IVIS) on days 3 and 10 after administration. As shown in Figure 1a, all of the Ad5/35-Luc vector remained at the injection site. In contrast, substantial amounts of the Ad5 vector migrated to the liver. This difference in vector distribution was confirmed by studies involving LacZ-expressing Ad5 and Ad5/35 vectors (data not shown). Studies on serum glutamic-oxaloacetic transaminase (GOT) and serum glutamic-pyruvic transaminase (GPT) levels revealed that mice injected with the Ad5-Luc vector had changes indicative of liver damage (Figure 1b). We also analyzed serum levels of key proinflammatory cytokines (IFN-γ and IL-6) on days 0, 3, and 10 after administration of virus vectors. The levels of IFN-γ and IL-6 were significantly elevated following administration of Ad5-Luc vector, but not of Ad5/35-Luc vector (Figure 1c). Thus, the hepatotoxicity caused by the Ad5 vector was circumvented by the use of an Ad5/35 vector.

Time-course study of HIV-specific immune responses in mice.

Ad5/35 vector can efficiently transfect antigen-presenting cells[18,21,26,27] and muscular cells (Figure 1a). In order to explore whether the virus vector can be used as a vaccine vector, we constructed an HIV Env gp160-expressing Ad5/35 vector (Ad5/35-HIV). The expression of HIV gp160 was confirmed by Western blotting (Figure 2a). The HIV Env gp160-expressing DNA vaccine (DNA-HIV) used in this study was reported previously.[28] The mice were immunized with 10^{10} vp of Ad5/35-HIV vector, and the HIV-specific cellular immune response was periodically monitored by the intracellular cytokine staining (ICS) assay. The assay has been widely utilized to distinguish the relative contributions of $CD8^+$ cells to the overall T-cell responses.[29] On day 3, HIV-specific IFN-γ-secreting $CD8^+$ T cells can be detected (Figure 2b) and peaked 2 weeks after immunization. On day 50 and month 7 after final immunization, 2.5 and 1.2% of HIV-specific IFN-γ-secreting $CD8^+$ T cells still persisted, respectively.

Mice were vaccinated with Ad5/35-HIV vector to explore the humoral immune response 7 weeks after the final immunization. The animals immunized with 10^{10} vp of Ad5/35-HIV vector developed a high-tittered anti-gp160 antibody (Ab) response (Figure 2c). The specificity of the Ab response was confirmed by Western blotting (Figure 2c, upper panel). The magnitude of this response was not significantly altered by preimmunization with the DNA-HIV vaccine (Figure 2c). DNA-HIV vaccination alone generated a low level of HIV-specific serum Ab (Figure 2c, bottom panel). HIV-specific neutralizing Ab was only detectable in the Ad5/35-HIV

