



Regulatory effects of the thymus on the IgE response of mice
by Rhet Lucy Schneller

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Abstract:

The regulatory effects of the thymus gland on the production of reaginic immunoglobulin E (IgE) were studied using congenitally athymic (nude) mice, phenotypically normal littermates (NLM) of nude mice and Balb/c mice. Antigen specific IgE was quantitated in CFW mice by the passive cutaneous anaphylaxis (PCA) assay. Alum, complete Freund's adjuvant and saline extract of Bordetella pertussis were used as adjuvants. Nude mice failed to produce specific IgE following both primary and secondary immunization with hen egg albumin (EA), crude ascaris extract and crude ascaris extract coupled to dinitrophenyl groups. In contrast, NLM and Balb/c mice made strong IgE responses.

Nude mice implanted with thymus glands from neonatal Balb/c or NLM donors made IgE responses equal to the responses of NLM mice. These data indicate that there is an absolute requirement for thymus-derived cells in the formation by mice of specific IgE.

Nude mice were passively sensitized intradermally with an IgE-positive serum; when challenged intravenously with specific antigen, these nude mice demonstrated strong PCA reactions. It is clear, therefore, that although nude mice cannot make specific IgE, they do have the mechanism necessary to elicit PCA reactions.

Attempts were made to show a negative regulatory effect of the thymus on IgE production by using splenectomized, 60Co-irradiated or antilymphocyte serum-treated mice. No enhancement of IgE production was observed in these experiments. In contrast to results obtained by others using rats, infection of immunized mice with the nematode *Nippostrongylus brasiliensis* did not potentiate specific IgE production.

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ABSTRACT

The regulatory effects of the thymus gland on the production of reagenic immunoglobulin E (IgE) were studied using congenitally athymic (nude) mice, phenotypically normal littermates (NLM) of nude mice and Balb/c mice. Antigen specific IgE was quantitated in CFW mice by the passive cutaneous anaphylaxis (PCA) assay. Alum, complete Freund's adjuvant and saline extract of Bordetella pertussis were used as adjuvants. Nude mice failed to produce specific IgE following both primary and secondary immunization with hen egg albumin (EA), crude ascaris extract and crude ascaris extract coupled to dinitrophenyl groups. In contrast, NLM and Balb/c mice made strong IgE responses. Nude mice implanted with thymus glands from neonatal Balb/c or NLM donors made IgE responses equal to the responses of NLM mice. These data indicate that there is an absolute requirement for thymus-derived cells in the formation by mice of specific IgE.

Nude mice were passively sensitized intradermally with an IgE-positive serum; when challenged intravenously with specific antigen, these nude mice demonstrated strong PCA reactions. It is clear, therefore, that although nude mice cannot make specific IgE, they do have the mechanism necessary to elicit PCA reactions.

Attempts were made to show a negative regulatory effect of the thymus on IgE production by using splenectomized, ⁶⁰Co-irradiated or antilymphocyte serum-treated mice. No enhancement of IgE production was observed in these experiments. In contrast to results obtained by others using rats, infection of immunized mice with the nematode Nippostrongylus brasiliensis did not potentiate specific IgE production.

INTRODUCTION

Properties of Homocytotropic Antibodies. In humans, reaginic antibodies, which are responsible for some allergic reactions, are in the immunoglobulin class IgE. IgE is a glycoprotein with a molecular weight of 200,000 and is synthesized by plasma cells predominantly in the respiratory and gastrointestinal tracts. It is capable of agglutinating red blood cells coated with antigen but possess no complement-fixing activity (1). It differs from other classes of immunoglobulin in that it is non-precipitating. It does not have the ability to cross the placenta and is not found in human colostrum (2). Zone electrophoresis has shown that IgE moves in the "fast" region while zone centrifugation in buffered sucrose gradient has shown that it sediments in the 7S region (2). Human IgE can sensitize the skin of humans, but not the skin of most other species, for immediate-type hypersensitivity reactions.

