

increased in 4 of the 5 actively treated subjects (ie, A1, A2, A3, and A5) for whom sera had been available. In contrast to Bet v 1-specific IgG levels, which remained high 48 weeks after the booster injection (ie, visit 13; week 96) (Fig 1, B, and Fig E2, A), birch pollen-specific IgE had strongly declined (Fig 1, E). Importantly, none of the actively treated subjects developed skin reactions to birch pollen at any time when tested by skin prick testing with birch pollen extract. The lack of birch pollen-specific skin reactions despite the presence of Bet v 1-specific IgE antibodies is most likely due to the protective effects of treatment-induced IgG antibodies, which were shown to also block IgE binding to Bet v 1 in patients with allergy (Fig 2). Thus, treatment of nonallergic subjects seems to have induced only clinically silent IgE sensitization. A summary of adverse events observed during the 2 years of the study is presented in Table E3 in this article's Online Repository at www.jacionline.org. These events were mild and classified as not related to the treatment. The only treatment-related side effects were mild (ie, local injection site reactions; n = 4), which occurred in 3 of the actively treated subjects (Table E3). Thus, vaccination of nonallergic subjects with recombinant hypoallergenic Bet v 1 derivatives was safe and well tolerated.

We are aware of only 1 other study in which a chemical conjugate consisting of a viral particle and a synthetic peptide derived from the major house dust mite allergen, Der p 1, was used to vaccinate nonallergic subjects.⁸ However, this study was conducted only over a period of 1 year and contained no placebo group, whereas our study reported that the synthetic vaccine induced allergen-specific IgG antibodies without inducing symptomatic allergic sensitization. One possible next first step could be to vaccinate children who have a clinically silent IgE sensitization and to assess whether this vaccination can prevent the development of allergic symptoms later in life. Our study is a small pilot study but may be considered as a first molecular approach toward preventive vaccination.

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IgE-class-specific immunosuppression in offspring by administration of anti-IgE to pregnant mice



To the Editor:

The presence of atopic dermatitis is known to increase the risk of sensitization to food allergens. However, it is noteworthy that infants who present with atopic dermatitis before the age of 3 months have the highest risk of developing food allergies, and thereafter the risk decreases even if atopic dermatitis is present. Accumulating evidence also suggests that environmental events that occur during the first months of life may lead to "atopic march," that is, typical progression of various allergic diseases, through sensitization to aeroallergens as well as food allergens.¹ Reversal of specific IgE sensitization in adult patients does not seem realistic, due to development of memory immune cell types. Therefore, prevention of IgE sensitization in the first months of life can be thought to be the key to reducing the risk of future allergy development.

In the early 1980s, before the discovery of cytokines that control IgE production, IgE isotype-specific inhibition of antibody production was intensively investigated. According