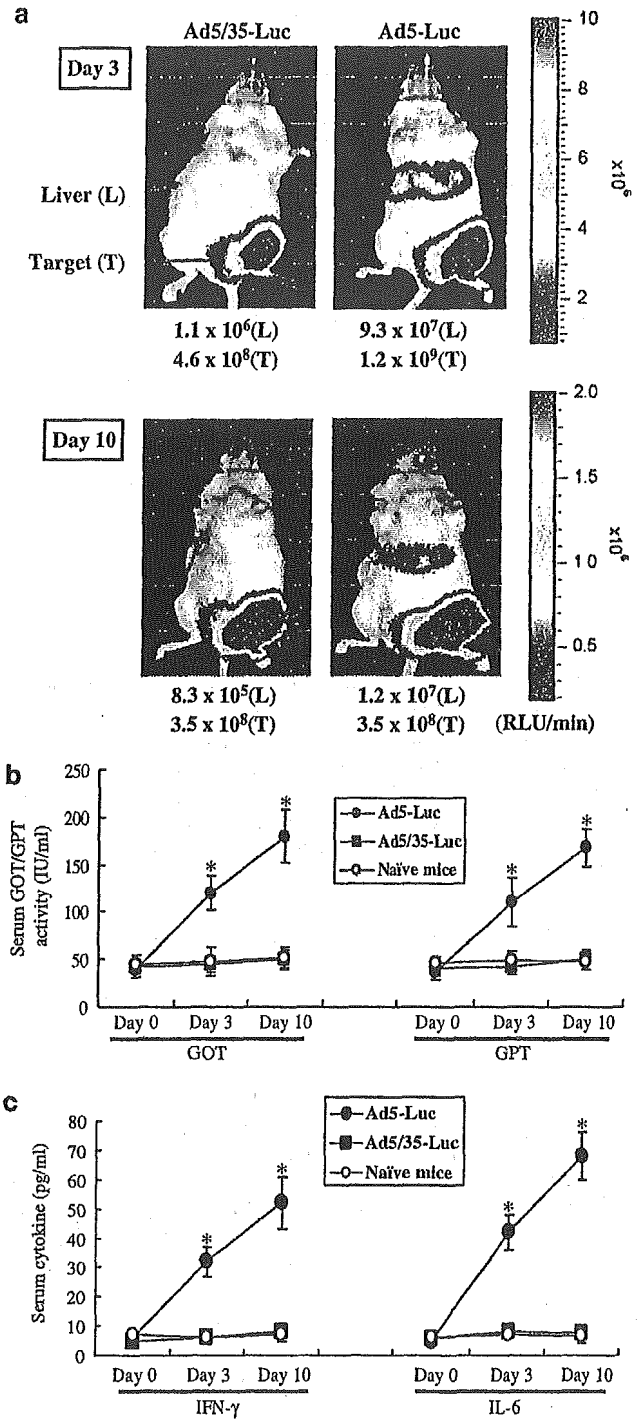

*Figure 1 Biodistribution and safety of Ad vectors. BALB/c mice were injected i.m. with 10^{11} vp of the Ad5-Luc or Ad5/35-Luc vector. (a) Using an IVIS CCD camera, vector distribution was detected after the addition of luciferin (3 mice/group) (expressed in relative light units (RLU)). One of the mice is represented and other mice used show the same pattern. (b) Serum GOP and GPT levels were measured on days 0, 3, and 10 after injection (5 mice/group). IU: international unit. (c) Serum IFN-γ and IL-6 levels were measured on days 0, 3, and 10 after injection (5 mice/group). *Mean values are significantly different between Ad5-Luc-administered mice and Ad5/35-Luc-administered mice or naïve mice at the same time point.*

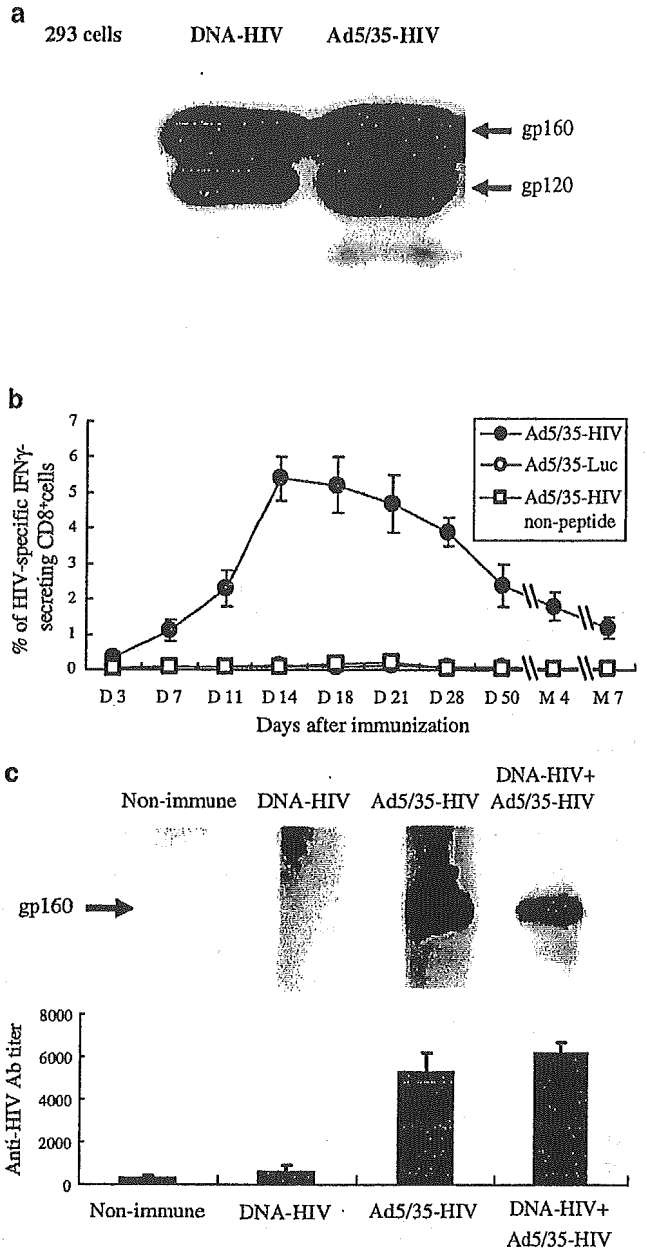

Figure 2 Time course of HIV-specific immune responses in mice. (a) HIV Env protein expression of DNA-HIV vaccine and Ad5/35-HIV on HEK293 cells was confirmed by Western blotting using an HIV Env-specific mAb. (b) Time-course study of cellular immune responses after a single i.m. injection of 10^{10} vp of Ad5/35-HIV vector (3 mice/time point). D: day; M: month. (c) HIV-specific Ab was detected by Western blotting using 100-fold diluted antisera (serum pool of 10 mice/group) (upper panel) and ELISA (10 mice/group) (bottom left panel).

vaccinated mice (1:186) and DNA prime/Ad5/35-HIV boosted mice (1:206).

