

RESEARCH ARTICLE

Intravenous IgA complexed with antigen reduces primary antibody response to the antigen and anaphylaxis upon antigen re-exposure by inhibiting Th1 and Th2 activation in mice

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Abstract

Context: Serum IgG, IgE and IgM have been shown to enhance the primary antibody responses upon exposure to the soluble antigens recognized by those antibodies. However, how IgA affects these responses remains unknown.

Objective: We investigated the effects of intravenously administered monoclonal IgA on the immune responses in mice.

Materials and methods: DBA/1J mice were immunized with ovalbumin in the presence or absence of anti-ovalbumin monoclonal IgA. The Th1 and Th2 immune responses to ovalbumin and the anaphylaxis induced by re-exposure to ovalbumin were measured.

Results: IgA complexed with antigen attenuated the primary antibody responses to the antigen in mice, in contrast to IgG2b and IgE. The primary antibody responses, i.e. the *de novo* synthesis of anti-ovalbumin IgG2a, IgG1 and IgE in the serum, and the subsequent anaphylaxis induced with re-exposure to ovalbumin were reduced by the co-injection of anti-ovalbumin monoclonal IgA at ovalbumin immunization. The Th1, Th2 and Tr1 cytokines interferon- γ , interleukin-4 and interleukin-10, respectively, released from ovalbumin-restimulated cultured splenocytes collected from allergic mice were also reduced by the treatment. The induction of interferon- γ and interleukin-4 secretion by splenocytes from ovalbumin-immunized mice stimulated *in vitro* with ovalbumin was also significantly reduced by the antigen complexed with anti-ovalbumin IgA.

Conclusion: These data suggest that the direct inhibition of Th1 and Th2 activation by anti-ovalbumin monoclonal IgA participates in the inhibition of the primary antibody responses. IgA plays important immunosuppressive roles under physiological and pathological conditions and is a promising candidate drug for the treatment of immune disorders.

Keywords

Anaphylaxis, IgA, immunosuppression, primary antibody response, Th1/Th2

History

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Introduction

Immunoglobulin A (IgA) is the predominant Ig isotype secreted by the mucosa, and its production in the body is the highest of all the Ig isotypes¹. Secretory IgA, a major form of IgA, which is secreted onto the mucosa, is generated by multiple processes^{2–4}. Plasma cells in the lymphatic organs closely associated with the mucosa produce dimeric and polymeric IgA, which are composed of two or several alpha heavy chains and light chains, respectively, covalently conjugated with one joining chain (J chain)⁴. The J chain in IgA, as in IgM (another polymeric immunoglobulin), promotes the dimerization and polymerization of these isotypes⁴. Although the presence of the J chain in IgM is

known to reduce its activating effects on complement⁵, dimeric and polymeric IgAs containing the J chain activate complement more effectively than does monomeric IgA⁶. The J chain also contributes to the transcytosis of dimeric IgA from the basolateral side of epithelial cells to their lumens because it binds to the polymeric immunoglobulin receptor (pIgR) expressed on these cells⁷. A cleaved portion of pIgR, called the “secretory component” (SC), and dimeric IgA form secretory IgA when it is secreted onto the mucosa. Secretory IgA is distributed onto the surface of the mucosa in response to the hydrophilic properties of SC⁸ and is protected from enzymatic cleavage by the wrapping properties of SC at this site^{9–11}. Secretory IgA cannot associate as effectively with the IgA receptor Fc α RI as dimeric IgA because of the steric hindrance afforded by SC^{12,13}. Therefore, secretory IgA exhibits non-inflammatory properties and is a convenient defense system on a wide range of mucosal surfaces. For example, secretory IgA prevents the invasion of commensal and pathogenic bacteria^{14,15} and considerably inhibits the

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penetration of antigens and toxins¹⁵ into the circulation, without inducing excessive systemic inflammatory responses.

In the serum, IgA is mainly composed of monomers in humans and dimers in mice, and is the second most common antibody among the Ig isotypes¹⁶. However, the role of IgA in the serum remains relatively unclear. Because the IgA receptor Fc α RI is genetically absent in mice, investigation of the functions of Fc α RI *in vivo* was difficult before the establishment of human IgA knock-in mice¹⁷ and human Fc α RI transgenic mice¹⁸. Only limited information on the functions of serum IgA *in vivo*, mediated by receptors such as pIgR¹⁹ and the asialoglycoprotein receptor (ASGPR)²⁰, has been obtained from mouse models. IgA-dependent protection was not induced against infection with *Streptococcus pneumoniae*²¹ in pIgR knockout mice, whereas IgA which recognizes the hepatitis A virus was shown to mediate the infection of the virus via ASGPR²².

Hjelm et al.²³ demonstrated the important functions of serum IgG, IgM and IgE. These isotypes enhance the primary antibody responses by inducing effective immune responses against antigens that penetrate the circulation. IgG1, IgG2a and IgG2b complexed with the recognized antigen enhance the primary antibody responses to the antigen, possibly through the Fc γ receptors, as IgG3 and IgM do by activating the complement pathway²³. IgE also enhances the antibody response by inducing B-cell activation via its association with CD23, the low-affinity IgE receptor Fc ϵ RII^{23,24}. In contrast, how the presence of IgA complexed with an antigen at immunization influences the primary and subsequent antibody responses against the antigen and the development of the pathogenic condition have not been clarified.

Therefore, in the present study, we established hybridomas producing anti-ovalbumin (anti-OVA) IgA monoclonal antibodies (OA-2 and OA-4) and investigated the mechanisms by which anti-OVA monoclonal IgA complexed with OVA reduces the primary antibody responses to the antigen in naïve mice compared with the promotion of these responses by anti-OVA IgG2b and IgE. We also examined the effects of OA-2 and OA-4 on the establishment of an allergic constitution, which causes anaphylactic shock following re-exposure to OVA, in mice. To clarify the mechanisms underlying the effects of IgA on primary antibody responses, we also investigated the differences in the Th1/Th2/Tr1 cytokine production by OVA-restimulated cultured splenocytes from mice administered OVA alone- or the OVA/OA-4 immune complex and by cultured splenocytes from OVA-immunized mice that were stimulated *in vitro* with OVA- or the OVA/OA-4 immune complex.