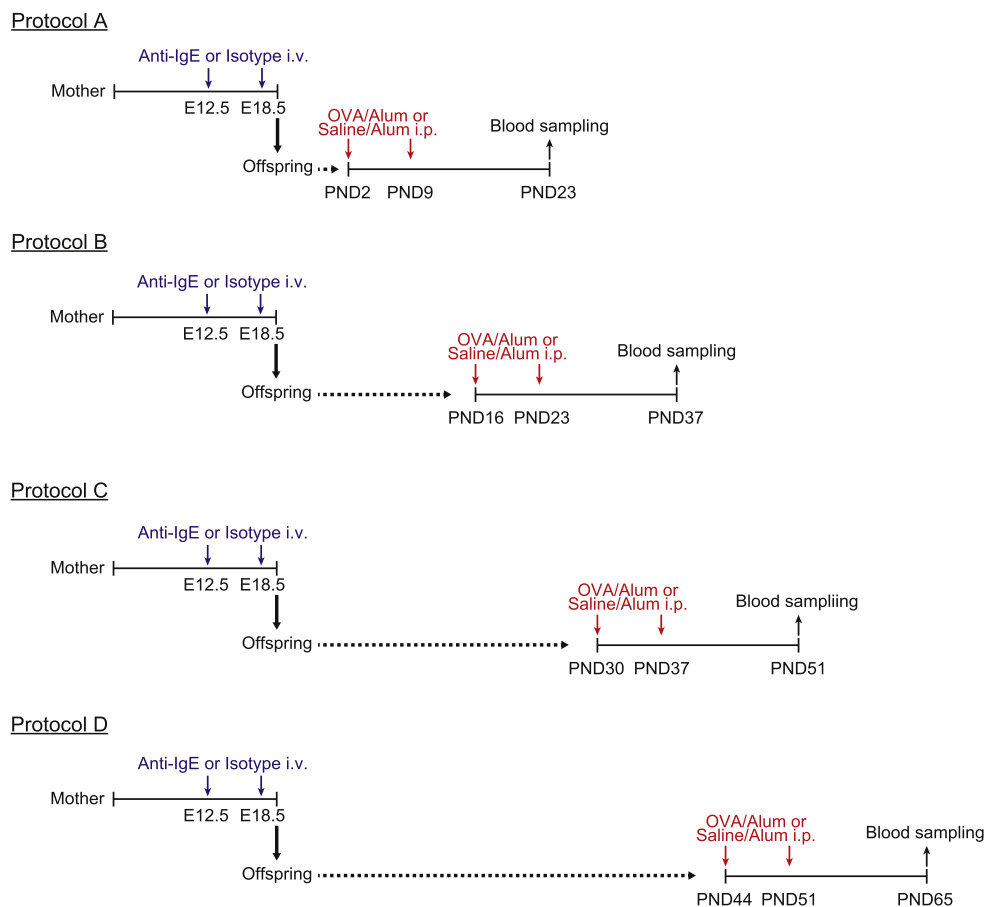


FIG 1. Experimental protocols. Pregnant mice were intravenously (i.v.) injected with anti-IgE antibody or isotype antibody on embryonic 12.5 days (E12.5) and E18.5. Offspring were intraperitoneally (i.p.) injected with OVA or saline emulsified with alum, twice at an interval of 1 week (ie, on PND2 and PND9 for protocol A, PND16 and PND23 for protocol B, PND30 and PND37 for protocol C, and PND44 and PND51 for protocol D). Sera were collected 14 days after the second injection.

to one report, inhibition was achieved in neonatal mice immunized with syngeneic IgE, which reactively induces production of anti-IgE antibodies.² However, because such an invasive intervention in human neonates is unrealistic, that report seems to have been forgotten. In this context, we reevaluated the feasibility of IgE class-specific inhibition of antibody production for a future clinical trial. Thus, as a proof of concept regarding prevention of allergic sensitization, we injected anti-IgE antibody into pregnant mice, because most anti-IgE antibodies belong to IgG class, and maternal IgG antibodies can be transferred to the fetus through the action of neonatal Fc receptor that is present in placental tissues.³

We used rat IgG₁ anti-mouse IgE antibody (clone R35-92; BD Biosciences, Tokyo, Japan) in the present study, because its efficacy in murine asthma models was reported to parallel the results reported for therapeutic anti-IgE (omalizumab) efficacy in allergic asthmatic human subjects.⁴ The effect of maternal administration of anti-IgE on antigen-specific IgE production in offspring was examined (see this article's [Methods](#) section in the Online Repository at www.jacionline.org) until adulthood at 9 weeks after birth by sampling blood. In brief, pregnant mice were injected intravenously with 100 μ g of anti-mouse IgE or isotype control at embryonic 12.5 days and embryonic

18.5 days. Ovalbumin (OVA) emulsified with alum was then injected intraperitoneally into the offspring on the corresponding postnatal day (PND), based on each of 4 different protocols (A–D) as shown in the legend to [Fig 1](#). Sera were collected 14 days after the second injection ([Fig 1](#)). Given that the serum half-life of IgG antibodies is 6 to 8 days in mice and 22 to 23 days in humans,³ it was judged that the time was long enough to examine the efficacy of anti-IgE as a preclinical study of preventing the development of allergies during early infancy.

When pregnant C57BL/6 mice were administered anti-IgE, production of IgE antibodies specific to the immunizing antigen (OVA) was suppressed even in offspring injected at PND44 ([Fig 2, A](#)). In BALB/c mice, suppression was seen in offspring injected at PND2, but not at the later ages ([Fig 2, C](#)). However, the serum levels of OVA-specific IgG-class antibody ([Fig 2, B and D](#)) and production of such cytokines as IL-4, IL-13, IL-17, and IFN- γ by spleen cells (see [Fig E1, A and B](#), in this article's Online Repository at www.jacionline.org) from the offspring were not altered. In infant mice of both strains, the manifestations associated with antigen challenge in a model of food allergy were ameliorated by the treatment (see [Fig E2](#) in this article's Online Repository at www.jacionline.org). However, this amelioration