Immune responses and challenge in mice 2 weeks after vaccination. There is growing evidence that cellular immunity contributes to protecting the host against HIV infection.[3,4,30,31] The ability of the Ad5/35 vector to trigger the activation and proliferation of antigen-specific T cells was monitored. Vaccination with the DNA-HIV vaccine induced the number of HIV-specific IFN-γ-secreting $CD8^+$ T cells to increase from background levels (<0.2–0.7%) ($P<0.05$) (Table 1). This was significantly less than the effect of vaccination with the Ad5/35-HIV vector (10^{10} vp/mouse) that increased the IFN-γ-secreting $CD8^+$ T cells to 5.5% ($P<0.05$). Priming with the DNA-HIV vaccine followed by an Ad5/35-HIV vector boost led to a further three-fold increase in the number of IFN-γ-secreting $CD8^+$ T cells ($P<0.05$).

A tetramer-binding assay was used to verify that the IFN-γ-secreting cells were MHC class I-restricted HIV-specific $CD8^+$ T cells.[32] A single immunization with Ad5/35-HIV vector elicited a significant increase in the number of tetramer-binding $CD8^+$ T cells (Table 1). When compared with DNA-HIV vaccination alone, immunization with the Ad5/35-HIV vector yielded five-fold more HIV-specific $CD8^+$ T cells ($P<0.05$). Priming with the DNA-HIV vaccine, followed by Ad5/35-HIV boosting, further increased the tetramer binding ($P<0.05$).

To examine the protective activity of the Ad5/35-HIV vector, immunized mice were challenged with 10^8 plaque forming units (PFU) of vPE16 2 weeks after final immunization. The animals that were vaccinated with the Ad5/35 vector alone or in combination with the DNA-HIV vaccine were completely protected from infection (Table 1); however, the DNA-HIV vaccination alone had little impact on the susceptibility to infection by vPE16.

Long-term cell-medicated immune responses and challenge in mice. The durability of these vaccine regimens was explored. HIV-specific cellular immune responses persisted through 7 months after final immunization (Table 1 and Figure 2b). To determine whether this immune response was protective, vaccinated mice were challenged with vPE16 (10^8 PFU/mouse) 7 weeks after final immunization. The viral load of Ad5/35-HIV-immunized mice was reduced by 10^5 as compared with that of the control mice ($P<0.05$). DNA-HIV vaccination by itself was not protective, but the combination of DNA-HIV priming and Ad5/35-HIV boosting yielded a prolonged and complete protection (Table 1).

Biodistribution of Ad in rhesus macaques

To study the biodistribution of Ad in monkeys, 10^{11} vp of Ad5-Luc and Ad5/35-Luc vectors was injected i.m. into two rhesus monkeys for each vector. The luciferase activity in the tissues was detected 3 days after administration, because high luciferase activity in the mouse liver was observed at that time point. Liver infection with Ad5 vector was 20- to 40-fold stronger than that with Ad5/35 vector (Figure 3a). It is important to note that the luciferase activity of the cerebellum and the posterior cerebrum in the monkeys that received the Ad5-Luc vector was two- and four-fold higher, respectively, than that of the monkeys that received the Ad5/35-Luc vector.

Immune response in rhesus monkeys after vaccination

To explore the immunogenicity of the Ad5/35-HIV vector in monkeys, two rhesus macaques were immunized i.m. with 10^{11} vp of Ad5/35-HIV vector. A detectable HIV-specific serum Ab response developed

Table 1 HIV-specific cell-mediated immune responses and virus challenge after vaccination

	Week 2			Week 7			Month 4	Month 7
	ICS (%)	*Tetramer (%)*	*Ovary viral titer*	*ICS (%)*	*Tetramer (%)*	*Ovary viral titer*	*Tetramer (%)*	*Tetramer (%)*
Nonimmune	0.1±0.1	0.1±0.1	$8\times10^{8}\pm35$	0.1±0.1	0.1±0.1	$1\times10^{9}\pm65$	0.0±0.0	0.0±0.0
DNA-Empty	0.1±0.1	0.1±0.1	$2\times10^{9}\pm45$	0.0±0.0	0.0±0.0	$8\times10^{8}\pm32$	0.0±0.0	0.0±0.0
Ad5/35-Luc	0.2±0.1	0.2±0.2	$2\times10^{9}\pm25$	0.0±0.0	0.0±0.0	$4\times10^{8}\pm46$	0.0±0.0	0.0±0.0
DNA-HIV	0.7±0.1	1.0±0.3	$6\times10^{6}\pm42$	0.4±0.2	0.6±0.1	$5\times10^{7}\pm51$	0.3±0.1	0.1±0.1
Ad5/35-HIV	5.5±0.3	5.2±0.3	ND	2.5±0.8	3.1±0.2	$2\times10^{3}\pm34$	2.5±0.5	1.2±0.4
DNA-HIV+Ad5/35-HIV	17.2±0.8	19.4±2.1	ND	8.2±1.2	8.9±0.8	ND	7.1±0.6	4.1±0.3

Mice were immunized with DNA plasmid or Ad5/35 vector, either alone or in combination. At 2 weeks, 7 weeks, 4 months, and 7 months after final immunization, HIV-specific cellular immune responses were detected by ICS assay and tetramer assay. The data represent the percentage of IFN-γ- or tetramer-positive $CD8^+$ T cells (5–10 mice/group). The backgrounds were less than 0.1% IFN-γ-secreting $CD8^+$ T cells when cells were stimulated with control peptide (influenza NP peptide, TYQRTRALV). The vaccinated mice (10 mice/group) were challenged with vaccinia virus vPE16 2 or 7 weeks after final immunization. At 6 days after the challenge, the vPE16 titer in mouse ovaries was measured. ND, not detectable.