Materials and methods

Antibodies

Hybridomas producing anti-OVA monoclonal IgA (OA-2 and OA-4), anti-OVA monoclonal IgG2b (O2B-3) and anti-OVA monoclonal IgE (OE-1) were established previously using PEG-1500 (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. Affinity chromatography was used to purify the anti-OVA antibodies. Briefly, the hybridomas were seeded at 2×10^6 cells/20 ml of RPMI-1640 (Wako Pure Chemical

Industries, Ltd, Osaka, Japan) containing 10% fetal bovine serum (BioWest SAS, Nuaillé, France) and penicillin (100 units/ml)–streptomycin (100 μ g/ml) (Wako Pure Chemical Industries, Ltd). After 4 days, the culture supernatants were applied to HiTrap NHS-activated HP columns (GE Healthcare UK Ltd, Buckinghamshire, UK) coupled with OVA. These columns were washed with phosphate-buffered saline (PBS) before the application of elution buffer (0.1 M glycine, pH 3). The eluent was mixed with neutralization buffer (1 M Tris–HCl, pH 8) and the anti-OVA-antibody-containing solution was ultrafiltered with a 3000 Da molecular cutoff membrane (Vivaspin 20; Sartorius Stedim Biotech GmbH, Goettingen, Germany). The buffer was then changed to PBS using a PD10 column (GE Healthcare UK Ltd). The purity of the purified anti-OVA antibodies was assessed with Laemmli's sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Other antibodies were purchased as follows: anti-phosphorylcholine (PC) monoclonal IgA (clone TEPC15; Sigma-Aldrich Co., St. Louis, MO); alkaline phosphatase-conjugated anti-mouse IgA (SouthernBiotech, Birmingham, AL); unlabeled anti-mouse IgG1 (Thermo Fisher Scientific, Inc., Rockford, IL); unlabeled anti-mouse IgG2a (Bethyl Laboratories, Inc., Montgomery, TX); alkaline phosphatase-conjugated anti-mouse IgG1 (X56) and IgG2a (R19-15) (BD Biosciences, San Jose, CA); alkaline phosphatase-conjugated anti-mouse IgG2b (Santa Cruz Biotechnology, Inc., Dallas, TX); anti-mouse IgE (LO-ME-3; GeneTex Inc., Irvine, CA) and alkaline phosphatase-conjugated anti-biotin (Vector Laboratories Inc., Burlingame, CA).

Animals

Female DBA/1J mice (8–12 weeks old; Charles River Laboratories Japan Inc., Yokohama, Japan) were maintained in a temperature-controlled environment with standard chow and water. Each group of 3–6 mice was housed in a separate cage. All animal procedures were approved by the Animal Ethics Committee of Kobe Pharmaceutical University.

Immunoblotting analysis and ELISAs to determine affinities for OVA, and competitive ELISAs to determine the differences in the epitopes recognized by two anti-OVA monoclonal IgAs

Affinity-purified OA-2 and OA-4 (0.1 mg/ml) were separated by SDS–PAGE with and without 2-mercaptoethanol (Sigma-Aldrich) on a 5–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan). The proteins were then transferred from the gel to a PVDF membrane (ATTO). After the membrane was blocked with SuperBlock Blocking Buffer in PBS (Thermo Fisher Scientific, Inc.), OA-2 and OA-4 were detected with alkaline phosphatase-conjugated anti-mouse IgA and NBT/BCIP (Bio-Rad Laboratories, Inc., Hercules, CA).

To compare the affinities of OA-2 and OA-4 for OVA, ELISA plates were coated with 100 μ l of OVA (100 μ g/ml; Sigma-Aldrich) in PBS at 4 °C for 20 h. After the plates were blocked with 1% casein (Sigma-Aldrich) in PBS, 100 μ l of the indicated concentrations of OA-2 and OA-4 were added to the wells. The OA-2 and OA-4 bound to OVA were detected

colorimetrically using alkaline phosphatase-conjugated anti-mouse IgA and *p*-nitrophenyl phosphate (Sigma-Aldrich).

To determine the differences in the epitopes recognized by the two anti-OVA monoclonal IgAs, the indicated concentrations of OA-2 or OA-4 were added to the wells of the OVA-coated and casein-blocked ELISA plates. After the unlabeled OA-2 or OA-4 was bound to the OVA-coated wells, biotinylated OA-2 or OA-4 was added to the wells. The biotinylated OA-2 or OA-4 bound to the OVA was then detected with alkaline phosphatase-conjugated anti-biotin antibody.

Immunization and parenteral antigen-induced anaphylaxis

Mice were immunized with an intravenous injection of OVA (40 µg) alone or with OVA (40 µg) incubated for 1 h at 37 °C with OA-4, OE-1 or O2B-3 (70, 140 or 280 µg) in 200 µl of PBS on Day 0. The mouse sera were collected on Day 11 or 14. Non-sensitized control mice were injected with 200 µl of PBS.

To investigate the preventive effects of anti-OVA IgA on the establishment of an allergic constitution, mice were sensitized with an intravenous injection of 40 µg of OVA, hemocyanin from *Limulus polyphemus* hemolymph (HEM; Sigma-Aldrich), or PC-keyhole limpet hemocyanin (PC-KLH; Biosearch Technologies Inc., Novato, CA) pre-incubated with or without OA-2, OA-4 or anti-PC monoclonal IgA (140 µg) for 1 h at 37 °C in 200 µl of PBS on Day 0. The mouse sera were collected on Day 11. The mice were then challenged with intravenous injections of 40 µg of OVA, HEM or PC-KLH in PBS on Days 14 and 36 and their rectal temperatures were measured²⁵. The mouse mast cell protease-1 (MMCP-1) concentrations in sera collected 1 h after the challenge on Day 14 were measured using a commercially available ELISA kit, following the manufacturer's instructions (eBioscience, Inc., San Diego, CA). The sera and spleens of these mice were collected 3 days later (Day 39).

In some experiments, naïve mice were immunized 1 h after the intravenous administration of OA-4 with an intravenous or intraperitoneal injection of the indicated amounts of OVA in PBS or OVA mixed with ImjectAlum (Thermo Fisher Scientific, Inc.).

Measurement of anti-OVA, anti-HEM and anti-PC-KLH antibodies in sera

The levels of anti-OVA IgA including OA-4 in sera diluted 1:50 or 1:5000 were determined by detecting the binding activities of anti-OVA monoclonal IgA to OVA as described above. Anti-OVA IgG2a, IgG2b and IgG1 levels were measured in the sera using OVA-coated plates, as previously described (antigen-coating method)^{25,26}, except when sera diluted 1:500 (for IgG2a and IgG2b) or 1:5000 (for IgG1) were used. Anti-OVA IgG2a and IgG1 levels were also determined with another ELISA using unlabeled anti-mouse IgG2a- or IgG1-coated plates (antibody-coating method) using sera diluted 1:50 (for both IgG2a and IgG1). This was similar to the previously reported ELISA used to detect anti-OVA IgE with unlabeled anti-mouse-IgE-coated plates²⁵. Similarly, anti-HEM and anti-PC-KLH IgG2a and IgG1 levels

were determined, except that HEM and PC-KLH were used as the coating antigens. Sera diluted 1:5, 1:50, 1:50 and 1:500 were used to determine the anti-HEM IgG2a, anti-HEM IgG1, anti-PC-KLH IgG2a and anti-PC-KLH IgG1 levels, respectively.

Measurement of cytokines secreted by cultured spleen cells

Splenocytes (5×10^6 cells/ml) from mice immunized with OVA and OA-2 or OA-4 were prepared and cultured for 3 days with OVA (100 µg/ml). The cytokines released from the splenocytes into the culture supernatants were determined using commercially available ELISA kits (eBioscience, Inc.). In another experiment, the effects of OA-4 on cells cultured *in vitro* were determined. Mice were immunized with intravenous injections of OVA (40 µg) in 100 µl of PBS on Days 0, 14 and 36. Three days later, their splenocytes (5×10^6 cells/ml) were collected and cultured for 3 days with OVA (100 µg/ml) pre-incubated for 1 h with the indicated concentrations of OA-4.

Statistical analysis

The Mann-Whitney *U*-test was used to compare differences between two independent groups. Tukey's *post hoc* test was only performed for multiple-group comparisons when the *p* value for one-way analysis of variance (ANOVA) was <0.05. A *p* value of <0.05, determined with the Mann-Whitney *U*-test and Tukey's *post hoc* test, was considered significant. For clarity, only comparisons with the OVA or PC-KLH group are shown in the figures.