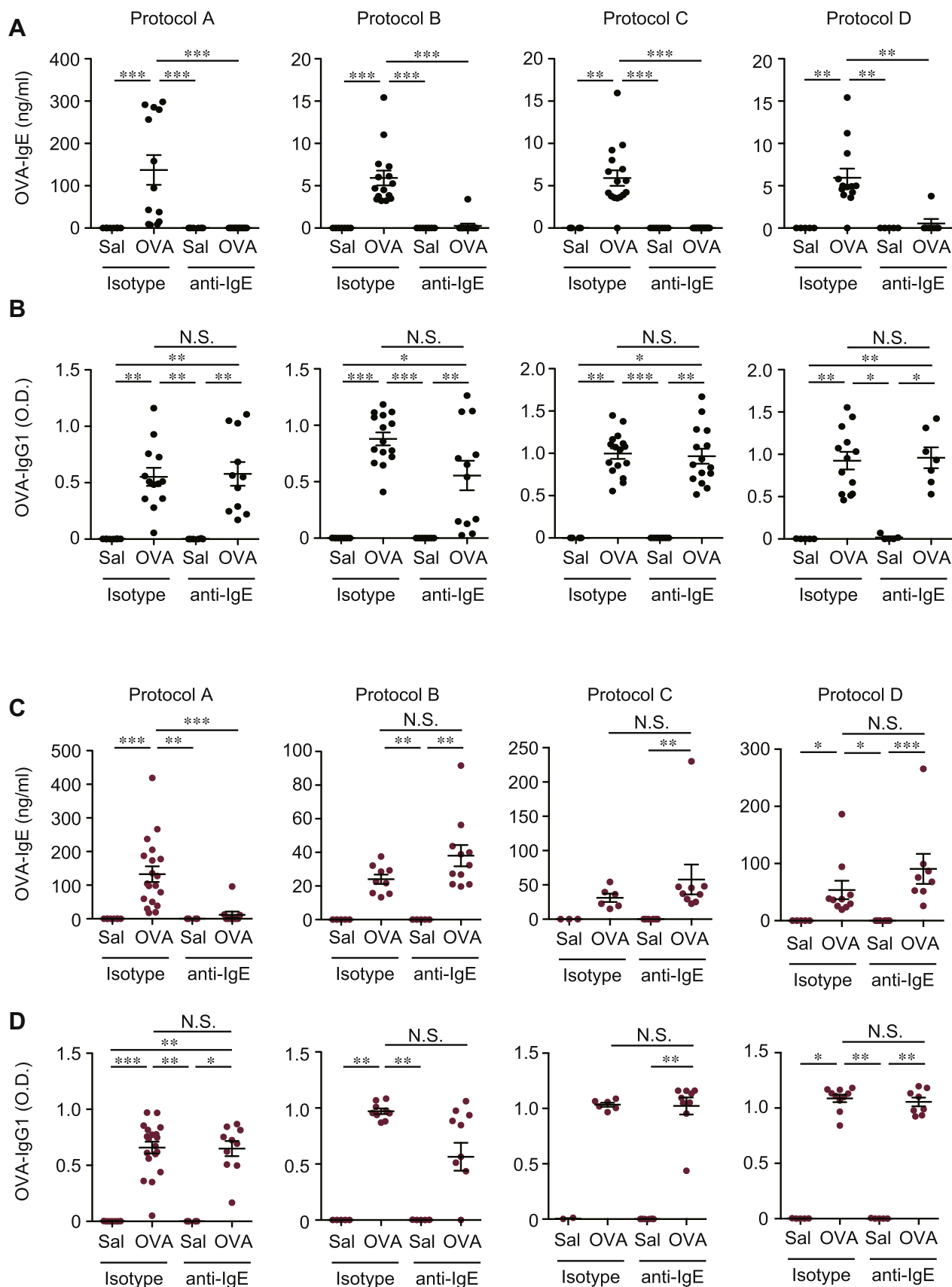


FIG 2. Production of antigen-specific IgE and IgG₁ antibodies by offspring that had been injected with anti-IgE during the fetal period. C57BL/6 mice (**A** and **B**) ($n = 5-16$ per group) or BALB/c mice (**C** and **D**, shown in dark red) ($n = 3-19$ per group) were used. The serum levels of OVA-specific IgE antibody (Fig 2, **A** and **C**) and OVA-specific IgG₁ antibody (Fig 2, **B** and **D**) are shown for each protocol (see legend for Fig 1). N.S., Nonsignificant; Sal, saline. N.S. for $P \geq .05$, * $P < .05$, ** $P < .01$, and *** $P < .001$.

was not clearly observed in adult mice, probably because they developed IgG (non-IgE)-mediated allergic reactions.