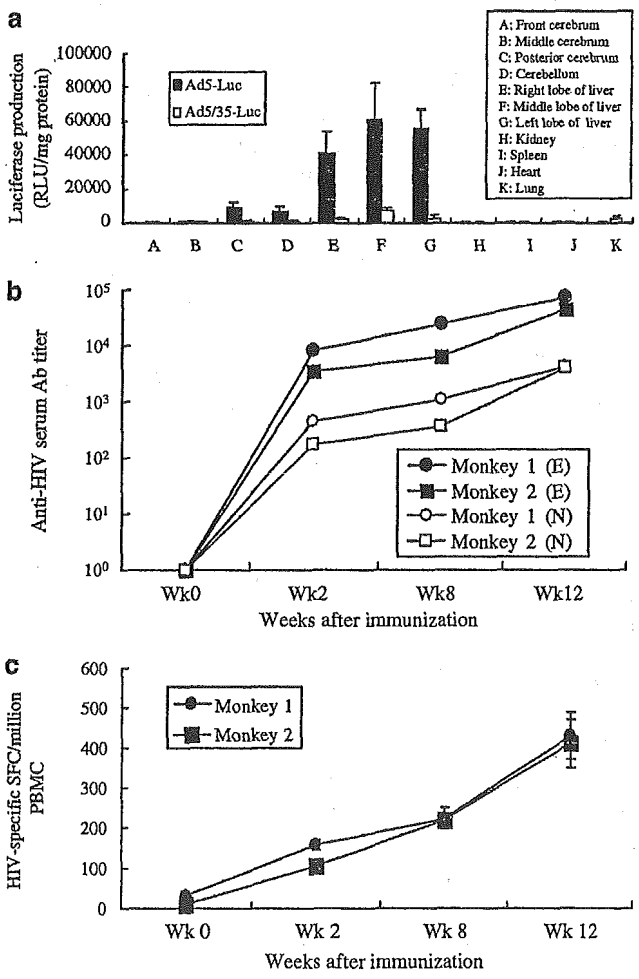

Figure 3 Biodistribution and HIV-specific immune responses in rhesus monkeys. Rhesus monkeys (2 monkeys/group) were administered i.m. 10^{11} vp of Ad5-Luc or Ad5/35-Luc vector. The luciferase activity in the organs of the monkey (expressed in RLU) was examined 3 days after administration (a). Rhesus monkeys were immunized i.m. with 10^{11} vp of Ad5/35-HIV vector at 0 and 8 weeks. PBMCs were isolated at weeks 0, 2, 8, and 12. HIV-specific Ab titers were measured in triplicate by ELISA (E) (●, ■) and neutralizing assay (N) (○, □) (b). The detecting limitation of the neutralizing assay was 100 ND_{50}/ml. PBMCs were stimulated with HIV Env gp120 protein, and the number of cells activated to secrete IFN-γ was determined in triple wells by ELISPOT (c). SFC: spot-forming cells.

within 2 weeks of immunization (Figure 3b). The animals were boosted at 8 weeks. After 4 weeks, titers in excess of 1:50 000 were achieved. Similar results were observed in neutralizing Ab. A increase in the number of HIV-specific IFN-γ-secreting T cells was also detected in the peripheral blood mononuclear cells (PBMCs) (Figure 3c). Boosting with Ad5/35-HIV vector further increased this T-cell response.

Effect of pre-existing immunity on vaccination

To evaluate the effect of the anti-Ad5 neutralization Ab (found in 60% of the adult human population)[17] on the Ad5/35 vector, the infectivity of the vector was examined after incubation with serially diluted serum from subjects with high titers of anti-Ad5 Abs (anti-Ad5 neutralizing titer = 1:64). As shown in Figure 4, the human antisera had 1:8 anti-Ad5/35 neutralizing titer and normal human sera against either Ad5 or Ad5/35 vector was less than 1:4. The sera derived from Ad5/35-HIV-immunized monkeys showed two-fold higher neutralizing Ab titer against Ad5/35 vector than Ad5 vector.

To examine the effect of pre-existing anti-Ad5 immunity on the activity of the Ad5/35 vector *in vivo*, mice were injected i.m. with 10^{10} or 10^{11} vp of Ad5-Luc. After 8 weeks, these animals were immunized with 10^{10} vp of Ad5-HIV or Ad5/35-HIV. The HIV-specific responses were detected by the tetramer assay 2 weeks after immunization. Although pre-existing immunity to Ad5 reduced the immune response elicited by both vectors, Ad5/35-HIV was significantly more immunogenic than Ad5-HIV ($P<0.05$; Figure 4).

