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ORAL MICROBIOLOGY
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Role of $CD4^+$ and $CD8^+$ T-cells in the induction of oral tolerance to *Actinomyces viscosus* in mice

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Mucosal presentation of *Actinomyces viscosus* results in the induction of antigen specific systemic suppressor cells in mice. The aim of the present study was to determine the phenotype of the suppressor cells responsible for the induction of oral tolerance to low doses of *A. viscosus*. When CD8 cell-depleted DBA/2 mice were intragastrically immunized and systemically immunized with *A. viscosus*, the delayed type hypersensitivity response was suppressed but not the levels of antigen specific serum antibodies. Adoptive transfer of orally tolerized $CD4^+$ cells to $CD4^+$-depleted mice resulted in suppression of delayed type hypersensitivity response but not of the levels of antigen specific serum antibodies. In contrast, adoptive transfer of orally immunized $CD8^+$ cells to $CD8^+$-depleted mice resulted in partially suppressed delayed type hypersensitivity response but significantly inhibited the levels of antigen specific serum antibodies. When orally tolerized $CD8^+$ cells were cocultured with systemically immunized $CD8^+$ cell-depleted spleen cells, splenic specific antibodies were inhibited. However, no suppression of splenic specific antibodies could be observed in the cultures containing orally tolerized $CD4^+$ cells and systemically immunized $CD4^+$ cell-depleted spleen cells. The results of the present study suggest that oral tolerance of humoral and cellular immunity induced by low doses of *A. viscosus* may be mediated by $CD8^+$ and $CD4^+$ cells, respectively.

Key words: *Actinomyces viscosus*; $CD4^+$ cells; $CD8^+$ cells; mice; oral; tolerance

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Mucosal administration of antigen followed by a subsequent systemic challenge with the same antigen can lead to the induction of a systemic immune suppression, known as oral tolerance (6, 10, 29). The precise mechanism is still controversial and various factors have been proposed that can induce oral tolerance, such as humoral factors, antigen–antibody complexes, serum suppressor factors, auto anti-idiotypic antibodies, and cellular components like suppressor T cells and suppressor B cells. The effector cells proposed for the induction of mucosal immune suppression (oral tolerance) are the $CD4^+$ and/or $CD8^+$ T cells (6, 10, 29).

Studies in mice have provided a better understanding of the induction of oral tolerance. For example, oral tolerance to retinal S-antigen or ovalbumin was still inducible in mice lacking of $CD8^+$ T cells, suggesting that activation of $CD8^+$ T cells may not be prerequisite in the induction of oral tolerance (1, 11, 36). Further, oral tolerance may be due to either clonal anergy or deletion (15, 17, 37). When mice were fed with myelin basic protein or collagen type II and then challenged with proteolipid protein or methylated bovine serum albumin, respectively, oral tolerance to myelin basic protein or collagen type II could be induced, suggesting that this immune suppression was mediated by bystander suppression (24, 40). These seemingly contradictory mechanisms of oral tolerance are now thought to be due to the antigenic dose. Feeding animals with high doses induced passive immune suppression mediated by deletion and/or anergy or receptor down-regulation, whereas, feeding with low doses stimulated active immune suppression mediated by CD4 and/or CD8 T cells (6, 10).

We have been studying tolerance to the oral bacterium *Actinomyces viscosus*, which is one of a group of the early pioneer bacteria that colonize tooth surfaces. *A. viscosus* has been implicated in dental root surface caries and gingivitis in humans (14, 41). Previously, we have shown that antigen specific oral tolerance in mice could be induced by gastric immunization with low doses of *A. viscosus*. This immune suppression was mediated by

antigen specific suppressor cells that originated from the Peyer's patches and then migrated to the spleen (31). The aim of this study was to elucidate the phenotype of these T-suppressor cells thought to be responsible for the induction of oral tolerance to low doses of *A. viscosus*.

Material and methods

Animals

The female 6–8-week-old DBA/2 mice used in this study were supplied by the Central Animal Breeding Centre, The University of Queensland. Institutional Ethics approval was granted for this project.

Antigen preparation

A. viscosus T14 was a kind donation from Dr. A. C. R. Tanner, The Forsyth Institute, Boston, MA, and was revived from frozen stocks and grown on Trypticase Soy Agar plates containing 5% horse blood. Colonies from plates were cultivated in Trypticase Soy Broth (TSB) medium (BBL, Microbiology System. Cockeyville, MD). Both plates and broth cultures were incubated at 37°C in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in anaerobic jars for 3–5 days. The purity of cultures was monitored by Gram stain and colony morphology on Trypticase Soy Agar plates. *A. viscosus* from Trypticase Soy Broth were harvested by centrifugation and then washed in sterile phosphate buffered saline (PBS). The bacteria were suspended in PBS and heat killed at 100°C for 15 min, then stored at −20°C. The protein concentration was determined by a BCA protein assay kit using bovine serum albumin (BSA) as a standard (Pierce Biotechnology, Rockford, IL).