A similar type of antibody, possessing properties of the human reaginic antibody, IgE, has been reported in mammals other than man by several investigators. Thus, reaginic IgE-like, homocytotropic antibodies (HTA) have been reported in several animal species including the guinea pig (3), rat (4), and mouse (5). Like the human reagin, these antibodies are heat-labile; heating the undiluted serum at 56°C for 30 minutes destroys their skin-sensitizing ability. Many species, including the mouse, also produce another homocytotropic antibody

which belongs to the IgG₁ subclass (5). IgG₁ does not persist cytophilically in the skin for long periods of time (hours, as compared to weeks for IgE) but does persist in the serum longer than the reaginic-type antibody. IgG₁ is heat-stable, so heating for 30 minutes at 56°C does not destroy its activity (5). It is, therefore, possible to distinguish between the activities of IgG₁, and IgE HTA in the passive cutaneous anaphylaxis (PCA) assay by heating serums or varying the time of antigen challenge after skin-sensitization. IgG₁-mediated reactions are typically developed 2 hr after passive sensitization, whereas IgE-mediated reactions are developed 72 hr after passive sensitization.

It is thought that IgE attaches to mast cells and causes the release of chemical mediators of the PCA reaction (1). Mota (6) also reported that rat mast cells can be sensitized passively with serum containing what he called "mast cell sensitizing antibody," which is comparable to human IgE. He found that sensitized mast cells release histamine and 5-hydroxytryptamine after the addition of specific antigen. It is thought that serotonin (5-hydroxytryptamine) is the main chemical mediator of anaphylaxis in the rat and the mouse (6).

In mice, IgE production depends on the antigen-adjuvant system as well as on the strain of animal used. Revoltella and Ovary (7) reported that three of six strains of mice immunized with DNP-haemocyanin and AL(OH)₃ were able to produce IgE. SWR/J, C57BL/6J and

A/HeJ made IgE, whereas CBA/J, DBA/J and SW made high levels of IgG₁ but no IgE (7). Clausen et al (8) found that the inbred strains of mice NBL/n, C57Bl/6J and C57BL/10J were far superior to the strains AKR/n, STR/n and RML in production of IgE when challenged with hen egg albumin (EA) and saline extracts from Bordetella pertussis (PE).

Several different antigens and adjuvants have been used to elicit IgE production. Using EA as antigen, Clausen, et al., were successful in stimulating IgE with SE as adjuvant, whereas incomplete Freund's adjuvant was superior in stimulating 2 hr PCA antibody, IgG₁ (9). Tumor antigens and alloantigens have also been used to induce IgE production in mice. Thus C57BL and Balb/c mice immunized with 1×10^6 viable tumor cells from the B16 melanoma established in C57Bl mice, responded with production of IgE antibody (10). Kind and Macedo-Sobrinho were able to get IgE production in mice in response to rabbit anti-mouse thymocyte serum (11).

Small doses of antigen such as pollen, which cause human allergies, seem to be best for production of IgE (12). In guinea pigs, Perini and Mota (13) demonstrated that low doses of EA together with low doses of lipopolysaccharide (LPS) were effective in stimulation of IgE production, and high doses of antigen and adjuvant resulted in high production of IgG₁.

It has also been reported that a state of tolerance can be induced

to the IgE antibody class. Katz and co-workers (14) showed that two pretreatments of mice with 500 μ g of the copolymer DNP-D-glutamic acid and D-lysine significantly reduced the animals' ability to respond to DNP-crude ascaris extracts (DNP-CAE) with production of IgE antibody. This tolerance could be transferred to syngeneic, unprimed, irradiated recipients with spleen cells from mice primed with DNP-CAE and alum and given two 500 μ g injections of DNP-D-glutamic acid and D-lysine 25 days later. Saverbronn Maia, et al, showed that three immunizations of 100 μ g EA significantly reduced IgE production of DBA/1J mice when immunized subsequently with 0.1 μ g EA.

Thymus Dependency of IgE. Thymus-derived cells (T cells) are necessary for production of IgM or IgG to the so-called "Thymus-dependent" antigens. Although most antigens, such as heterologous proteins and heterologous erythrocytes, are thymus dependent, there are some "thymus-independent" antigens, such as endotoxin, pneumococcal polysaccharide, and Vi antigen.