Discussion

This study demonstrates that an Ad5/35-HIV vector vaccine induces strong cellular and humoral immune responses with minimal toxicity in mice and rhesus macaques. A prime–boost strategy involving the DNA-HIV vaccine and the Ad5/35-HIV vector generated protective immunity against viral infection in mice.

A widely used HIV vaccine should have high immunogenicity, low cost of production, and low or no pathogenicity. Replication-defective Ad5 is one of the best vectors for HIV vaccine development. Vaccination

with recombinant Ad5 has achieved great success in inducing protection against virus infection in several animal models.[4,15,33] Ad5 is well characterized, and its subclinical disease association in humans is well known.[34,35] However, a majority of the human population (more than 60%) is infected with the Ad5 virus.[17,36,37] The neutralizing Ab and the cellular immune responses against the Ad5 fiber and capsid may reduce the efficacy of the Ad5 vector when it is used in a clinical trial.[37,38] The switching of the Ad serotypes[37,39] and the use of animal Ads[40–44] enables the partial bypass of the pre-existing immune responses to Ad5 viruses. However, there are a few drawbacks: lack of knowledge regarding the biology of these viruses, including tropism on human cells; potential difficulties in manufacturing; and the possibility of *in vivo* recombination with other human viruses leading to unknown diseases. Animal Ad vectors may induce the antigen-specific responses as strongly as Ad5 in animal models.[43] However, their immunogenicity in humans is still unknown. This study used a chimera Ad5 vector with Ad35 fiber, which relates with cell tropism. The Ad5/35, similar to Ad5, has a high productive titer in tissue culture cells, because it is commonly known that human subgroup B Ads, such as Ad5, have a considerably higher titer as compared with other subgroup viruses, including Ad35. Nevertheless, the virus displayed the cell tropism of Ad35. We explored the immunogenicity of the Ad5/35 vector encoding HIV Env gene in both mice and non-human primates. The results indicate that the Ad5/35-HIV vector elicited strong HIV-specific humoral and cellular immune responses that conferred protective immunity (Table 1 and Figure 3b and c). Coupled with the evidence that an Ad5/35 vector transduces human dendritic cells more efficiently as compared with an Ad5 vector,[18,21,26,27] these findings suggest that the Ad5/35-HIV vector is a promising candidate for human trials.

Another concern regarding the use of the Ad5 vector in clinical trials is its strong tropism to hepatocytes that is caused by the high expression of CAR in the hepatocytes. Our experiments showed a high expression of the Ad5 vector in the liver in both mice and non-human primates after i.m. administration, but not of the Ad5/35 vector (Figure 1). In contrast to Ad5 vector, Ad/35 vector did not elevate the levels of serum markers (GOT/GPT) of hepatotoxicity and key proinflammatory cytokines (IFN-γ and IL-6) in mice (Figure 1b and c). These results demonstrate that, as a vaccine vector, Ad5/35 vector is safer than Ad5 vector. However, low expression of Ad5/35 vector in monkey liver was still detected after i.m. administration of Ad5/35-Luc vector to monkeys (Figure 3a). It may have resulted from low capacity of Ad5/35 to infect liver nonparenchymal cells, but not liver parencymal cells.[20] Interestingly, we found a certain magnitude of Ad5 vector expression in the posterior cerebrum and cerebellum of monkeys; however, the Ad5/35 vector was not expressed (Figure 4a). Nevertheless, in the present experiment, we could not precisely define the location of the Ad5-infected cells or determine whether the infection potentially causes local inflammation or toxicity. However, potential brain infection after Ad5 vector administration is a safety concern because intranasal administration of the Ad5 vector has been reported to result in the infection of the central nervous system.[45]

In this study, the effect of pre-existing anti-Ad5 immunity on the Ad5/35 vector was explored along with several immunization protocols as follows. (1) Both *in vitro* and *in vivo* studies demonstrate that the Ad5/35 vector is significantly less susceptible to neutralization by anti-Ad5 Abs as compared with a conventional Ad5 vector (Table 2 and Figure 4). The administration or infection of Ad can induce immune responses against the Ad hexon, penton, and fiber antigens. The exchange of fiber can partially reduce the inhibition of the