Elisa

Antibody levels were measured using an indirect ELISA method (30). Briefly, 50 µl of a 50 µg/ml suspension of heat-killed *A. viscosus* in PBS was pipetted into wells of a 96-well microtiter plate (Nunc. Roskilde, Denmark). The plate was centrifuged at 1750 × *g* for 5 min at 4°C, then 100 µl of cold 0.25% glutaraldehyde in PBS was added to all wells and left for 15 min at room temperature. The plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and also washed three times between all the following steps. Nonspecific binding was blocked by the addition of 300 µl of PBS-T and 1% skim dried milk. The plates were left overnight at 4°C. Samples were diluted to appropriate dilutions in PBS-T-0.1% skim dried milk and 100 µl was added to triplicate wells. Mouse anti-*A. viscosus* hyperimmune serum was added to all wells at a standard dilution of 1 : 1000. This dilution represented a 50% binding level and was taken as 100 ELISA Units (EU). The total immunoglobulin (Ig), IgA, IgM and IgG antibody specific isotypes were detected using biotinylated sheep antimouse whole Ig (Promega, Madison, WI) and antimouse IgA (Promega) at 1 : 5000 dilution and anti-IgM (Promega) and IgG (Promega) at 1 : 20,000 dilution and the plates were incubated for 60 min at room temperature. Streptavidin horseradish peroxidase (Amersham Int., Buckinghamshire, UK) was diluted at 1 : 10.000 and 100 µl was added to all wells. Following incubation for 30 min, 150 µl/well of substrate containing 0.0075% H_2O_2 and 2.5 mM *o*-Tolidine hydrochloride substrate in 100 mM phosphate citrate buffer (pH 4.5) were added to all wells. The blue color reaction was stopped after 10 min by 50 µl/well of 3 M HCl and the optical density (OD) was measured at 450 nm using a Titretek Multiscan (ICN-Flow Laboratory, Costa Mesa, CA). The OD readings of the background (PBS-T-0.1% skim dried milk instead of sample) were subtracted from all readings. The ELISA units were calculated by dividing the OD of the sample by that of the standard OD and then multiplying by 100 (30).

Delayed type hypersensitivity

Delayed type hypersensitivity was measured using footpad swelling as described previously (30). Briefly, 1 week after the last intraperitoneal injection of saline or heat-killed *A. viscosus*, mice were challenged intradermally, by giving a 5 µl injection of a 100 µg/ml suspension of *A. viscosus* in PBS into the left hind footpad using a fine needle attached to a Hamilton syringe (Hamilton Co., Reno, NV). Sterile saline 5 µl was injected into the right hind footpad as a negative control. The dorso-ventral thickness of the hind footpad was measured using a dial micrometer (Mitutoyo, Kawasaki-Shi, Japan). Measurements were taken before challenge and subtracted from the reading of footpad swelling after 24 h.

Serum antibody levels

Mice were sacrificed 24 h after footpad challenge. Blood was collected by cardiac puncture and the serum separated by centrifugation and stored at −20°C. Serum antibody levels to *A. viscosus* were determined using ELISA as described above. Serum was diluted 1 : 300 in PBS-skim dried milk and assayed in triplicate wells. Positive and negative controls were included in each assay.

Experimental procedures

Monoclonal antibody preparation

Rat antimouse CD4 clone GK1.5 (isotype IgG2b) (5), mouse antimouse CD8 clone 49–11.2 (isotype IgG2a) (16) and mouse anti-*Fusobacterium nucleatum* clone FN4 BA4 (isotype IgG2a) (2) were grown in 24-well plates (Nunc) in RPMI 1640 medium (CSL, Melbourne, Australia) containing 1% penicillin-streptomycin (CSL), 1% glutamine (Sigma Co., St. Louis, MO), 1% of 5×10^{-3} mercaptoethanol (Sigma) and 10% heat-inactivated fetal calf serum (CSL) at 37°C in 5% CO_2 in air. The cells were grown to confluence, harvested by centrifugation, washed, and re-suspended in sterile PBS. Pristane-treated BALB/c nu/nu mice were injected intraperitoneally with 0.5 ml of sterile PBS containing 1.5×10^6 cells of clone GK1.5 or 49–11.2. Cells from clone FN4 BA4 were also injected intraperitoneally into pristane-treated female BALB/c conventional mice. After 4–5 weeks, ascites fluid from these mice was harvested. Following removal of peritoneal cells and lipid, the antibodies were partially purified by ammonium sulfate precipitation, filter sterilized and stored at −20°C freezer until used. Protein concentration was determined by a BCA protein assay kit (Pierce). Institutional approval had been granted to generate ascetic fluid using this procedure.