Until recently, only speculations have been made on the T-cell collaboration with B cells in relationship to the IgE response. For example, Okumura and Tada (16) demonstrated that neonatal thymectomy of rats caused a reduction in the production of IgE in response to dinitrophenol-crude Ascaris extract (DNP-CAE).

The most meaningful data supporting T-cell dependency of the IgE

response is seen in the work of Michael and Bernstein (17). They found that congenitally athymic (nude) mice failed to respond with IgE production to EA, bovine serum albumin, and human gamma globulin, whereas their normal thymus-bearing littermates did respond to these antigens. However, nude mice that had received thymus cells from normal Balb/c mice were able to make an IgE response to these antigens.

Some unpublished work of Reed and Munoz further indicates that IgE responses may be thymus dependent. They found that normal littermates to nudes could make an IgE response to EA and SE, bovine gamma-globulin and SE and Keyhole Limpet hemocyanin, whereas nude mice did not respond to these antigens. They also measured the IgE response to two antigens known to be thymus independent for an IgM response, namely, SIII and endotoxin. Neither nudes nor their normal littermates made any IgE response to these antigens.

Thymus Regulation of the IgE Response. IgE production may be thymus dependent but may also be regulated by the thymus. Tada and co-workers (16), for example, demonstrated that while neonatal thymectomy of rats decreases the IgE response, adult thymectomy increases this response. They showed a similar enhancement of IgE production with 400 rad X-irradiation (18), splenectomy (16), some immunosuppressive drugs (19) and antithymocyte treatment (20). One explanation for such an enhanced effect is the "turning off" of suppressor T-cells or the

destruction of T-cells, allowing B-cells to produce IgE in an unregulated manner.

Tada and Okumura (21) demonstrated that HTA formation can be suppressed by passive administration of homologous antibody against the same antigen. This suppression appeared selective in that the HTA response was affected, whereas the IgM response was not. They suggested that blocking antibody in the passively administered serum acts by suppressing the synthesis of reagin rather than by preventing the combination of allergen and reagin (21). Their reasons for this conclusion were: the fact that the amounts of passive antibody used were much less than those needed to cover all of the antigenic determinants; and also the fact that IgE was suppressed while hemagglutinating antibodies were not.

Okumura and Tada (22) have used a hapten-carrier molecule to induce reagin formation. By transferring thymocytes from rats hyper-immunized with crude *Ascaris* extract (CAE) or DNP-CAE into recipients that were producing high titers of HTA, they achieved a drastic reduction of HTA within two days of transfer. Because this effect only took place when the carrier was the same in donor and recipient, they concluded that carrier-specific, thymus-derived lymphocytes can negatively regulate the HTA formation which is known to be hapten-specific (23). They further investigated this negative control to find what they

called an "antigen-specific T-cell factor" which they felt was responsible for the suppression of IgE production. They treated the thymocyte extracts from hyperimmunized donors with different nucleases and proteinases and found that the T-cell factor was not digestible by RNase but is destroyed with trypsin and pronase, therefore, they concluded, the T-cell factor was of protein nature. Its activity sedimented slower than IgG hemagglutinin in sucrose density gradient ultracentrifugation and was electrophoretically located in regions corresponding to alpha and beta globulins. They suggest, therefore, that the T-cell factor may be a unique receptor of the T-cell and not an unusual immunoglobulin (24).

In working with rats, White and Holm (25) demonstrated that administration of equine antilymphocyte serum before immunization with KLH yielded an enhancement of early synthesis of reaginic antibody but giving equine antilymphocyte serum after administration of KLH resulted in late synthesis enhancement (25). Antilymphocyte serum pretreatment was suppressive for KLH agglutinin production.

Kind and Macedo-Sobrinho (11) have reported a similar regulatory control of the thymus on IgE formation in mice. They found that X-irradiation (450 rad) resulted in sustained level of IgE production in mice treated with rabbit anti-mouse thymocyte serum. Splenectomy and thymectomy did not result in increased IgE production.

Effects of Nippostrongylus brasiliensis on IgE Production.