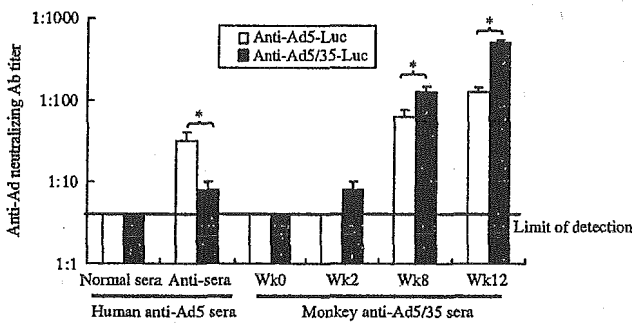

*Figure 4 Effect of pre-existing antiviral immunity. Ad5-Luc and Ad5/35-Luc vectors were incubated with an equal volume of serially diluted normal human sera (No. 2, anti-Ad5 neutralization titer <1:4), human antisera (No. 2, anti-Ad5 neutralization titer = 64), or monkey antisera from Figure 3b and c (No. 2) in triplicate and were subsequently added to infected Vero cells in a 96-well plate at 10^7 vp/well. The luciferase activity was measured 48 h after infection. The neutralizing titer was calculated with limited serum dilution when the luciferase activity in the Ad-infected cells was equal with the background. Average and standard deviations for three independent experiments are shown. *Mean values are significantly different between groups.*

Table 2 Effect of pre-existing antiviral immunity

	Prime	*Anti-Ad5 neutralizing Ab titer*	*Boost*	*Tetramer assay (%)*
Control	Non	<1:4	Ad5-HIV (10^{10} vp/mouse)	4.8±0.2
			Ad5/35-HIV (10^{10} vp/mouse)	5.1±0.2
Low dose	Ad5-Luc (10^{10} vp/mouse)	1:102	Ad5-HIV (10^{10} vp/mouse)	2.3±0.4 *
			Ad5/35-HIV (10^{10} vp/mouse)	4.6±0.6 *
High dose	Ad5-Luc (10^{11} vp/mouse)	1:248	Ad5-HIV (10^{10} vp/mouse)	0.5±0.1 *
			Ad5/35-HIV (10^{10} vp/mouse)	2.6±0.4 *

After 8 weeks, naive mice or mice pretreated with 10^{10} or 10^{11} vp of Ad5-Luc vector (6 mice/group) were immunized with 10^{10} vp of Ad5-HIV or Ad5/35-HIV vector. At the time of vaccination, anti-Ad5 neutralizing titers were measured in Ad5-Luc-treated mice. At 2 weeks after vaccination, the HIV-specific responses were detected by an HIV-specific tetramer assay. *Mean values are significantly different between the groups.

pre-existing immunity against the parent Ads (Table 2 and Figure 4). The exchange of other genes, including hexon and penton, may further reduce the inhibition of pre-existing anti-Ad5 immunity. (2) We also explored the immune responses by using the same vector for prime/boost. When the mice were immunized i.m. with 10^{10} vp of Ad5/35-HIV 1–3 times at 4-week intervals, the HIV-specific cell-mediated immune responses were detected by the tetramer assay. An increased response was observed after the second immunization but not after the third immunization (data not shown). High anti-Ad5/35 neutralizing Ab after second immunization may block the Ad5/35-HIV infection (Figure 4). These results are in agreement with the data from our study on monkeys (Figure 3b and c) and with that from studies by other groups,[33,43] and other virus such as MVA vector may be applicable for further boost if high immune responses are required. The DNA-HIV vaccine prime/Ad5/35-HIV vector boost regimen greatly increased HIV-specific cell-mediated immune responses (Table 1 and Figure 3b and c), but not the humoral immune response (Figure 2c). This suggests that DNA vaccination enhances adenoviral recombinant-induced cell-mediated immunity rather than humoral immunity, as described by other groups.[4,38] Furthermore, DNA vaccine priming can reduce the humoral response to the adenoviral antigens and can counterbalance the impaired B-cell response to the antigen expressed by the adenoviral recombinant in mice that are preimmune to Ad.[46] This DNA prime/Ad5/35 boost regimen might be highly suitable for use in humans with previous exposure to the Ad5 virus. (3) To examine the immunogenicity and protective immunity of the Ad5/35 vector, we used HIV-1 IIIB in this study, because the strain has been well studied and we can compare our data from the new vector with that from other studies. For the clinical trail, we have constructed DNA vaccine and Ad5/5 vector expressing ENV and GAG of a Clade C HIV-1 isolate and similar results with HIV-1 IIIB strain were obtained in a mouse model (manuscript in preparation).

Considered together, using Ad5/35 vector, we developed an HIV vaccine with a higher immunogenicity and low pathogenicity. The Ad5/35-HIV vaccine induced strong HIV-specific immune responses in both BALB/c mice and rhesus monkeys. Priming with a DNA-HIV vaccine followed by Ad5/35-HIV boosting yielded protection against viral infection in mice. The Ad5/35 vector may be a promising vaccine for human clinical trial.