In humans, anti-IgE omalizumab has been shown to reduce IgE production by B cells stimulated with IL-4 and CD40 L *in vitro*, presumably by apoptosis triggered by binding to membrane-bound IgE receptor (mIgE) in the proximal domain but not in the antigen-recognizing domain on B cells.⁵ Because the level of IgE in the perinatal period remains very low, the only target of anti-IgE in the fetal immune system is considered to be mIgE⁺ B cells, which are barely detectable *in vivo*. However, in adults, it is thought that anti-IgE molecules mostly act on free IgE or IgE bound to the low-affinity receptor, CD23. This may be one of the reasons why anti-IgE exerted a profound inhibitory effect on IgE production in neonatal mice having low baseline levels of IgE. We measured the level of serum total IgE at each PND in naive C57BL/6 and BALB/c mice. As expected, the total serum IgE level remained undetectable until PND30 in low-IgE-responder C57BL/6. However, in high-IgE-responder BALB/c, the mean level was higher than the normal human serum value (100–200 ng/mL)⁶ on PND16 and after (see Fig E3 in this article's Online Repository at www.jacionline.org). This might explain the contrasting results seen in the 2 strains regarding the duration of the effect of anti-IgE (see Fig E4 in this article's Online Repository at www.jacionline.org). One may speculate that anti-IgE therapy does not efficiently suppress IgE responses in high IgE responders. However, it should also be noted that the total IgE levels stay very low during infancy in human.⁷

Although similar results were consistently obtained in 3 independent experiments, we were surprised that the blocking effect of anti-IgE lasted until PND44 in C57BL/6 mice. Although the mechanisms are unclear, one possible explanation is that even at very low concentrations anti-IgE might act on mIgE⁺ B cells if the IgE level is very low. Also, the levels of OVA-specific IgE in neonatal period were considerably high compared with the levels in other settings (Fig 2). It might be due to upregulation of type 2 immunity during the neonatal period,⁸ although the results of OVA-specific cytokine production did not sufficiently support this thought (Fig E1). Given that the offspring whose mothers had received anti-IgE and the controls whose mothers had received isotype were not littermates, possible effects of the microbiome as well as the genetic background should be investigated in future studies. At any rate, further investigation of the mechanism is necessary to fully understand the present results. However, we believe that human clinical studies should be prioritized because the use of omalizumab in expectant mothers with asthma was reported to be safe to both the mothers and their children.⁹ A randomized controlled clinical trial targeting expectant mothers having high risk for allergic diseases might be necessary in the future. But in the meantime, our hypothesis might be verified by a cohort study of expectant mothers with severe asthma and their infants by setting the acquisition of IgE antibodies by the children as the primary outcome and setting the mothers who are treated with omalizumab as the exposure group.

In conclusion, administration of anti-IgE into pregnant mice caused IgE-class-specific immunosuppression in offspring, and the effect was lasting at least until 9 weeks after birth in C57BL/6 mice having low baseline IgE levels. Future clinical studies of using anti-IgE to expectant mothers may be expected, aiming at primary prevention of allergies.

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METHODS

Mice

Wild-type (C57BL/6J and BALB/c) mice were purchased from Japan SLC, Inc (Shizuoka, Japan). All mice were housed in a specific-pathogen-free environment at the National Research Institute for Child Health and Development. The study was approved by the Review Board of the Institute, and all experiments were conducted according to the ethical guidelines of the institute.

Sensitization protocol

Pregnant mice were injected intravenously with 100 μ g of rat IgG₁ anti-mouse IgE (R35-92; BD Bioscience, Franklin Lakes, NJ) or rat IgG₁ isotype control (R3-34; BD Bioscience) at embryonic 12.5 days and embryonic 18.5 days. Offspring at PND2, PND16, PND30, and PND44 were injected intraperitoneally with OVA (Sigma-Aldrich, St Louis, Mo) at 5 μ g/g mouse body weight after emulsification with alum (Imject; Thermo Fisher Scientific, Waltham, Mass) (OVA:alum = 1:1), 2 times with a 1-week interval. Sera were collected 14 days after the second injection.