Results

Differences in the epitopes recognized by two anti-OVA monoclonal IgAs, OA-2 and OA-4, whose structures and affinities for OVA were similar

Immunoblotting showed that purified OA-2 and OA-4, produced by established hybridomas were similarly composed of IgA dimers and polymers, with lower amounts of IgA monomers (Figure 1A). Their binding to OVA in ELISAs was also similar at concentrations of 3–3000 ng/ml (Figure 1B). The binding of the biotinylated anti-OVA IgAs to OVA was strongly inhibited by the same unlabeled antibodies (Figure 1C; biotinylated OA-2 versus OA-2 and biotinylated OA-4 versus OA-4). In contrast, unlabeled OA-4 failed to affect the binding of biotinylated OA-2 to OVA (Figure 1C; biotinylated OA-2 versus OA-4). Unlabeled OA-2 significantly, but only slightly, inhibited the binding of biotinylated OA-4 to OVA (Figure 1C; biotinylated OA-4 versus OA-2). These results indicate that OA-2 and OA-4 have similar structures and affinities for OVA, but recognize different epitopes on OVA.

OA-4 complexed with OVA reduced the *de novo* synthesis of IgGs against the antigen in naïve mice

Because anti-OVA monoclonal IgAs OA-2 and OA-4 are mainly composed of dimers and polymers (Figure 1A), similar to naturally produced IgA in the sera of mice, we investigated the functions of serum IgA in mice with

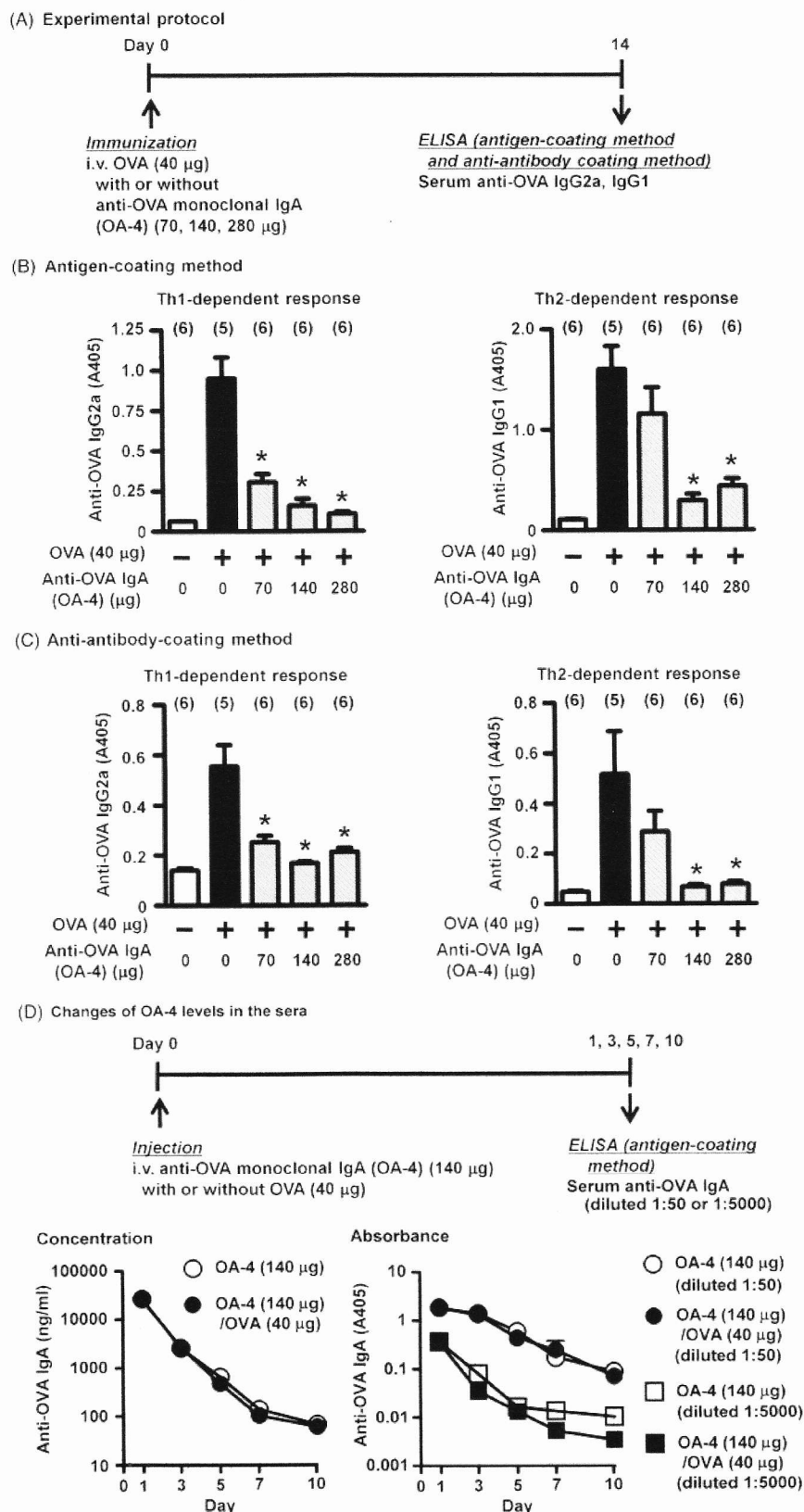
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Figure 2. Anti-OVA monoclonal IgA OA-4 complexed with OVA, administered at immunization attenuated the primary antibody responses to the antigen in a concentration-dependent manner. Serum anti-OVA IgG2a and IgG1 in DBA/1J mice immunized according to the protocol (A) were measured with two ELISAs, using the antigen-coating method (B) and the antibody-coating method (C), as described in detail in the "Materials and methods" section. We also measured the changes in OA-4 levels in the sera of OA-4-injected mice ($n = 8$) and those in the anti-OVA IgA levels (including injected OA-4 and *de novo*-synthesized antibody) in the sera of OA-4/OVA complex-injected mice ($n = 7$) within the indicated periods (D). Bars and symbols show the means $\pm$ SEM. The numbers of mice used are shown in parentheses above the columns (B and C). The data are representative of two independent experiments (B and C) and are from two independent experiments (D). * $p < 0.05$ versus the OVA group (Tukey's *post hoc* test after one-way ANOVA).



mice injected with OA-4 alone (Figure 2D). Considering that the diluted (1:50) sera from both groups of mice at Day 10 gave low absorbance values in the ELISA (<0.1 at 30 min after substrate addition), the influence of residual serum OA-4

at Day 10 or after on the ELISA detecting anti-OVA IgG2a and IgG1 should have been small and discountable. These results suggest that the anti-OVA IgG2a and IgG1 levels detected with both types of ELISA were accurate and also that

the primary antibody responses to OVA were attenuated by OA-4 complexed with the antigen.

