Biomedicine & Pharmacotherapy 70 (2015) 294–298

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Original article

The effect of immune status, age and genetic background on induction of oral tolerance to *Actinomyces viscosus* in mice

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ARTICLE INFO

Article history:
Received 27 November 2014
Accepted 30 December 2014

Keywords:
Age
A. viscosus
Genetic
Immune status
Oral tolerance

ABSTRACT

The aim of the present study was to determine the effect of immune status, age and genetic background on the induction of oral tolerance to *Actinomyces viscosus*. Suppression of delayed type hypersensitivity (DTH) response and antigen-specific serum antibody levels could be induced in DBA/2 mice intragastrically and systemically immunized with *A. viscocus*, suggesting the induction of oral tolerance. In contrast, this immune suppression could be abrogated if the animals had been systemically immunized prior to the induction of oral tolerance with the same bacterium. Long-term systemic immunization prior to intragastric immunization with *A. viscocus* suppressed DTH response only. Cell transfer of this group of animals also suppressed DTH response in the donors, indicating the action of suppressor cells for inhibition of DTH response. Furthermore, oral tolerance to *A. viscocus* failed to occur in mice aged at 3 days and 1, 2, 4, 6 and 36 weeks old. Mice bearing H-2^d haplotype were the most susceptible to oral tolerization, followed by H-2^b and H-2^k. Therefore, the results of the presence study suggest that the induction of oral tolerance to *A. viscosus* in mice may be dependence on the immune status and genetic background but not age.

1. Introduction

Oral tolerance or mucosal immune tolerance is an antigen-specific systemic immune suppression following mucosal presentation of antigen [1,2]. While this immune phenomenon may be a part of host defenses against mucosal antigens, it has also been shown to be a potential immunotherapy to, at least, autoimmune diseases [1,2]. It has been well recognized that the induction of oral tolerance is affected by a number of different factors. For example, when mice were systemically primed with antigen, oral immunization with the same antigen failed to suppress the existing immune response, suggesting that oral tolerance is dependence on the immune status [3,4]. These observations were further confirmed by the fact that oral immunization failed to be suppress the ongoing antigen-induced arthritis in rats [5]. Furthermore, oral immunization with ovalbumin (OVA) or myelin basic protein (MBP) in rodent neonates, but not in rodent young adults, failed to induce oral tolerance [6–8], suggesting that the induction of oral tolerance to proteins may be determined by age. With respect to genetic background, certain mice strains bearing different H-2 haplotypes do have the capability of inducing oral tolerance more efficiently than other mice strains [9–11].

Actinomyces viscosus, a commensal gram-positive facultative anaerobic bacterium, is among the first organisms to colonize on the tooth surface and has been associated with root caries [12,13] and gingivitis in humans [14]. Our previous studies indicated that gastric intubation with *A. viscosus* in mice results in the induction of oral tolerance and antibody production by Peyer's patch cells, concomitantly [15,16]. Furthermore, suppression of systemic antibody production and delayed type hypersensitivity (DTH) following gastric intubation with *A. viscosus* are mediated by $CD8^+$ and $CD4^+$T suppressor cell, respectively [17,18]. Since oral tolerance is dependence on factors such as age and genetic background, the aim of the present study, therefore, was to test the hypothesis that the induction of oral tolerance to *A. viscosus* in mice may be determined by the immune status, age and genetic background.

2. Materials and methods

2.1. Animals

Female 6–8 weeks old C57BL/6 (H-2^b), Balb/C (H-2^d), DBA/2 (H-2^d), and C3H/HeJ (H-2^k) mice used in this study were supplied

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http://dx.doi.org/10.1016/j.biopha.2014.12.039
0753-3322/

by the Central Animal Breeding Centre, The University of Queensland. Institutional Ethics approval was granted for this project.

2.2. Antigen preparation

A. viscosus T14 was a kind donation from Dr. A.C.R. Tanner, The Forsyth Institute, Boston, MA and grown at 37 °C in atmosphere of 80% N_2, 10% CO_2 and 10% H_2 in anaerobic jars as previously described [15]. The purity of cultures was monitored by Gram stain and colony morphology on Trypticase Soy Agar plates. After harvesting by centrifugation and then washing 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).