Several investigators have reported a potentiated reagin response following nematode infection. Thus, Block, et al (26) demonstrated that rats infected with Nippostrongylus brasiliensis larvae secondary to immunization with EA or KLH plus alum exhibited increased reagin production of short duration. The potentiated response was only found in IgE production and not in the other classes of antibody. Jarrett, et al (27), in the rat, showed that both antigen and adjuvant must precede infection with N. brasiliensis for a potentiated response, which could rise as much as 100-fold. They showed that no potentiated response occurred if PE was substituted for the parasite, leading them to conclude that the N. brasiliensis did not act as an adjuvant.

The Thymus as the Origin of the Mast Cell. It has been hypothesized by Csaba et al (28) and also by Ginsberg (29) that the thymus is the origin of the mast cell. Pritchard, Viklicky and Sima (30), however, studied the connective tissue of the skin and the lymph nodes of nude mice and found what they proposed to be mast cells. Because the nude mouse is congenitally athymic, they concluded that the origin of the mast cell could not possibly be the thymus. If nude skin was able to elicit a PCA reaction, there would be good evidence in support of Pritchard's conclusion.

The following experiments were designed to evaluate the role of the thymus in IgE production.

MATERIALS AND METHODS

Animals. Balb/cJ mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Congenitally athymic (nude) mice (nu/nu) (31) and their phenotypically normal littermates (NLM; +/-nu or +/+) were obtained from the Montana State University Colony in which the mutant gene, nu, is being placed onto a Balb/c background by backcross-intercross mating scheme (32).

Balb/cJ, nude and NLM mice, 1-3 months of age, were immunized by various schemes designed to induce the production of HTA. No attempt was made to distinguish between the HTA response of males and females.

CFW female mice, 1-3 months of age, obtained from Carworth Farms, Portage, Mich., were used as serum recipients in PCA assays.

All animals were maintained in a clean environment and were given acidified-chlorinated water and autoclaved Purina 5010 feed.

Antigens. Hen egg albumin (EA), 5-times crystallized, was obtained from Miles Laboratories Inc., Kankakee, Ill. The crystallized EA was stored at -20°C . For immunizations, EA was dissolved at the appropriate concentration in saline or phosphate buffered saline (PBS). Fresh EA solution was prepared for each experiment.

Crude Ascaris Extract. Dr. Herbert J. Morse, Ill, Laboratory of Microbial Immunity, National Institutes of Health, Bethesda, Maryland,

made and provided the crude ascaris extract (CAE). CAE was prepared by homogenizing saline-washed Ascaris organisms; dialyzing the homogenate against several changes of PH 8.2 borate buffered saline and clarifying dialysate by centrifugation (10,000 x G - 30 minutes). CAE was stored in small aliquates at -20°C. For use, CAE was thawed and diluted to the desired concentration in saline.

DNP-Ascaris. Dinitrophenylated crude Ascaris extract (DNP-CAE) was also made and provided by Dr. H. Morse. The DNP group was combined with ascaris extract in the ratio of 48 DNP/10⁵ daltons.

Vi-antigen (Vi) was purified by Dr. F. Jarvis, Idaho State University, Pocatello, Idaho, by a continuous-flow paper curtain electrophoresis procedure (33). This antigen, a polymer of N-acetyl galactosaminuronic acid (33), was provided by Dr. J. T. Ulrich of Idaho State University. The lyophilized Vi was stored at 4°C. for use in experiments, Vi was dissolved in saline.

Adjuvants. Alum was prepared as follows: 75 grams of aluminum ammonium sulfate (Humco Laboratory, Texarkana, Texas) was added to 180 ml of distilled water and then 75 ml of 1 N NaOH was slowly added with continuous mixing. The resulting precipitate was washed 8 times with sterile distilled water and was finally resuspended in sterile distilled water at a concentration of 60 mg/ml. This adjuvant was mixed by pipetting with the different antigens used prior to immunizations.

Pertussis Extract. A saline extract of Bordetella pertussis (PE) was made (9) and provided by Dr. John Munoz, Rocky Mountain Laboratory, Hamilton, Montana. The lyophilized extract was stored at 4°C and was dissolved in saline for use as an adjuvant. At times it was mixed with antigen before immunization.