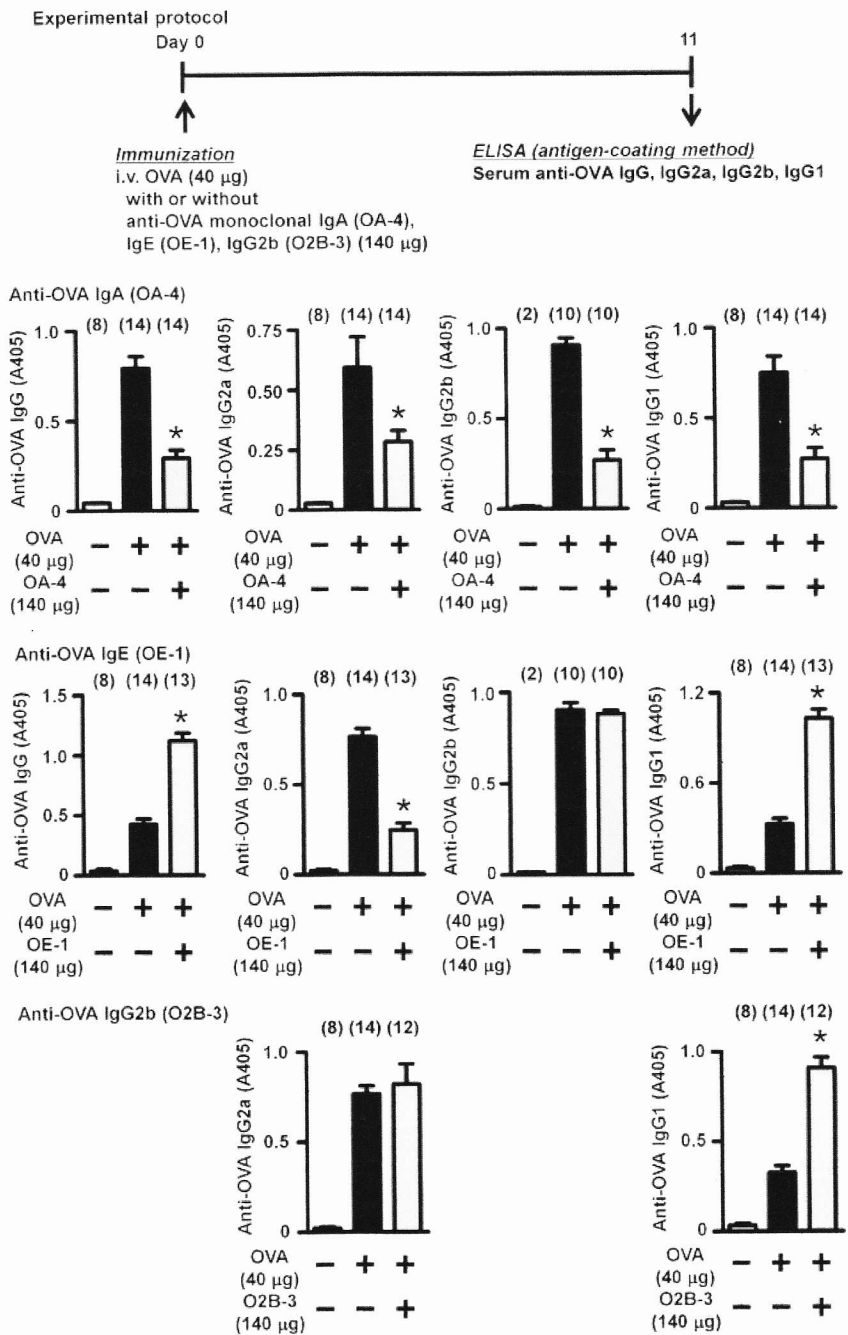
To clarify whether the suppressive effect of IgA complexed with the antigen on the primary antibody responses was isotype specific, the effects of the optimal dose (140 µg) of OA-4 and the same dose of anti-OVA IgE (OE-1) or IgG2b (O2B-3) complexed with OVA on the primary antibody responses to the antigen were determined. OA-4 attenuated the *de novo* production of all the isotypes measured, including Th1-dependent anti-IgG2a and IgG2b and Th2-dependent anti-OVA IgG1. In contrast, anti-OVA IgE OE-1 complexed with OVA promoted the primary antibody responses, evident as increased levels of anti-OVA IgG and IgG1 in the sera. However, the anti-OVA IgG2a levels were lower in mice

injected with OVA incubated with OE-1 than in those injected with OVA alone (Figure 3), while the mice in both groups produced similar levels of anti-OVA IgG2b (Figure 3). Anti-OVA IgG2b O2B-3 complexed with OVA also promoted the primary antibody responses, evident as increased levels of anti-OVA IgG1 in the sera (Figure 3).

OA-2 and OA-4 complexed with OVA prevented the establishment of an allergic constitution against the antigen and anaphylaxis after antigen re-exposure by reducing anti-OVA IgE production and the activation of T cells against OVA

To investigate whether IgA prevents the development of an allergic constitution by inhibiting the primary antibody

Figure 3. In contrast to OA-4, the anti-OVA monoclonal IgE OE-1 and anti-OVA monoclonal IgG2b O2B-3 complexed with OVA enhanced the primary antibody responses to the antigen. Mice received OVA alone or OVA complexed with OA-4, OE-1 or O2B-3 according to the protocol. Serum anti-OVA IgG, IgG2a, IgG2b and IgG1 were measured in these mice with the antigen-coating method. Bars show the means + SEM. The numbers of mice used are shown in parentheses above the columns. The data are from two independent experiments. **p* < 0.05 versus the OVA group (Mann-Whitney *U*-test).



responses, we measured the effects of a one-shot injection of OVA complexed with OA-2 or OA-4 compared with the effect of an injection of OVA alone on Day 0 on the anaphylactic response induced by re-exposure to OVA alone on Days 14 and 36 (Figure 4A). The hypothermia, an indicator of an anaphylactic response, induced by the first OVA challenge on Day 14 was severe, moderate and weak in mice immunized on Day 0 with OVA alone, OVA with OA-2 and OVA with OA-4, respectively (Figure 4B). The concentration of serum MMCP-1 released from activated mast cells was also significantly reduced by the co-administration of OA-4 with OVA on Day 0 (Figure 4B). Consistent with this result, the increases in anti-OVA IgG2a and IgG1 levels on Day 11 induced by OVA immunization on Day 0 were moderately and strongly reduced by pre-incubation of the antigen with OA-2 and OA-4, respectively, before immunization on Day 0 (Figure 4B). The inhibition of anaphylaxis by the antibody seemed to be caused by the attenuation of the immune responses, i.e. by the attenuation of *de novo* anti-OVA antibody production and not by the neutralization of the challenged OVA on Day 14 by residual OA-4 in the serum, because the serum OA-4 concentration was quite low (<100 ng/ml) at 10 days after the injection (Figure 2D).