Materials and methods

Recombinant vectors

E1,E3-deletion, replication-defective recombinant viruses were constructed with an Ad generation kit (Avior Therapeutics Inc., Seattle, WA, USA).[21] Briefly, a 5.2k bp *SalI*/*Pst*I fragment containing CAG promoter-HIV_{IIIB} rev/env gp160-polyA was isolated from pCAGrev/env.[28] A shuttle plasmid (pLHSP) containing Ad5 positions 22–342, Ad5 3523–5790, *Escherichia coli ori*, and ampicillin-resistant gene was obtained from Avior Therapeutics Inc. (Seattle, WA, USA). The 5.2k bp blunted fragment was subcloned into blunted *Eco*RI site of pLHSP plasmid vector to generate pLHSP-HIV shuttle plasmid. The pLHSP-HIV shuttle plasmid was linearized with *Pac*I and transfected with E1,E3-deletion, chimeric Ad5 or 5/35 genome to human embryonic kidney (HEK293) cells using calcium precipitation method to generate recombinant virus, Ad5-HIV and Ad5/35-HIV, respectively. The recombinant virus (Ad5/35-HIV, Ad5-HIV) was propagated in HEK293 cells and purified by two repetitions of the CsCl methods described elsewhere.[47] The total concentration of virions in each preparation was calculated from the optical density at 260 nm (OD_{260}), using the formula $1\ OD_{260} = 1 \times 10^{12}$ vp/ml. The HIV_{BH8} gp160-expressing replication-competent vaccinia virus (WR strain, vPE16; HIV_{BH8} gp160 has 97.32% amino-acid homology with HIV_{IIIB} gp160) was obtained from the AIDS Research and Reagent Program, National Institutes of Health, Rockville, MD, USA (Cat. No. 362). The vPE16 vectors were propagated in CV1 cells. The Ad5-Luc and Ad5/35-Luc vectors expressing luciferase coding gene were described previously.[26] The DNA-HIV vaccine (pCAGrev/env) containing HIV_{IIIB} *rev* and *env* genes has been previously reported.[28]

Biodistribution of Ad5 and Ad5/35 vectors in vivo

The experiment was performed as previously described.[48,49] In brief, the Ad5-Luc or Ad5/35-Luc vectors (10^{11} vp/mouse) were injected i.m. into BALB/c mice. On days 3 and 10, the mice were anesthetized with a 2% isofluorane/air mixture, and a single dose of 150 mg/kg luciferin in normal saline was administered intraperitoneally. The CCD images were obtained using a cooled *in vivo* imaging system (IVIS) CCD camera (Xenogen, Alameda, CA, USA) and analyzed. To study the viral biodistribution in primates, two rhesus monkeys (2 years old, male) were administered i.m. 10^{11} vp of Ad5-Luc or Ad5/35-Luc. At 3 days after administration, the luciferase activity was detected in the monkey organs (brain, liver, kidney, spleen, heart, and lung) using the Luciferase Assay Systems (Promega, Madison, WI, USA). Serum GOT and serum GPT were measured in mouse or monkey sera at the Kitayama-Rabesu Institute (Ina, Nagano, Japan). The concentration of serum IFN-γ and IL-6 was measured using the IFN-γ and IL-6 ELISA kits (Biosource, Camarillo, CA, USA), respectively, according to the manufacturer's protocol.

Animal immunization

Female BALB/c mice (8-week-old; H-$2D^d$) were purchased from Japan SLC Inc., Shizuoka, Shizuoka-ken, Japan. The mice were immunized with an i.m. injection of 100 μg of pCAGrev/env or pCAGempty plasmid DNA in phosphate-buffered saline (PBS) at 0, 1, and 2 weeks and were boosted with 10^{10} vp of Ad5/35-HIV or Ad5/35-Luc vector at week 3. For the time-course study, the mice were administered a single i.m. injection of 10^{10} vp of Ad5/35-HIV vector per mouse. To study the effect of pre-existing antiviral immunity on vaccination, the mice were injected i.m. with 10^{10} or 10^{11} vp of Ad5-Luc vector and then immunized 8 weeks later with 10^{10} vp of Ad5-HIV vector or Ad5/35-HIV vector. For rhesus monkey immunization, 10^{11} vp of Ad5/35-HIV vector was injected i.m. into two rhesus monkeys (2 years old, male) at weeks 0 and 8.

Intracellular cytokine staining assay

IFN-γ-secreting $CD8^+$ T cells were detected by the protocol recommended by the manufacturer (Cytofix/CytoPerm Plus kit, PharMingen, San Diego, CA, USA). In brief, lymphocytes were isolated from the mouse spleen. A single cell suspension was incubated with 10 μg/ml of the HIV V3 peptide (NNTRKRIQRGP GRAFVTIGKIGN) for 24 h at 37°C. At 2 h before the end of incubation, 1 μg/ml of GolgiPlug was added. The cells were washed with staining buffer (3% fetal calf serum (FCS), 0.1% sodium azide (NaN_3) in PBS), blocked with 4% normal mouse sera, and stained with phycoerythrin (PE)-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen). The cells were then suspended in 250 μl of Cytofix/Cytoperm solution at 4°C for 20 min, washed with Perm/Wash solution, and stained with anti-mouse IFN-γ Ab conjugated with fluorescein isothiocyanate (FITC) (PharMingen) at 4°C for 30 min, followed by flow cytometric analysis.