Lipopolysaccharide (LPS) was extracted from Escherichia coli 0113 by the hot phenol-water method (34) and was provided by Dr. J. A. Rudbach, University of Montana, Missoula, Mt. LPS was dissolved in PBS at a concentration of 1 mg/ml and stored at -20°C until diluted in PBS for use in experiments.

Complete Freund's Adjuvant (CFA) was purchased from Difco Laboratories, Detroit, Michigan.

Antilymphocyte Serum. Rabbit anti-mouse lymphocyte serum (RAMLS), lot 13096, was purchased from Microbiological Associates, Bethesda, Maryland. Other lots were not used in this experiment.

Nematodes. In some experiments designed to potentiate HTA production, mice were injected subcutaneously with 600 L₃ larvae of a mouse-adapted strain of the nematode Nippostrongylus brasiliensis (35).

Irradiation. Mice and rats were given 400-450 rad whole-body irradiation using a Picker 60 Cobalt therapy unit. To insure uniform

dose during irradiation, the mice were held in a plexiglass cage placed on top of a rotating turntable. The dose rate was approximately 75 rad/min.

Splenectomy. Spleens were removed surgically from Balb/c mice at approximately six weeks of age. The mice were anesthetized with the proper dose of Nebutol (36). The spleen was ligated and cauterized before removal. The peritoneum and skin were sutured separately. Care was taken not to tear the spleen and not to leave any spleen remnants in the mice.

Thymus Grafts. One month old nude mice were anesthetized with Nebutol (36) prior to implantation of two to four thymus glands from donors less than three days of age. Implants were made under the fascia in the axillary region of the nude. Either Balb/c or NLM were used as thymus donors. Only thymus-grafted nudes that outwardly appeared healthy were used in experiments.

Thymus Cell Injections. Thymocyte preparations were made by screening young adult Balb/c thymuses through stainless steel 80-mesh screens into PBS containing 1% fetal calf serum (PBS-FCS). The thymocytes were then washed once with PBS-FCS. Viability was determined by a Trypan Blue Exclusion test, and 1.4×10^8 viable cells contained in 0.7 ml were injected slowly into the tail vein of recipient nude mice.

Immunization Procedures. To obtain HTA, mice were given varying doses of antigen and adjuvant on day 0. Antigen and adjuvant were always mixed prior to immunization. CFA was mixed with antigen with a double syringe while all other adjuvants used were mixed with a pipette. The injections were usually given intraperitoneally, although there were some experiments in which the route of injection was intravenous.

The animals were bled on days 10 and 20 after initial immunization of antigen alone because I found that adjuvant has a deleterious effect on antibody production if given at this time. The animals were then bled on day 30, 10 days after the booster injection. Serum was collected from individual animals and stored separately at -30°C because I and others (37) have found that reaginic antibody denatures at 0°C .

Passive Cutaneous Anaphylaxis. A pool was made from the serum of individual animals within a given experimental group. Two-fold serial dilutions of the serum pool were made in saline. 0.05 ml of a given serum dilution was injected intradermally on the back of a recipient CFW mouse. The hair was clipped from the back of recipient mice 24 hours prior to the intradermal injection. Recipient mice were injected intravenously with 0.2 ml of antigen solution (1% solution of EA, 0.2 mg DNP-CAE or 0.2 mg CAE) in saline containing 0.5% Evans Blue dye. Thirty minutes after intravenous challenge with antigen and Evans

Blue the animals were killed by cervical dislocation and the skin on the back was incised and inverted. A positive reaction was noted by a blue circular area of the skin at the injection site. Vertical and horizontal measurements were recorded. An area less than 4mm x 4mm was recorded as a negative reaction, whereas an area greater or equal to 4mm x 4mm was recorded as a positive reaction. Reactions were given a subjective rating of 1+ to 4+ determined by the depth of color, a 4+ reaction being the deepest blue. The endpoint was defined as the last dilution giving a positive reaction.