The administration of OA-4 complexed with OVA on Day 0 effectively reduced the immune responses against the antigen, i.e. reduced the severity of the hypothermia induced by the second OVA challenge on Day 36 (Figure 4C). On Day 39, the anti-OVA IgG2a and IgG1 and Th2-dependent anti-OVA IgE levels in mice injected with the OA-4/OVA complex were significantly lower than those in the mice that received OVA alone (Figure 4C). To clarify whether the inhibitory effects of IgA complexed with antigen on antibody production were mediated by the prevention of T-cell activation, the antigen recall responses to OVA in the splenocytes from these mice were determined. The production of the Th1 cytokine interferon- γ (IFN- γ), the Th2 cytokines interleukin-4 (IL-4) and IL-13, and the Tr1 cytokine IL-10 by the OVA-restimulated cultured splenocytes was clearly suppressed by OA-2 or OA-4 complexed with OVA at immunization on Day 0 (Figure 4D). Therefore, the prevention of Th1/Th2 cell activation against OVA may be associated with the inhibitory effects of anti-OVA IgA complexed with OVA on the *de novo* synthesis of anti-OVA antibodies, including IgG2a, IgG1 and IgE. In contrast, simultaneous injections of OA-4 and HEM did not affect the antibody responses to the antigen, i.e. the upregulation of anti-HEM IgG2a or IgG1 levels on Day 39 (Figure 4E).

Production of the Th1/Th2 cytokines IFN- γ and IL-4, but not the secretion of the Tr1 cytokine IL-10, by OVA-restimulated cultured splenocytes was reduced by *in vitro* restimulation with the immune complex of OA-4 with OVA

We investigated whether *in vitro* OA-4 modified the profiles of the cytokines secreted by OVA-restimulated splenocytes (Figure 5). OA-4 pre-incubated with OVA and added to the culture medium reduced the OVA-induced production of IFN- γ and IL-4, but not IL-10, by the splenocytes (Figure 5). These results suggest that the direct inhibition of Th1/Th2

activation, but not the induction of the Tr1 response, is one of the possible mechanisms underlying the attenuation of the primary antibody responses by OA-4 complexed with the antigen.

Anti-PC monoclonal IgA, TEPC15, complexed with PC-KLH attenuated the primary antibody responses to the antigen, but the antibody did not attenuate the responses to OVA

To further investigate whether IgA suppresses the primary antibody responses in an antigen-specific manner, we compared the effects of a commercially available anti-PC monoclonal IgA (TEPC15) on the immune responses to PC-KLH and OVA. TEPC15 complexed with PC-KLH and injected on Day 0 (Figure 6A) reduced the primary antibody responses to the antigen, i.e. reduced anti-PC-KLH IgG2a and IgG1 levels in the serum on Days 11 and 39 (Figure 6B and C). Moreover, the severity of the hypothermia induced by the intravenous re-injection of PC-KLH was decreased on Day 36 (Figure 6C). In contrast, the simultaneous injection of TEPC15 and OVA did not affect the primary antibody responses to OVA; there was no reduction in anti-OVA IgG2a or IgG1 levels (Figure 6D) or in the severity of the hypothermia induced by re-exposure to OVA (Figure 6D). Based on the results shown in Figures 4 and 6, the primary antibody response to a certain intravenously injected antigen was specifically attenuated when the antigen was complexed with IgA.

Pre-injection of OA-4 prevented the primary antibody responses induced by a subsequent intravenous injection of OVA or intraperitoneal injection of OVA with alum adjuvant

With the protocol shown in Figure 7(A), mice were intravenously injected with OA-4 1 h before immunization with an intravenous OVA injection on Day 0. Pretreatment with antibody before immunization reduced the severity of the hypothermia induced by the re-injection of OVA on Day 14 and the anti-OVA IgG2a and IgG1 produced on Day 11 (Figure 7B). The intravenous pre-injection of OA-4 also inhibited the primary antibody responses to a subsequent intraperitoneal injection of OVA with an alum adjuvant (Figure 7C). Thus, the intravenous injection of IgA significantly attenuates the immune responses induced by subsequent antigen exposure, although IgA complexed with antigen before its administration inhibits the primary antibody responses to the antigens more effectively.

Discussion

We have demonstrated here that the IgA isotype complexed with the recognized antigen uniquely attenuates the primary antibody responses, whereas IgG and IgE amplify the responses to the antigen. Based on the presence of counter-acting cytokines, such as the immunoactivating Th1/Th2 cytokines and the immunosuppressive Th3/Tr1 cytokines²⁷, immunoregulation by immunoactivating IgG, IgE, and IgM and immunosuppressive IgA is a feasible fundamental system. Several antigen-specific immunosuppressive antibodies