2.3. Elisa

Antibody levels were measured using an indirect ELISA method [15]. Briefly, 50 μL of a 50 μg/ml suspension of *A. viscosus* in PBS was pipetted into wells of a 96-well microtitre plate (Nuncl, Roskilde, Denmark). The plate was centrifuged at 1750 × g for 5 minutes 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 (RT). The plates were washed three times and also washed three times between all other steps (below) and non-specific binding was blocked by the addition of 300 μL of PBS containing 0.05% Tween-20 (PBS-T) and 1% skim dried milk (SM). The plates were left overnight at 4 °C. After washing for three times in PBS-T, samples were diluted to appropriate dilution in PBS-T-0.1% SM and 100 μL were added to triplicate wells. A mouse anti-*A. viscosus* hyperimmune serum was used on each plate 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 anti-mouse whole Ig and anti-mouse IgA at 1:5000 and anti-IgM and IgG at 1:20,000 and the plates were incubated for 60 min at room temperature. These antibodies were purchased from Promega, Madison, WI. Streptavidin horseradish peroxidase (Amersham International, Buckinghamshire, UK) 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 Lab, Costa Mesa, CA). The OD readings of the background (PBS-TSM instead of sample) were subtracted from all readings. The ELISA units (EU) were calculated by dividing the OD of the sample by that of the standard OD and then multiplying by 100 [15].

2.4. Delayed type hypersensitivity (DTH)

DTH was measured using footpad swelling as described previously [15]. Briefly, 1 week after the last intraperitoneal injection of saline or heat-killed *A. viscosus*, mice were challenged intradermally, by giving a five microlitre injection of a 100 μg/mL suspension of heat-killed *A. viscosus* in PBS into the left hind footpad using a fine needle attached to a Hamilton syringe (Hamilton Co., Reno, Nevada). 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 hours.

2.5. Immunization procedure

In order to assess the effect of immune status on the *A. viscosus*-induced oral tolerance, DBA/2 mice were divided into five groups as illustrated in Table 1, each consisting of three to five mice. Intragastric (ig) challenge was carried out by passing a thin flexible tubing attached to a rounded end needle into the stomach of the mouse. *A. viscosus* was given as a 100 μg of bacterial suspension in 0.2 mL of PBS containing 7.5% sodium bicarbonate (PBS-SB). Groups I, II and III were intraperitoneally injected with 100 μL of PBS weekly for 2 weeks, whereas groups IV and V were injected with 100 μL of PBS containing 100 μg of bacterial antigen at the same time. One week later, groups I and II were intragastrically challenged with PBS-SB only for 2 consecutive days and repeated 3 days later, whereas groups III and IV were intragastrically challenged with bacterial suspension at the same time. Group V was intragastrically challenged for 14 consecutive days. One week after the last gastric intubation, group I mice were intraperitoneally challenged with 100 μL of PBS weekly for 2 weeks, whereas the remaining groups of animals were intraperitoneally injected with 100 μg of bacterial suspension in 100 μL of PBS at the same time.

In order to determine the effect of age on the *A. viscosus*-induced oral tolerance, DBA/2 mice aged 3 days and 1, 2, 4, 6, and 36 weeks old were intragastrically challenged with 100 μg of bacterial suspension in 0.2 mL of PBS-SB for 2 consecutive days and repeated 3 days later. One week after the last intragastric challenged, mice were intraperitoneally injected with 100 μg of bacterial suspension in 100 μL of PBS weekly for 2 weeks. As the control (group C), female 6 weeks old mice were sham intragastrically intubated and, then intraperitoneally injected with 100 μg of bacterial suspension in 100 μL of PBS weekly for 2 weeks.

In order to assess the effect of genetic background on the *A. viscosus*-induced oral tolerance, each of the female 6 weeks old C57BL/6 (H-2^b), Balb/C (H-2^d), DBA/2 (H-2^d), and C3H/HeJ (H-2^k) mice strains was divided into 2 groups. Group 1 was sham gastrically challenged, whereas group 2 was gastrically challenged with bacterial suspension as above. Subsequently, all animals were intraperitoneally injected with bacterial suspension. The immunization procedure was carried as above.

2.6. Adoptive transfer experiment

DBA/2 mice were systemically immunized and long-term gastrically intubated with bacterial suspension. One week after the last gastric intubation, mice were sacrificed and the spleens were removed. The adoptive transfer experiment was carried out

Table 1
The effect of immune status on the induction of oral tolerance to *Actinomyces viscosus*.

Group	Injection			
	Intraperitoneal (at day −14, −7, 0)	Intragastric (at day +7, +8, +11)	Intraperitoneal (at day)	
			+18	+25
I	PBS	PBS	PBS	PBS
II	PBS	PBS	Av	Av
III	PBS	Av	Av	Av
IV	Av	Av	Av	Av
V	Av	Av[a]	Av	Av

Av: heat-killed *A. viscosus*; PBS: saline.
[a] Intragastric injection for 14 consecutive days. Each group consisted of 3–5 mice.