Cell depletion in vivo and immunization procedure

Mice were divided into five groups as illustrated in Table 1, each group consisting of three to five mice. Group I, II, and III mice were injected intraperitoneally with 200 µl of sterile PBS for 3 consecutive days and weekly for 4 weeks. Using the same experimental protocol, group IV and V mice were injected with 200 µl of sterile PBS containing 1 mg of anti-*F. nucleatum* and antimouse CD8 monoclonal antibodies, respectively. Previous studies have shown that giving mice an intraperitoneal injection of 1 mg of antimouse CD4 or CD8 monoclonal antibodies for 3 consecutive days and weekly for 4 weeks completely depleted the T-cell CD4 and CD8 subpopulations for

Table 1. Induction of oral tolerance to heat-killed *A. viscosus* in CD8 cell-depleted mice

Group	Injection						
	Intraperitoneal (at days −3, − 1, 0)	Intragastric (at days +1, +2, +5, +7)	Intraperitoneal at day				
			+ 13	+ 14	+ 21	+ 28	
I	PBS	PBS	PBS	PBS	PBS	PBS	PBS
II	PBS	PBS	PBS	Avenue	PBS	Avenue	PBS
III	PBS	Avenue	PBS	Avenue	PBS	Avenue	PBS
IV	Anti-Fn Anti-Fn	Avenue	Anti-Fn	Avenue	Anti-Fn	Avenue	Anti-Fn
V	Anti-CD8 Anti-CD8	Avenue	Anti-CD8		Avenue	Anti-CD8	Avenue Anti-CD8

Anti-Fn, anti-*F. nucleatum* antibody; anti-CD8, anti-CD8 cell antibody; Avenue, heat-killed *A. viscosus*; PBS, phosphate buffered saline. Each group consisted of three to five mice.

more than 4 weeks after the last antibody injection (12, 30). Gastric feeding of *A. viscosus* for 2 days after the last three consecutive injections of saline or monoclonal antibodies induced tolerance. Group I mice were sham intragastrically immunized with 200 µl of saline containing 7.5% sodium bicarbonate at days 1, 2, and 5. One week later, mice were sham intraperitoneally immunized with 100 µl of saline weekly for 2 weeks. Using the same protocol, group II mice were sham intragastrically immunized and systemically immunized with 200 µl of saline containing 100 µg of heat-killed *A. viscosus*. Group III–V mice were intragastrically and systemically immunized (intraperitoneally) with 200 µl of saline containing 100 µg of heat-killed *A. viscosus*. One week after the last systemic immunization, the delayed type hypersensitivity response was determined, mice were sacrificed and blood taken. The levels of serum antibodies to heat-killed *A. viscosus* were assessed by ELISA.

Adoptive transfer of splenic CD4 and CD8 T-cells

Mice were sham gastrically immunized or gastrically immunized with heat-killed *A. viscosus* as above. One week after the last immunization, animals were sacrificed and the spleens removed. Spleen cell suspensions were prepared by passage through stainless steel grids. Adherent cells were removed by incubating the cell suspension in sterile Petri dishes for 30 min at 37°C in RPMI-1640. Nonadherent cells from each spleen suspension were collected and washed by centrifugation. Macrophage contamination was determined by Giemsa staining. The above procedure was repeated until no macrophage contamination was observed. The Ig-expressing cells were also removed by a panning method (39). Briefly, 50×10^6 viable cells in 10 ml of culture medium were placed in sterile Petri dishes coated with rabbit antimouse immunoglobulin (Dako, Carpinteria, CA) and incubated for 1 h at 4°C. The nonadherent Ig^- cells were carefully harvested and washed. The purity of the cells was assessed by flow cytometry (Becton Dickinson, Mountain View, CA). With this procedure, cell suspensions with less than 1% Ig^+ cells were obtained. A nonlytic cell separation was used to obtain CD4 or CD8 T cells from these cell suspensions as previously described (21). Briefly, 50×10^6 viable cells in 10 ml of culture medium were placed in sterile Petri dishes coated with rat antimouse CD4 T cells (clone GK 1.5) or mouse antimouse CD8 T cells (clone 49–11.2) and incubated for 1 h at 4°C. After harvesting and washing, the purity of the CD4 or CD8 cell suspensions was assessed by flow cytometry (Becton Dickinson). This procedure yielded more than 99% purity for both $CD4^+$ and $CD8^+$ T cells.