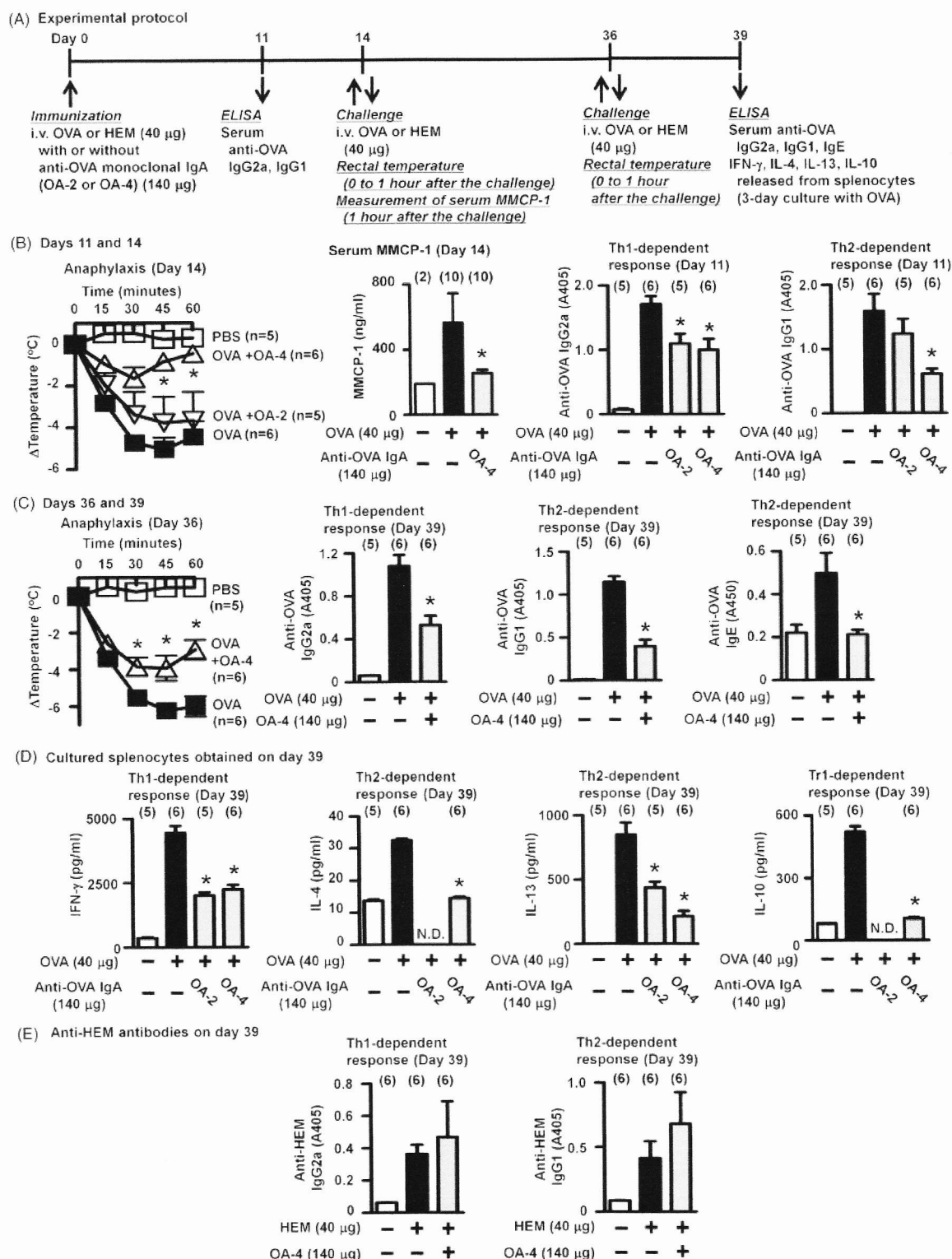
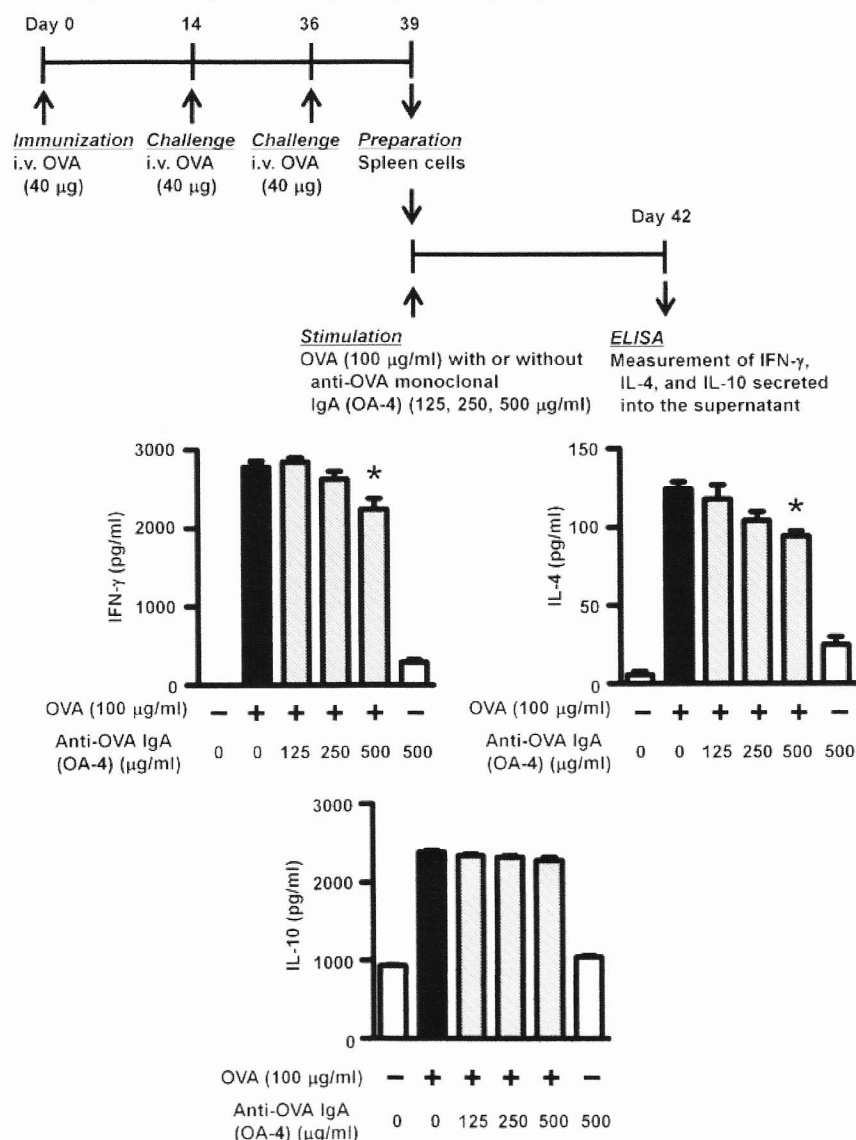


Figure 4. Single administration of anti-OVA monoclonal IgA (OA-2 or OA-4) complexed with OVA at immunization prevented the establishment of an allergic constitution causing hypothermia (an anaphylactic reaction) upon re-exposure to the antigen by inhibiting anti-OVA IgG2a, IgG1 and IgE production and the sensitization of T cells to OVA. DBA/1J mice were immunized and challenged according to the protocol (A). The reduction in rectal temperature caused by OVA challenge was monitored on Days 14 (B) and 36 (C). MMCP-1 concentrations in the serum on Day 14 were determined with a sandwich ELISA (B). Serum anti-OVA IgG2a, IgG1 and IgE levels were measured on Days 11 (B) and 39 (C) with antigen-coated ELISAs (for IgG2a and IgG1) or an antibody-coated ELISA (for IgE). Splenocytes collected on Day 39 were cultured with OVA (100 µg/ml) for 3 days to determine their secretion of IFN-γ, IL-4, IL-13 and IL-10 (D). Serum anti-HEM IgG2a and IgG1 were measured on Day 39 with antigen-coated ELISAs (E). Symbols and bars show the means + SEM, respectively, for the indicated numbers of mice (B, C and E) and three cultures of cells pooled from the indicated numbers of mice, shown in parentheses (D). The data are representative of two independent experiments. * $p < 0.05$ versus the OVA group for rectal temperature in (B) and (C) (Mann-Whitney U -test). * $p < 0.05$ versus the OVA group for MMCP-1 concentrations in (B), antibody levels in (C) and (D) (Tukey's *post hoc* test after one-way ANOVA). ND, not determined.

Figure 5. Production of the Th1/Th2 cytokines IFN- γ and IL-4, but not the secretion of the Th1 cytokine IL-10, by OVA-restimulated cultured splenocytes was reduced by *in vitro* restimulation with the immune complex of OA-4 with OVA. Concentrations of the Th1 cytokine IFN- γ , the Th2 cytokine IL-4 and the Th1 cytokine IL-10 were measured in the supernatants of splenocytes from OVA-immunized mice cultured for 3 days with OVA alone or with OVA complexed with OA-4. Bars show the means $\pm$ SEM of 2–3 cultures of cells pooled from three mice. Data are representative of two independent experiments. * $p < 0.05$ versus the OVA group (Tukey's *post hoc* test after one-way ANOVA).



have already been reported, but only in atypical cases. For example, the primary antibody responses to large particulate antigens, such as sheep red blood cells, were suppressed by serum IgG, which bound to the cells²³. This phenomenon has been used in the clinical treatment of Rhesus prophylaxis. This fact suggests that immunosuppression by antibodies is a possible therapy for allergic and autoimmune diseases if it can be applied under various conditions. However, the intravenous administration of IgG is unsuitable for the treatment of the allergies commonly induced by soluble antigens, such as OVA in food allergies, because the isotype does not suppress the immune responses to soluble antigens, but instead promotes them²³. Although sialylated IgG exceptionally attenuates the immune responses to the recognized antigen²⁸, the potential ability of sialylated IgG1 to induce anaphylaxis²⁹ may discourage its use for the treatment of allergic diseases. In contrast, serum IgA neutralizes antigens absorbed into the circulation and suppresses anaphylaxis³⁰. Alternatively, the transfer of maternal IgG when antigen-immunized mothers are breastfeeding

reduces the sensitization to the antigen in their offspring³¹. The oral administration of purified IgG to mouse neonates increased its concentration in their sera and reduced the immune responses induced by subsequent immunization with the antigen recognized by the antibody³². Therefore, maternal IgG given via placental transport or lactation can suppress the immune responses. Similarly, IgY transferred by yolk sac inoculation has been shown to act as an immunosuppressive antibody³³. However, these results were obtained from experiments with infants and this immunosuppressive system might be absent and unavailable in adults with fully developed immune responses. Compared with the antibodies discussed above, the favorable characteristics of IgA as a therapeutic drug for the treatment of allergies are clearly demonstrated in the present study. Therefore, the suppression of the immune responses to soluble antigens in adults could be achieved by the intravenous administration of IgA. Collectively, antibodies with an immunosuppressive function appear to shift from maternal IgG in infants to *de novo*-synthesized IgA for soluble antigens (as well as IgG for large