Measurement of antigen-specific immunoglobulin levels in sera

Ninety-six-well plates (Thermo Fisher Scientific) were coated with 10 μ g/mL OVA at 4°C overnight. After washing with PBS containing 0.05% Tween 20 (Promega, Madison, Wis), the wells were blocked with PBS containing 10% FCS. Optimally diluted serum samples (IgE = 1:25; IgG₁ = 1:1000) were applied, and the plates were incubated at room temperature for 1 hour. After washing, biotinylated anti-mouse IgE (R35-118; BD Biosciences) and IgG₁ (A85-1; BD Biosciences) were added, and the plates were incubated at room temperature for 1 hour. Then, after washing, horse radish peroxidase-conjugated streptavidin (Merck, Kenilworth, NJ) was added, followed by incubation at room temperature for 1 hour. For enzymatic reaction, TMB

substrate (SeraCare Life Sciences Inc, Milford, Mass) was used as a substrate, and 1 mol/L H₂SO₄ was used as a stop solution. The absorbance at 450 nm was measured using a plate reader (FlexStation 3; Molecular Devices, San Jose, Calif). The levels of OVA-specific IgE in the sera were normalized by using an anti-OVA mAb (TOS-2; provided by Mamoru Kiniwa, Taiho Pharmaceutical, Saitama, Japan) as the standard antibody.

OVA-specific cytokine production by spleen cells

Spleen cells were harvested from each mouse 3 days after OVA challenge. Spleen cell suspensions were prepared using a 70- μ m cell strainer (Corning, Corning, NY), followed by erythrocyte lysis with a lysing buffer (BioLegend, San Diego, Calif). The spleen cell suspensions were cultured in 48-well plates at 2×10^6 /mL in RPMI1640 (Nacalai Tesque, Kyoto, Japan) containing 10% FCS (Biological Industries, Cromwell, Conn), 100 U/mL penicillin, and 100 mg/mL streptomycin (Thermo Fisher Scientific) in the presence and absence of 200 μ g/mL OVA. Culture supernatants were harvested after 72-hour incubation, and the levels of cytokines in the culture supernatants were determined using ELISA kits (Thermo Fisher Scientific) according to the manufacturer's instructions.

OVA challenge

Five days after blood collection, each mouse was intraperitoneally challenged with OVA at 10 μ g/g mouse body weight. The rectal temperature was measured using a digital thermometer (TD-300; Shibaura Co., Ltd, Saitama, Japan) at 0, 10, 20, 30, 40, 50, and 60 minutes after the challenge.

Statistics

Differences between groups were examined for statistical significance by the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Statistical evaluations were performed using GraphPad Prism software (GraphPad Software, Inc, San Diego, Calif).