Recipient mice were injected intraperitoneally with 1 mg of either partially purified anti-CD4 or anti-CD8 antibodies for 3 consecutive days. Splenic CD4 T cells (40×10^6 cells in 100 µl of sterile PBS) from animals sham gastrically immunized or gastrically immunized with *A. viscosus* were adoptively transferred to CD4 T-cell-depleted recipients via the lateral tail vein. Using a similar method, splenic CD8 T cells from these animals were transferred to CD8 T-cell-depleted recipients. Twenty-four hours after cell transfer, all recipients were intraperitoneally immunized with 100 µl of sterile PBS containing 100 µg of *A. viscosus*. This systemic immunization was repeated 1 week later. Each group of recipients consisted of three mice. One week after the last immunization, the delayed type hypersensitivity response and the serum antibody levels were assessed as above.

Cell cocultures

Mice were immunized intraperitoneally with 100 µg of bacterial suspension in 100 µl of sterile PBS weekly for 2 weeks. One week after the last immunization, mice were injected intraperitoneally with anti-CD4 or anti-CD8 T-cell antibodies as described above. Two days after the last antibody injection, mice were sacrificed and spleen cells were obtained In other groups, animals were sham gastrically immunized or gastrically immunized with *A. viscosus* as above. One week after the last gastric immunization, mice were sacrificed and the spleens removed. Single cell preparations were prepared and the CD4 T cells and CD8 T cells were purified as above. Culture medium 1 ml containing 1×10^6 CD4 T cells and CD8 T cells was prepared from animals that had been gastrically immunized with *A. viscosus*. These cells were cocultured with equal volumes of medium and CD4- and CD8-depleted spleen T cells, respectively, from systemically immunized mice. As a control, CD4 and CD8 T cells from sham gastrically immunized animals were cocultured with CD4 and CD8 depleted splenic T cells, respectively, from systemically immunized mice. A cell suspension of 2 ml was added to each well of 24-well plates (Nunc), which were then incubated for 10 days at 37°C in 5% CO_2 in air. On day 3, 4 µg of *A. viscosus* cells were added to each well. The levels of splenic antibodies specific to *A. viscosus* were determined by ELISA as described above.

Statistical analysis

Data obtained from the experiments in which CD8 T cells were depleted prior to gastric intubation were analyzed by a one way analysis of variance followed by Fisher's least squares test. Data from cell transfers *in vivo* were analyzed using Student's t-test. Data from the coculture experiments were analyzed by repeat measurement test. All statistical analyses were carried out using a statistical software package (Minitab, Inc., State College, PA).

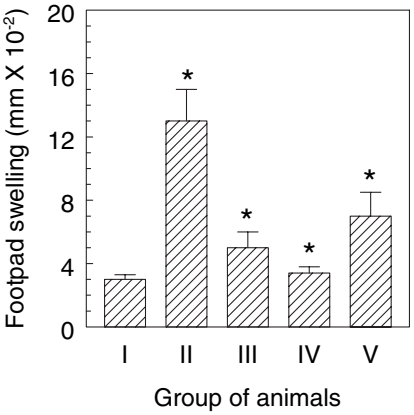

Fig. 1. Delayed type hypersensitivity response in CD8 T-cell-depleted mice following gastric immunization with heat-killed *A. viscosus.* See Table 1 for explanation of abbreviations. * $P < 0.05$.

Results

CD8 T-cell depletion *in vivo*

The delayed type hypersensitivity response in mice injected with anti-CD8 antibodies prior to gastric immunization with heat-killed *A. viscosus* is depicted in Fig. 1. The delayed type hypersensitivity response after gastric immunization with heat-killed *A. viscosus* (group III) was suppressed compared with that in group II mice ($P < 0.05$). A suppressed response could also be seen in mice injected with anti-*F. nucleatum* antibodies prior to gastric immunization with heat-killed *A. viscosus* (group IV) compared with group II mice ($P < 0.05$). Interestingly, the delayed type hypersensitivity response was also inhibited in mice injected with anti-CD8 antibodies prior to gastric immunization with heat-killed *A. viscosus* (group V) compared with that in group II mice ($P < 0.05$). Similarly, serum specific antibody isotypes in group III and IV were suppressed compared with those in group II mice ($P < 0.05$) (Fig. 2). Unlike the delayed type hypersensitivity response, the levels of serum antibodies in group IV were comparable to those in group II mice ($P > 0.05$).