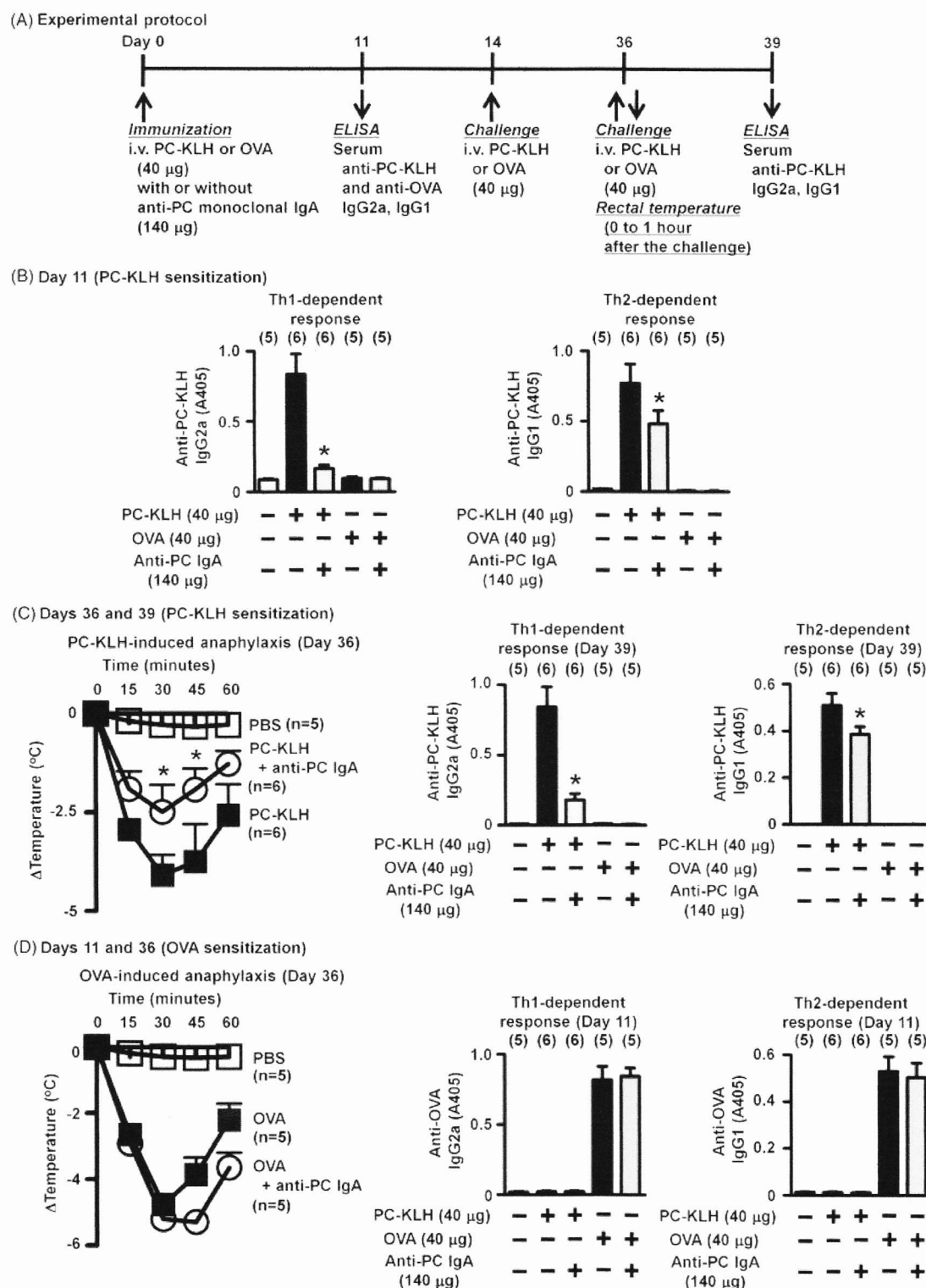
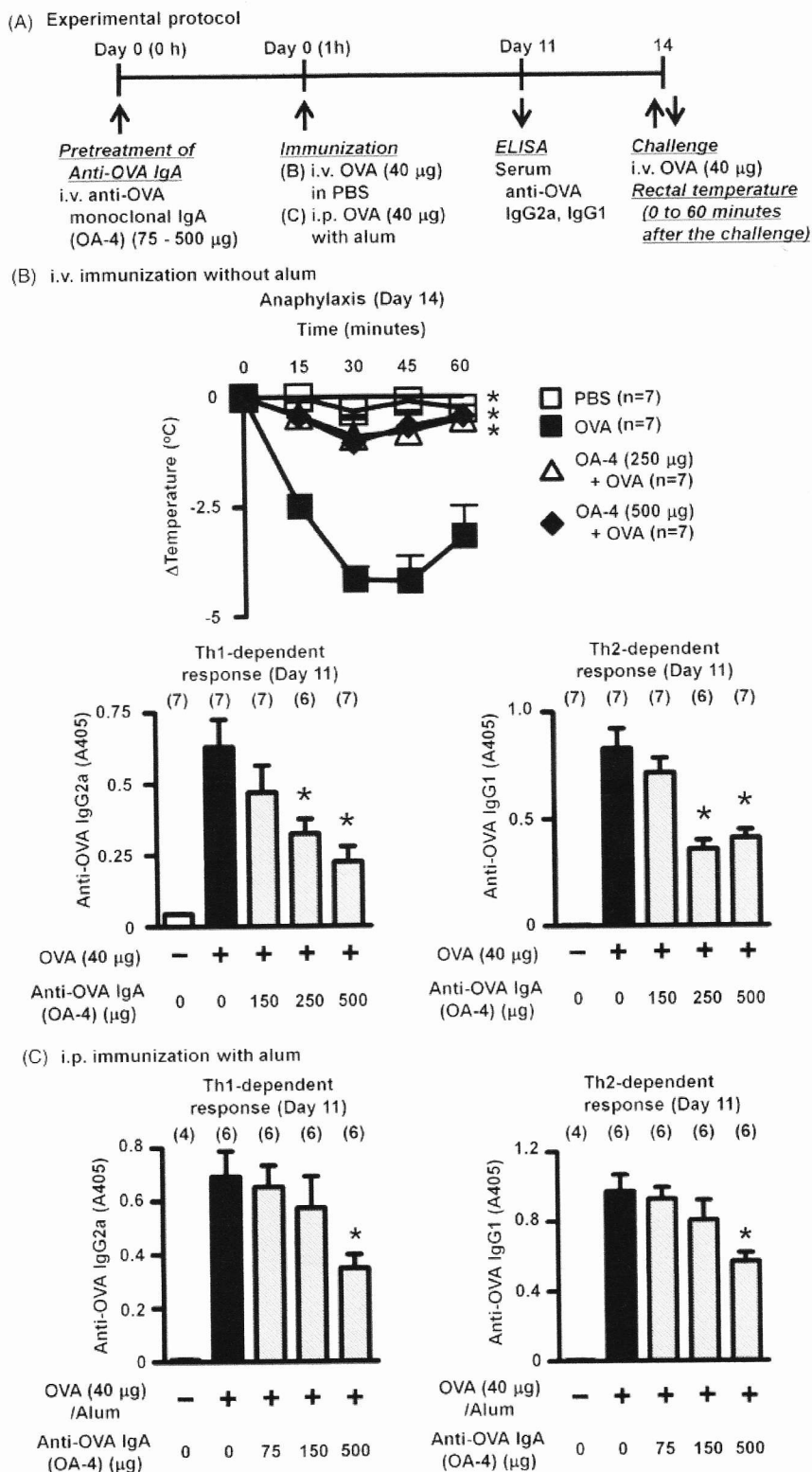