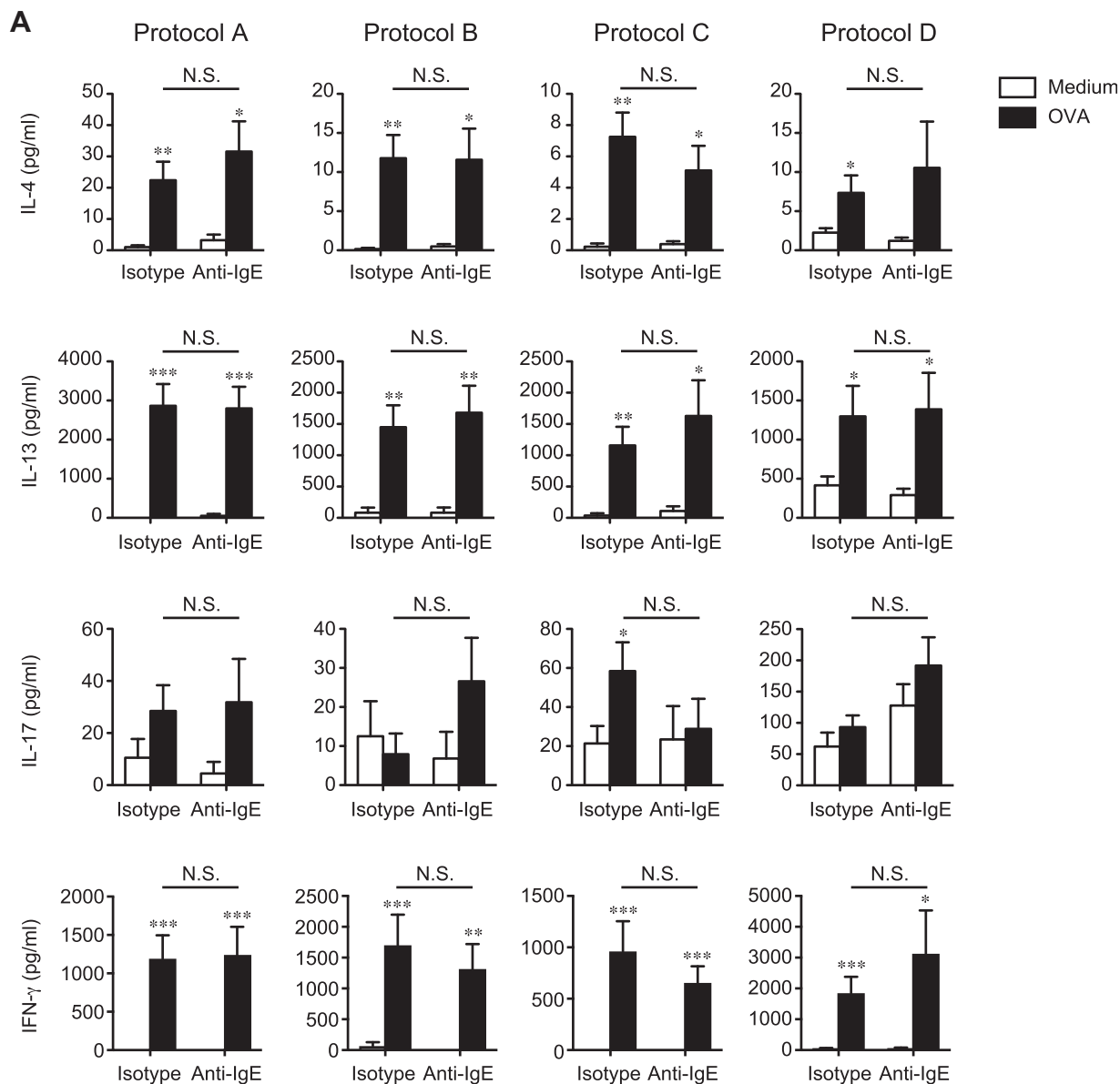


FIG E1. Antigen-specific cytokine production by spleen cells from the offspring. Spleen cells were obtained from C57BL/6 mice (A) or BALB/c mice (B, shown in dark red) at the same time as blood sampling according to each protocol shown in the legend to Fig 1. The cells were cultured with OVA or medium alone for 72 hours. ELISA was performed to determine the levels of cytokines in the culture supernatants. Data show the mean $\pm$ SEM ($n = 5-10$). * $P < .05$, ** $P < .01$, and *** $P < .001$ vs the corresponding value for the culture with medium alone. N.S., No significant difference ($P \geq .05$) between the indicated groups.

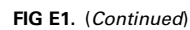


FIG E1. (Continued)