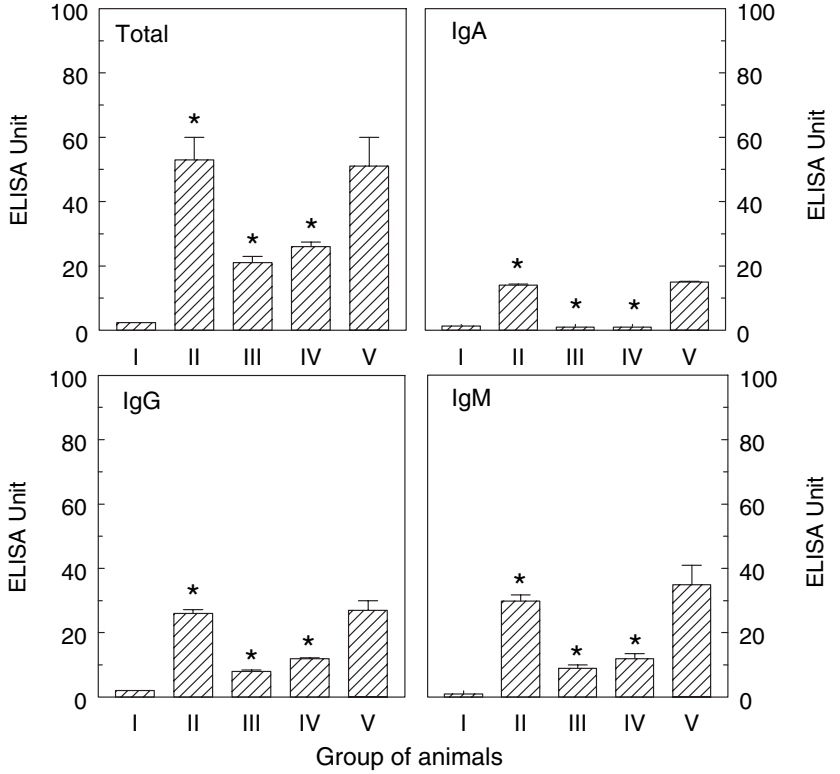

Fig. 2. Serum IgA, IgG, and IgM specific antibodies in CD8 T-cell-depleted mice following gastric immunization with heat-killed *A. viscosus.* See Table 1 for explanation of abbreviations. * $P < 0.05$.

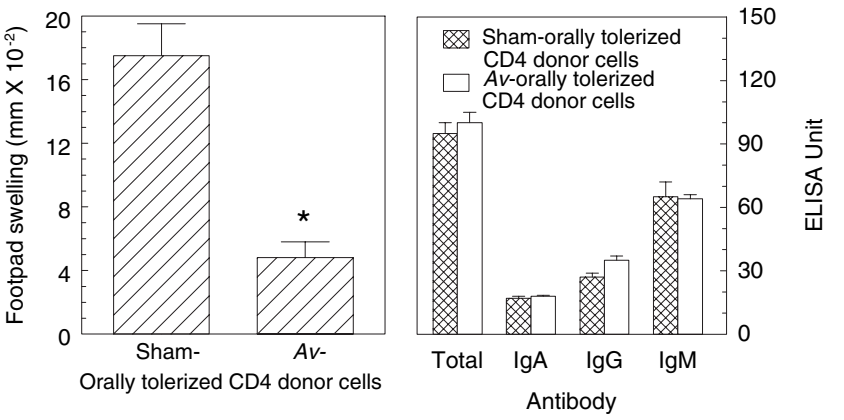

Fig. 3. Immune response in CD4 T-cell-depleted mice injected with CD4 T cells from mice gastrically immunized with heat-killed *A. viscosus.* Mice were gastrically immunized with *A. viscosus* or PBS. One week after the last gastric immunization, splenic CD4 T cells were obtained and transferred to the CD4 T-cell-depleted recipients. Twenty-four hours later, all recipients were intraperitoneally immunized with heat-killed *A. viscosus* and immunization was repeated 1 week later. Delayed type hypersensitivity response and serum specific antibody levels were determined 1 week after the last immunization by footpad swelling and ELISA, respectively. * $P < 0.05$.

Cell transfers

When CD4 T cells from animals gastrically immunized with heat-killed *A. viscosus* were injected into CD4 T-cell-depleted recipients, the delayed type hypersensitivity response in the recipients was significantly lower than that in animals transferred with CD4 T cells from animals sham gastrically immunized ($P < 0.05$) (Fig. 3). In sharp contrast, the levels of serum specific-antibody isotypes in the former recipients were comparable to those in the latter animals ($P > 0.05$) (Fig. 3). Furthermore, recipients injected with CD8 T cells from animals gastrically immunized with heat-killed *A. viscosus* showed a slightly reduced delayed type hypersensitivity response and significantly suppressed levels of serum specific-antibody isotypes when compared with mice injected with CD8 T cells from sham gastrically immunized animals ($P < 0.05$) (Fig. 4).