Figure 6. Single administration of anti-PC monoclonal IgA complexed with PC-KLH at immunization prevented the establishment of an allergic constitution causing anaphylactic shock upon re-exposure to the antigen by inhibiting sensitization in an antigen-specific manner. DBA/1J mice were immunized with an injection of PC-KLH, PC-KLH complexed with anti-PC monoclonal IgA (TEPC15), OVA or OVA mixed with TEPC15, and were then challenged according to the protocol (A). Serum anti-PC-KLH IgG2a and IgG1 were measured in the PC-KLH-immunized mice on Days 11 (B) and 39 (C) with antigen-coated ELISAs. The reduction in rectal temperature caused by PC-KLH injection was measured in mice on Day 36 (C). Serum anti-OVA IgG2a and IgG1 in OVA-immunized mice on Day 11 and the reduction in rectal temperature caused by OVA injection on Day 36 were measured (D). Symbols and bars show the means $\pm$ SEM, respectively, of the indicated numbers of mice, shown in parenthesis (B, C and D). Data are representative of two independent experiments. * $p < 0.05$ versus the PC-KLH group (Mann-Whitney U -test).

Figure 7. Pre-injection of OA-4 effectively prevented subsequent OVA sensitization. DBA/1J mice were injected with OA-4 followed by two immunization methods (A). Serum anti-OVA IgG2a and IgG1 levels in mice immunized with an intravenous injection of OVA (B) or an intraperitoneal injection of OVA mixed with alum adjuvant (C) were measured on Day 11. The reduction in rectal temperature caused by the OVA injection was measured on Day 14 (B). Symbols and bars show the means $\pm$ SEM, respectively, of the indicated numbers of mice, shown in parenthesis (B and C). Data are representative of two independent experiments. $*p < 0.05$ versus the OVA group for rectal temperature in (B) (Mann-Whitney *U*-test). $*p < 0.05$ versus the OVA group for antibody levels in (B) and (C) (Tukey's *post hoc* test after one-way ANOVA).



particulate antigens) in adults. One of the limitations of this study is that our results suggesting the suppressive effects of dimeric and polymeric serum IgA on the primary antibody responses in mice cannot be extrapolated to assign the same functions to the monomeric IgA in human serum. In the future, it must be clarified that the immunosuppressive effects

of IgA are shared among these species, and the forms of IgA (monomeric, dimeric or polymeric) that are essential for this distinctive effect must be identified.

Our study suggests that the inhibition of the primary antibody responses by IgA complexed with the antigen involved is partly mediated by the direct inhibition of Th1 and

Th2 activation, but not by the promotion of the Th1 responses. This result is consistent with a report that secretory IgA, but not dimeric IgA, induced IL-10 production in dendritic cells through specific ICAM-3-grabbing non-integrin receptor-1 in mice³⁴. Alternatively, two mechanisms that suppress the primary antibody response to particulate antigens by IgG³⁵ might also be involved in the inhibitory effect of OA-4 on sensitization against to soluble antigens. The first of these mechanisms is “epitope masking”. This mechanism may only be partly associated with the inhibition of the primary antibody responses by OA-4 complexed with the antigen because OVA has multiple epitopes and OA-4 is considered to mask only one of them. The inhibition of T-cell activation by OA-4 supports the limited participation of epitope masking because epitope masking by IgG suppresses the primary antibody responses but not T-cell activation³⁵. The second mechanism is the “induction of suppressive signaling”. Undefined molecules carrying the immunoreceptor tyrosine-based inhibitory motif on T and/or B cells might be bound by IgA and mediate its inhibitory effects, as FcγRIIb does with exposure to the IgG immune complex³⁶.

The attenuation of the primary antibody responses by the pre-injection of OA-4 also indicates both the preventive effects of therapeutically administered exogenous IgA on the establishment of immune disorders and the potentially important role of endogenous IgA on the antibody responses that occur under physiological and pathological conditions. However, further studies are required that investigate the effects of IgA on the immune responses in various situations, such as in already-sensitized mice expressing IgE, IgG and IgM that recognize the same antigen, as in humans with allergic or autoimmune diseases. To this end, we are now investigating the effects of the intravenous pre-injection of OA-4 on intravenous OVA-induced anaphylaxis and the subsequent antibody responses in OE-1-sensitized mice. In these experiments, OA-4 attenuated OE-1-enhanced *de novo* anti-OVA antibody production without affecting anaphylaxis (manuscript in preparation), suggesting that IgA also has the potential ability to suppress the antibody responses in sensitized mice. Our future research will focus on identifying the effect of OA-4 in a mouse model of food allergy. Recently, we reported the possible use of IgA in vaccines³⁷. The addition of IgA to a subcutaneously injected vaccine directed against a contaminating antigen reduced the anaphylactic response and further immune responses to the contaminating antigen without affecting the efficacy of the vaccine in mice. The results presented here suggest that the suppression of the further immune response in the vaccinated mice was induced, at least partly, by the direct inhibition of Th1 and Th2 activation by IgA that penetrates into the circulation from the skin.

Conclusions

In conclusion, IgA complexed with antigen attenuated the primary antibody responses to the antigen in mice, at least partly by directly inhibiting Th1/Th2 activation in the spleen, thereby blunting the severity of the anaphylactic shock induced by re-exposure to the antigen. These results suggest that IgA modulates the immune responses under both

physiological and pathological conditions and confirms the decades-old hypothesis that IgA exerts an immunosuppressive effect. Therefore, IgA may be a potential drug for the preventive and definitive treatment of immune disorders, especially allergies and autoimmune diseases.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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K.Y. planned and performed all the experiments described herein and drafted the article. K.M. and T.N. assisted K.Y. with establishing the IgA-producing hybridomas and the preliminary animal experiments using ovalbumin. A.M. and M.Y. assisted K.Y. with the animal experiments using ovalbumin and phosphorylcholine-keyhole limpet hemocyanin. Y.I., A.I. and A.K. assisted K.Y. with the *in vitro* experiments including the ELISAs and the purification of the IgA monoclonal antibodies. S.Y. acted as the laboratory supervisor for K.Y. and assisted in the preparation and proofing of this article.

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