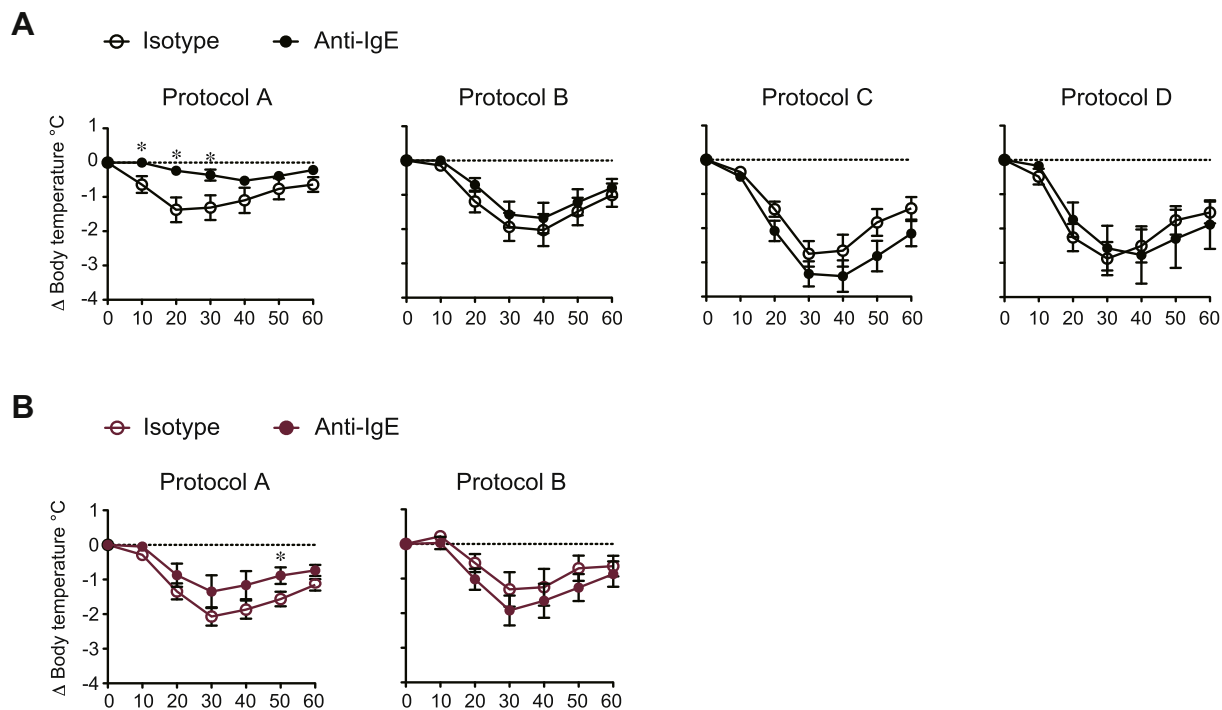


FIG E2. The changes in body temperature of mice after intraperitoneal challenge with OVA in C57BL/6 mice (**A**) and BALB/c mice (**B**, shown in *dark red*) ($n = 8-18$ per group). Data represent the mean $\pm$ SEM. $*P < .05$. We did not perform further experiments using BALB/c mice in protocols C and D, because we realized that IgG₁-dependent anaphylaxis might occur when the level of anti-OVA IgG₁ was increased, and that this phenomenon would occur in mice but not in humans.

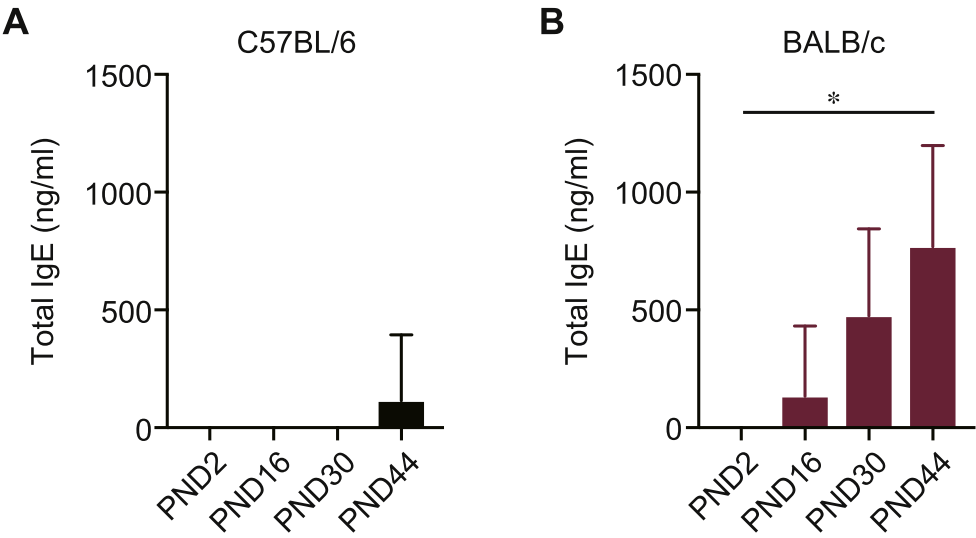
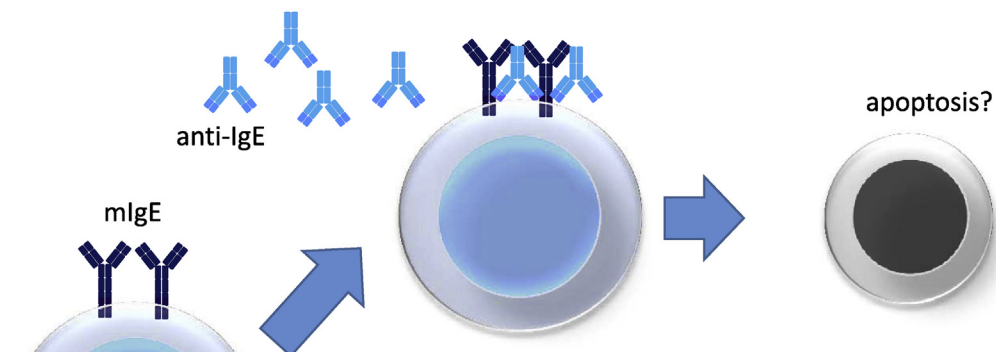
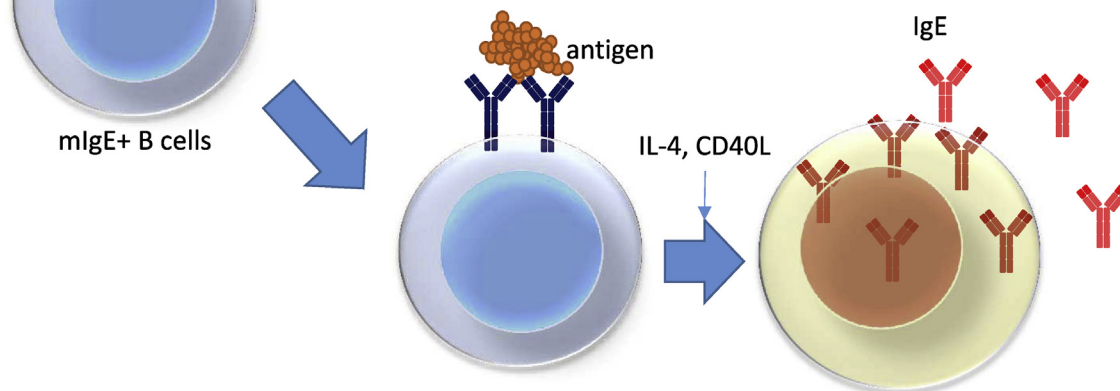