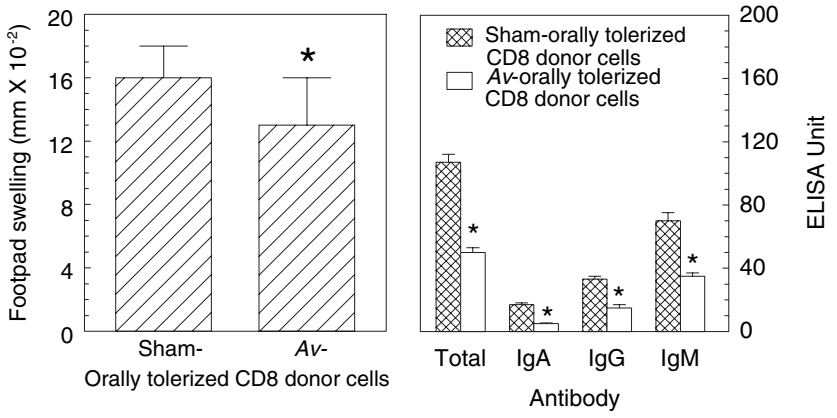

Fig. 4. The immune response in CD8 T-cell-depleted mice injected with CD8 T cells from mice gastrically immunized with heat-killed *A. viscosus.* Mice were gastrically immunized with heat-killed *A. viscosus* or phosphate buffered saline. One week after the last gastric immunization, splenic CD8 T cells were obtained and transferred to the CD8 T-cell-depleted recipients. Twenty-four hours later, all recipients were intraperitoneally immunized with heat-killed *A. viscosus* and immunization repeated 1 week later. Delayed type hypersensitivity response and serum specific antibody levels were determined 1 week after the last immunization by footpad swelling and ELISA, respectively. * $P < 0.05$.

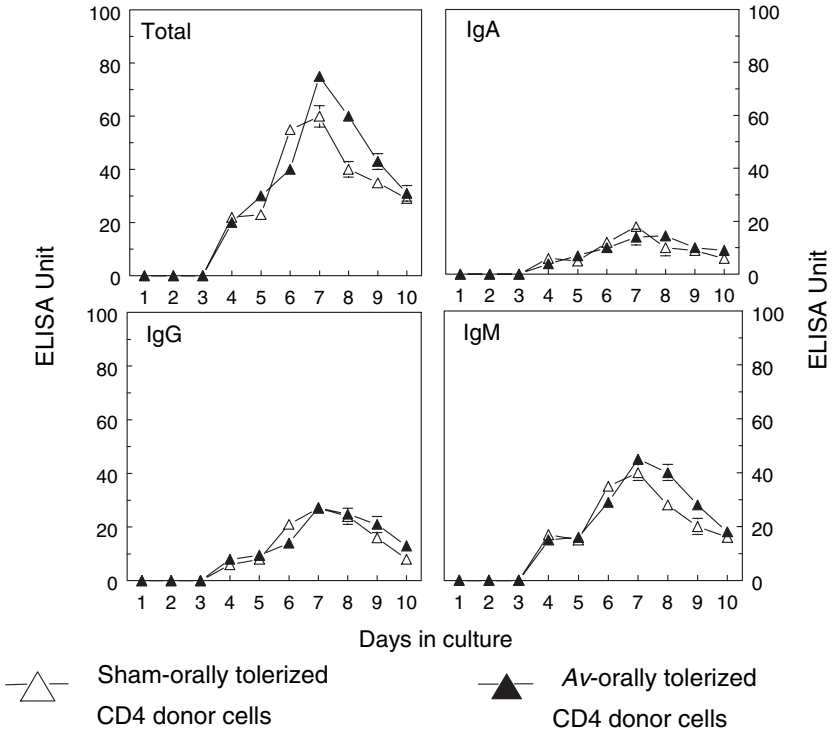

Fig. 5. Splenic specific antibody production in the cultures containing CD4 T cells from orally tolerized immunized mice and CD4-depleted T cells from systemically immunized mice. CD4 T cells were purified from spleens of mice gastrically immunized with PBS or heat-killed *A. viscosus.* Another group of mice was intraperitoneally immunized with heat-killed *A. viscosus* and then injected with anti-CD4 antibodies to obtained CD4 T-cell-depleted spleen cells. Equal numbers of purified CD4 T cells and CD4 T-cell-depleted spleen cells were cocultured and 4 μg of heat-killed *A. viscosus* suspension was added in each well on day 3 in the culture. Specific antibody production was determined from the culture supernatant by ELISA.

Cell cocultures

CD4 T cells from gastrically immunized animals were cocultured with spleen cells depleted of CD4 T cells from animals systemically immunized with heat-killed *A. viscosus.* The results showed that the production of splenic specific antibodies were comparable to those of cultures of cells from animals that were sham gastrically immunized and systemically immunized ($P < 0.05$) (Figs 5 and 6). However, suppression of splenic specific antibodies was seen in cocultures of CD8 T cells from animals gastrically immunized and CD8 T-cell-depleted spleen cells from animals systemically immunized ($P < 0.05$) (Fig. 6).