Tetramer assay

The tetramer assay used a PE-conjugated H-$2D^d$/p18 tetramer (RGPGRAFVTI), as previously described.[28] In brief, splenocytes were isolated from mice and incubated for 30 min at 4°C with 4% normal mouse serum in PBS. Cells were stained with FITC-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen) for 30 min at 4°C. After washing twice with the staining buffer (3% FCS, 0.1% NaN_3 in PBS), the cells were incubated with the tetramer reagent for 15 min at 37°C, followed by flow cytometric analysis (Becton Dickinson).

Recombinant vaccinia virus used for the challenge study

Using vPE16 vaccinia virus, the virus challenge experiment was performed as described previously.[28] Vaccinated female mice were intraperitoneally challenged with 10^8 PFU of vaccinia virus vPE16 at 2 or 7 weeks after the final immunization. At 6 days after challenge, the mice were killed, their ovaries were sonicated, and the vPE16 titer was determined by serial 10-fold dilution on a plate of CV1 cells. Infected cells were detected by staining with crystal violet and plaques were counted at each dilution.

Detection of HIV-1-specific Ab

The HIV-1-specific Ab was detected by the Western blotting method and the enzyme-linked immunosorbent assay (ELISA). By Western blotting method, the HIV envelope glycoprotein gp160-coated membrane from the New Lav Blot 1 kit (Bio-Rad, Marnes-la-Coquette, France) was incubated with a 100-fold dilution of mouse serum followed by an affinity-purified horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin (ICN Pharmaceuticals Inc., OH, USA). HIV gp160 protein was detected using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech).

ELISA was performed as described elsewhere.[10] To summarize, 96-well microtiter plates were coated with 10 μg/ml of HIV_{IIIB} gp120 protein (donated by AIDS Research and Reference Reagent Program, National Institutes of Health) and incubated overnight at 4°C. The wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. They were then treated with 100 μl of serially diluted antisera and incubated for an additional 2 h at 37°C. The bound immunoglobulin was quantified using an affinity-purified HRP-labeled anti-mouse Ab or anti-monkey Ab (both from Sigma). The mean Ab titer was expressed as the reciprocal of the serial serum dilution that exceeded the assay background by 2 s.d.

The HIV-specific neutralizing titer of immune mice or monkeys was also measured. The serially diluted antisera were incubated with 200–300 blue spot-forming units (BFU) of $HIV\text{-}1_{LAI}$ at 37°C for 1 h. The mixture was incubated with confluent MAGIC5 cells (from Dr Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan),[50,51] Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and 0.2 mg/ml of G418 in a 96-well plate at 37°C for 2 days. The cells were fixed with fixing solution (1% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min and stained with staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride, 0.4 mg/ml X-gal in PBS) at 37°C for 18–24 h. The staining was stopped by removing the staining solution and the cells were washed twice with PBS. The blue spot in each well was counted after the staining, and the neutralizing titer was calculated as (1−(% infection/% infection of control wells)) × 100. The 50% neutralization dose (ND_{50}) is defined as the concentration of the Ab that reduced the number of infected cells by 50%. The detecting limitation of the assay was 100 ND_{50}/ml.

ELISPOT assay

The frequency of HIV-specific IFN-γ-secreting cells in monkeys was determined using an ELISPOT assay kit (U-Cytech, Utrech, The Netherlands) according to the manufacturer's manual. In brief, 2×10^5 monkey PBMCs were stimulated in triplicate wells with 1 μg/ml of the HIV_{IIIB} gp120 protein for 16 h at 37°C. Nonstimulated cells were used to assess the background. The cells were transferred to an anti-IFN-γ Ab-coated 96-well plate and incubated for 5 h at 37°C. The cells were removed and 200 μl/well of ice-cold deionized water was added to lyse the remaining PBMCs. Subsequently, the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with biotinylated anti-IFN-γ Ab for 1 h at 37°C. After 10 washings with PBS-T, 50 μl of gold-labeled anti-biotin Ab was added and incubated for 1 h at 37°C. The plate was washed 10 times with PBS-T, and 30 ml of activator solutions was added. The plate was incubated in the dark for 30 min at room temperature to develop spot formations. After 30 min incubation, the plate was washed with deionized water and air-dried; spots were counted by a computer-assisted video image analysis. The results were expressed as spot-forming cells (SFC) per million cells.

Ad-specific neutralizing assay

Ad5-Luc or Ad5/35-Luc vector (10^7 vp) was incubated with an equal volume of serially diluted normal human sera (anti-Ad5 neutralizing titer <1:4), human antisera (anti-Ad5 neutralizing titer = 1:64), or monkey sera (at weeks 0, 2, 8, and 12 after immunization with Ad5/35-HIV vector) at 37°C for 2 h. The mixture was incubated with confluent Vero cells in a 96-well plate at 37°C for an additional 48 h. The luciferase activity was detected by Luciferase Assay Systems (Promega, Madison, WI, USA).

The neutralizing titer was calculated with limited serum dilution when the luciferase activity in the Ad-infected cells was equal with the background.

Data analysis

All values were expressed as means ± standard error (s.e.). Statistical analysis of the experimental data and controls was conducted with one-way factorial analysis of variance. Significance was defined at $P<0.05$ in the statistical analysis.

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