FIG E3. Total IgE in the sera from naive mice in BALB/c (**A**) and BALB/c mice (**B**, shown in *dark red*). Total IgE in each serum was determined by ELISA. n = 4-6 per group. The mean $\pm$ SEM are shown. **P* < .05.

A Fetal/Neonatal period (with anti-IgE)



B In the presence of antigen (without anti-IgE)



C Adulthood (with anti-IgE)

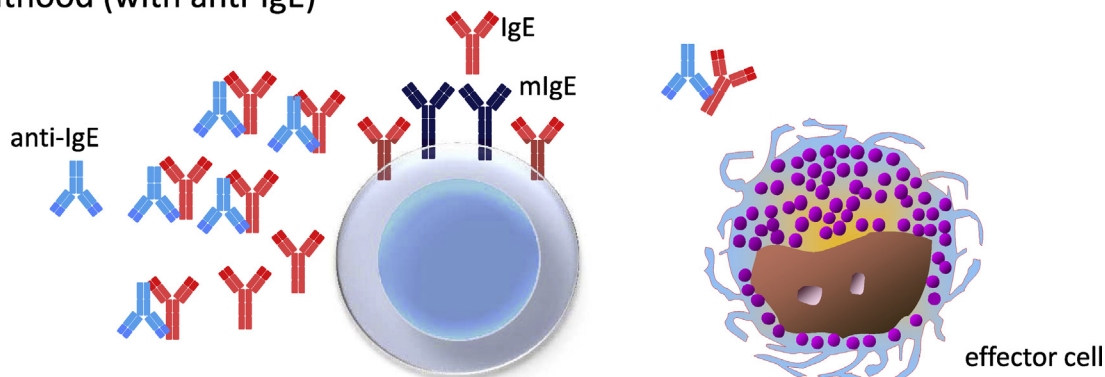


FIG E4. One possible explanation as to why anti-IgE blocks IgE production during perinatal period but not afterwards. **A**, When anti-IgE is present in mice almost lacking IgE, that is, like a fetus, it may induce apoptosis of mIgE⁺ B cells by binding to the membrane proximal domain of the receptor. **B**, In the presence of antigen, IL-4, and CD40L, mIgE⁺ B cells can produce IgE. **C**, When anti-IgE is present in mice having considerable levels of IgE, binding of anti-IgE to mIgE may be blocked by excessive IgE. Meanwhile, anti-IgE acts on effector cells by blocking binding of IgE to Fcε receptors.