Discussion

In a previous study we showed that oral tolerance to heat-killed *A. viscosus* in mice was transferable by cells and not by serum, suggesting that this immune tolerance was mediated by antigen specific suppressor cells (31). The results of this present study further demonstrated that the induction of tolerance to low doses of *A. viscosus* was mediated by cells with the phenotype $CD4^+$ and $CD8^+$ T-suppressor cells. It became evident that when CD8 T-cell-depleted mice were gastrically immunized with heat-killed *A. viscosus*, suppression of serum antiheat-killed *A. viscosus* antibody levels could be abrogated. On the other hand, suppression of the delayed type hypersensitivity response in this group of animals remained intact. To confirm these findings, transfer of CD8 T cells from gastrically immunized animals into CD8 T-cell-depleted recipients also resulted in suppressed serum specific antibody levels, but not the delayed type hypersensitivity response. Suppressed splenic specific antibody production was also seen in cultures containing CD8 T cells from gastrically immunized animals and CD8 T-cell-depleted splenocytes from the systemically immunized mice. These results suggest that gastric immunization with heat-killed *A. viscosus* may induce activation of CD8 T cells, which in turn suppress the production of specific antibody following systemic challenge with heat-killed *A. viscosus*. In previous studies, conflicting results have been reported (9, 19, 21, 25) where the transfer of $CD8^+$ T cells from spleens of mice gastrically immunized with myelin basic protein (MBP) (19), S-antigen (25) and human thyroglobulin (9) resulted in the induction of oral tolerance in the recipients. In contrast, oral tolerance remained intact when low doses of ovalbumin (1, 11), S-antigen (36), and MBP (3) were injected into mice that had been treated with anti-CD8 cell antibodies prior to gastric immunization This suggests that CD8 T cells may not be required in the induction of this immune tolerance. In our study, *A. viscosus*-induced oral tolerance in

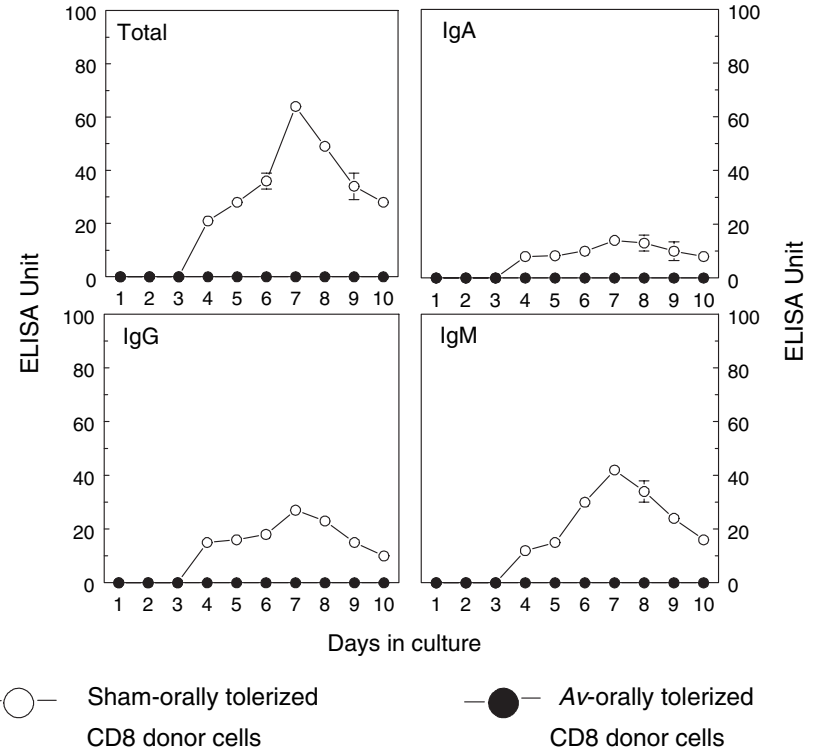

Fig. 6. Splenic specific antibody production in the cultures containing CD8 T cells from gastric tolerized immunized mice and spleen cells depleted of CD8 T cells from systemically immunized mice. CD8 T cells were purified from spleens of mice gastrically immunized with phosphate buffered saline or heat-killed *A. viscosus.* Another group of mice was intraperitoneally immunized with *A. viscosus* and then injected with anti-CD8 cell antibodies to obtained spleen cells depleted of CD8 T cells. Equal numbers of purified CD8 T cells and spleen cells depleted of CD8 cells were cocultured and 4 μg of heat-killed bacterial suspension was added to each well on day 3 in the culture. Specific antibody production was determined from the culture supernatant by ELISA.

the humoral immune limb of CD8 T-cell-depleted mice could still be observed. The exact reason for this discrepancy remains to be determined but it may be due to different types of antigens. When bacterial antigens are given gastrically, they may induce different systemic immune profiles when compared with nonbacterial antigens (38). How CD8 T cells could suppress only serum specific antibodies in mice gastrically immunized with heat-killed *A. viscosus* is not known at this stage. One possibility is that CD8 T cells activated following gastric immunization may directly inhibit B-cell activation, as suggested previously (20). Alternatively, gastrically activated CD8 T cells may secrete cytokines such as tumor growth factor beta, which in turn down-regulate B-cell activation (18).