It should be noted that two different HTAs are measured by the PCA assay. If the recipient animals are allowed to wait two hours before challenge with antigen and Evans Blue dye, then IgG₁ is detected. IgG₁ is heat stable, has a short duration in the skin and a long duration in the serum. In contrast, if antigen and Evans Blue dye are injected 72 hours after skin sensitization, the PCA reaction detects IgE, which is heat labile, has a longer duration in skin but a shorter duration in the serum.

RESULTS

IgE Production in the Mouse. Heat sensitivity is a good property to use to distinguish between heat-stable IgG₁ and heat-labile IgE. Thus, it was determined from heating undiluted serum that the 2-hour PCA reaction remained while the 72-hour PCA diminished. This same result was found in sera from both antigens used for immunization and from serum collected after a primary immunization on day 10 and a secondary immunization collected on day 30 (Table I). It should be noted that the 72-hour response remained if the serum was not heated. These results suggest that the antibody observed at 72-hours is IgE. Because of these results, I routinely used the 72-hour PCA response as a measure of IgE.

Preliminary Studies on the Ability of Different Antigens to Elicit an IgE Response in Mice. Several different antigen-adjuvant combinations were tested for their ability to elicit IgE antibody production in Balb/c mice (Table II). Mice immunized with 10 μ g CAE plus 50 μ g PE failed to produce IgE (line A), even when boosted with 10 μ g CAE on day 20. Negative results were also obtained with 125 μ g EA plus 50 μ g PE (line B) and 10 μ g CAE plus 1 μ g LPS (line C); again, booster injections were not effective. CAE, however, was a good antigen to elicit IgE when used with either CFA or alum adjuvants. 100 μ g CAE plus 20 mg alum (line D) gave 72 hr PCA reactions on day 10 after a primary immunization and day 30 after a secondary immunization. A slightly higher

TABLE I

Effects of heating a homocytotropic antibody positive serum from Balb/c mice, 30 minutes at 56°C as demonstrated by passive cutaneous anaphylaxis reactions

Immunization Day 0	Immunization Day 20	Heated	No. of Serum in Pool	Day Serum Collected	Sensitization Period	PCA ^h Titer of Serum Pool
10 µg EA ^a , Al ^b	10 µg EA	+	5	30	2 hr	256 ^c
same ^f	same	+	5	30	72 hr	neg ^g
same	same	-	7	30	72 hr	128
10 µg CAE ^d , CFA ^e	none	+	5	20	2 hr	64
same	same	+	5	20	72 hr	neg
same	same	-	7	20	72 hr	128

^aEA = hen egg albumin

^bAl = 10 mg alum

^c256 = titer reciprocal of serum pool

^dCAE = crude ascaris extract

^eCFA = complete Freund's adjuvant in equal volume as antigen

^fsame = same serum as above

^gneg = not detected at 1/8 dilution

^hPCA = passive cutaneous anaphylaxis

titer on day 10 was obtained if the dose was reduced to 10 μ g CAE plus 10 mg alum (line E). When CFA was used (line F), the mice produced IgE on days 10 and 20, and also after a booster injection. Very good primary titers were obtained with 10 μ g DNP-CAE plus 10 mg alum (line G), and also after a booster injection of DNP-CAE.

Egg albumin with alum as adjuvant was by far the easiest compound to use and also elicited high titers of IgE on days 10, 20 and 30. The optimal dose was 10 μ g EA plus 10 mg alum (line H). CFA was also effective with 125 μ g EA in production of IgE on days 20 and 30 although the titers are much lower than if alum was used as adjuvant.

After all these tests were completed, it was decided that 10 μ g EA plus 10 mg alum (line L) was the best antigen-adjuvant combination with which to observe the IgE response.

Thymus Dependency of the IgE Response. An attempt was made to determine whether or not IgE exhibits the usual thymus-derived cell (T-cell)-bursa-equivalent (B cell) collaboration required for production of other immunoglobulin classes. To test for such a requirement, attempts were made to induce IgE production in the nude mouse (Table III). Because this animal lacks T cells from birth, it provides an excellent system in which to observe thymus dependency. Both nude and NLM mice were immunized with 10 μ g EA plus 10 mg alum, and were boosted with 10 μ g EA on day 20. The NLM responded on days 10, 20 and 30 with