Failure of CD8 T cells to induce oral tolerance to heat-killed *A. viscosus* in mice could mean that delayed type hypersensitivity suppression after gastric immunization may be mediated by CD4 T cells. Indeed, transfer of CD4 T cells obtained from mice gastrically immunized with heat-killed *A. viscosus* to CD4 T-cell-depleted recipients did result in suppression of the delayed type hypersensitivity response, but not of serum specific antibody levels. It has been well documented that gastric immunization with antigens such as beta-lactoglobulin (34), collagen type II (8), and ovalbumin (26) resulted in suppressed activation of interferon gamma-producing T-helper type I (Th1) cells and enhanced activation of interleukin (IL)-4, IL-10 and tumor growth factor beta-producing Th2 cells. This suggests that suppression of Th1 T-cell activation after gastric immunization may be due to the action of Th2 T-cell-derived IL-10, IL-4, and/or Th3 T-cell-derived tumor growth factor beta (8, 34). In this respect, Rizzo and colleagues (28) provided evidence that the suppressed delayed type hypersensitivity response and reduced inflammation score of experimental autoimmune uveitis seen in mice gastrically immunized with interphotoreceptor retinoid-binding protein could only be achieved in the presence of both IL-4 and IL-10, indicating that oral tolerance of the delayed type hypersensitivity response may be mediated by IL-4 and IL-10. Therefore, one may speculate that the suppressed delayed type hypersensitivity response after gastric immunization with low doses of heat-killed *A. viscosus* may also be mediated IL-10- and/or IL-4-secreting CD4 T cells. This requires further clarification as the cytokine profiles in mice after gastric immunization with this oral bacterium were not assessed in the present study.

Recently, a regulatory $CD25^+CD4^+$ T-cell subset that secretes high levels of IL-10 and tumor growth factor beta and may act as suppressor T cells and play a role in controlling the immune response has been reported ([7] for review). Indeed, gastric immunization with ovalbumin (33, 42), beta-lactoglobulin (35) or human HSP60-derived uveitogenic peptides (27) was shown to result in oral tolerance to the respective antigens and to induce a high number of these regulatory CD4 T cells. Interestingly, transfer of orally activated $CD25^+CD4^+$ T cells led to a suppression of the delayed type hypersensitivity response in recipients, suggesting that this regulatory CD4 T-cell subset may play a crucial role in oral tolerance of the cellular immune limb (42). Gastrically activated CD4 T cells which mediated suppressed delayed type hypersensitivity responses were found in the present study and the existence of these regulatory CD4 T cells cannot be ruled out.

Activation of both CD4 and CD8 T cells in the intestinal Peyer's patch after gastric immunization has been well documented (6, 10). The results of the present study further demonstrated that heat-killed *A. viscosus*-induced oral tolerance of the cellular and humoral immune limb was mediated by $CD4^+$ T cells and $CD8^+$ T cells, respectively, suggesting that the delayed type hypersensitivity and humoral immune response are regulated independently by distinct T-cell subsets. A similar suggestion has also been put forward by da Silva and colleagues (4), who showed that humoral immune suppression could only be observed in mice susceptible to ovalbumin-induced oral tolerance but not in resistant mice. Delayed type hypersensitivity unresponsiveness could be induced in both susceptible and resistant mice, but this was abrogated by cyclophosphamide pretreatment in the latter (4). The authors also concluded that oral tolerance of both the delayed type hypersensitivity and antibody response was independently regulated.

Extrapolation of the results of the present study to the immunopathogenesis of periodontal disease remains speculative. There is an increase in the number of *A. viscosus* isolated from the dental plaque of patients with periodontal disease, particularly those not responding to periodontal treatments (14, 41). The number of peripheral blood T cells specific to *A. viscosus* has been shown to be slightly increased in patients with gingivitis and decreased following treatment (22, 23), suggesting that this oral bacterium may play a role in the immunopathogenesis of periodontal disease. Because this bacterium is part of the oral microbial community it is possible that the bacterium is constantly being swallowed. This may induce activation within the mucosal immune system or oral tolerance. If so, both CD4 and CD8 T cells activated by *A. viscosus* via the intestinal sites may also contribute to the altered CD4 : CD8 T-cell ratio seen in inflamed gingival tissues (13, 32).

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