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IgE Antibody Responses Induced by Repeated Administration of Antigens without Adjuvant[1,2] (41444)

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Abstract. It is generally considered that the induction of IgE antibody responses in laboratory animals to parenterally injected antigens requires the simultaneous administration of an adjuvant such as $Al(OH)_3$ gel or *Bordetella pertussis* vaccine. In this study, we have been able to induce IgE antibody formation in mice to antigens such as heterologous whole serum or ovalbumin by giving multiple intraperitoneal injections of these substances without any added adjuvant. The IgE responses were measured by the passive cutaneous anaphylaxis (PCA) test performed in rats. The abrogation of PCA activity by heat treatment of undiluted serum for one hour at 56° was confirmed. We propose that this represents an ideal model to study induction of IgE antibody formation.

The production of IgE antibody responses in mice usually requires the simultaneous administration of the "IgE adjuvants," aluminum hydroxide [$Al(OH)_3$][4] gel or *Bordetella pertussis* vaccine or extracts with antigen such as ovalbumin (OVA) (1). Certain exceptions to this have been previously reported. IgE antibody to rabbit serum proteins could be induced by the intraperitoneal (ip) injection of rabbit anti-mouse thymocyte serum (2). Furthermore, subcutaneous injection of concanavalin A (Con A) (3) or painting with the contact sensitizing agents picryl chloride or oxazolone (4) induced serum IgE responses to these respective substances in mice. In addition, worm infection causes high IgE responses to worm antigens (5). In each of the above cases, however, the eliciting antigen has some special property, other than immunogenicity, that may influence antibody formation. Anti-thymocyte serum has been shown to affect regulatory T cells (6) and to enhance IgE formation (7). Con A is mitogenic for T lymphocytes (8), and worm infection enhances IgE responses polyclonally (9). Contact sensitizing agents have the ability to bind to skin proteins.

In this study we show that mice are able to produce IgE antibody responses to two soluble antigen systems, OVA and normal rabbit serum (NRS), when given multiple, ip injections of the antigen without administration of any adjuvant. This finding is important because neither antigen system is known to have any of the special properties mentioned above.

Materials and Methods. *Animals.* BALB/c mice were used throughout. Outbred albino rats (ARS Sprague–Dawley or Holtzman Co., Madison, Wisc.) were kept in our own facilities for at least 2 weeks before experimental use and were older than 8 weeks when used.

Antigens. OVA (5× crystallized, lot 830018, Calbiochem, La Jolla, Calif.) was dissolved in physiological phosphate-buffered saline at the appropriate concentration. NRS was purchased from Colorado Serum Company, Denver, Colorado. Both antigens were stored at −20° and repeatedly thawed and refrozen for use.

[1] Most of these studies were done while J. K. M. was a doctoral candidate in the Department of Microbiology at Montana State University, Bozeman, Montana. See J. K. Manning, Regulation of IgE Antibody Responses in Mice, Doctoral Thesis, Montana State University, 1977.

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[4] Abbreviations used: $Al(OH)_3$, aluminum hydroxide; Con A, concanavalin A; id, intradermal; NRS, normal rabbit serum; OVA, ovalbumin; PCA, passive cutaneous anaphylaxis; HA, hemagglutinating antibody; DTT, dithiothreitol.

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Measurement of IgE antibody. Mouse IgE antibody was measured by the passive cutaneous anaphylaxis (PCA) test performed in rats (10–13). A series of twofold dilutions of mouse serum in saline was injected intradermally (id) using 30-gauge needles (MPL, Inc., Solopak) into the back skin of shaved rats to minimize nonspecific trauma. After 48 hr, the rats were injected intravenously with the appropriate antigen dissolved in Evan's blue dye solution (5 mg OVA dissolved in 0.5 ml 1% Evan's blue or 2 ml of NRS in which 5 mg Evan's blue dye was dissolved). Thirty minutes later, the rats were killed, and the skin was reflected for examination. PCA titers were defined as the reciprocal of the last dilution giving a discrete blue spot at least 4 mm in diameter on the underside of the skin. For the anti-OVA response, a standard antiserum dilution series, for which the PCA titer was predetermined by testing on several rats, was placed on each rat to validate the assay system. Each PCA test rat displayed a PCA titer for the standard antiserum that was within one twofold dilution of the predetermined PCA titer. For both the id and iv injections, the rats were sedated by an intramuscular injection of 0.04–0.06 ml Innovar-vet (Pitman-Moore Inc., Washington Crossing, N.J.). The identity of PCA antibody as IgE is verified by its activity in rat skin (12, 13) and loss of PCA activity upon heating undiluted serum (14) at 56° for 30–60 min.

Hemagglutinating antibodies. Hemagglutinating antibody (HA) titers to OVA were determined by V-bottom microtiter plates as described by Wegmann and Smithies (15) by utilizing sheep erythrocytes coated with OVA by $CrCl_3$ treatment. Dithiothreitol (DTT) resistant and sensitive antibodies were determined by performing the assays in the presence and absence of 0.2 *M* DTT.

Immunizations. Adult mice were injected ip with antigen on Days 0, 2, 4, 7, 9, 11, and 14 and were bled at various intervals thereafter. Mice received either 10 μg OVA or 0.25 ml of NRS at each injection. Neonatal mice were injected ip with progressively increased doses of OVA (0.02–10 μg) or NRS (0.05–0.10 ml) antigen starting within 24 hr of birth and then repeated at 2- to 7-day intervals. These mice were bled 6–7 weeks later.

Serum collection. At the appropriate times after immunization, blood was collected from the tail into plastic microcentrifuge tubes. The clotted blood was rimmed and left for several hours or overnight at 4°. The serum was collected after centrifugation and stored in ¼- or ½-dram vials at −70°. Serum stored in this manner demonstrated stable PCA activity up to 2 years after collection.

Endotoxin measurement. Samples of our antigen preparations and saline diluent were analyzed for endotoxin content by the Limulus amebocyte lysate assay (Pyrotell, Associates of Cape Cod, Mass.) using the Abbott MS-2 research instrument.

Results. *IgE antibody responses to OVA.* BALB/c mice are capable of producing IgE antibody to OVA. Using various preparations of OVA and $Al(OH)_3$ gel and several hundred BALB/c mice ranging in age from 2 to 6 months we found that immunization with one ip injection of 10 μg OVA mixed with 1 mg $Al(OH)_3$, as reported by Vaz *et al.* (16), consistently resulted in an anti-OVA IgE titer within the range of 128–512, which was present 10 days after immunization and persisted for at least 5 months thereafter. One ip injection of OVA without $Al(OH)_3$ gel does not induce any detectable IgE antibody.

Anti-OVA PCA antibody activity could also be detected in adult BALB/c mice given multiple, ip injections of 10 μg OVA alone without simultaneous administration of adjuvant (Table I). These data show that a heat labile, PCA reactive antibody was induced in 9 of 10 mice immunized with such a protocol resulting in titers from 8 to 64. The antibody activity was not detectable on Day 11 but was present on Day 18 (4 days after the last injection of OVA). The mice were bled subsequently without further injection of OVA. It is interesting that seven of the nine animals which displayed a PCA titer at Day 18 had levels of PCA antibody 78 days later which were within one dilution of the Day 18 titer.

TABLE I. IgE RESPONSES OF MICE IMMUNIZED BY MULTIPLE INJECTIONS[a] OF OVALBUMIN (OVA) WITHOUT ADJUVANT

Mouse	Anti-OVA PCA[b] responses			Anti-OVA PHA[c] response		
	Day 18	Day 32	Day 78	Day 18 −DTT/+DTT	Day 32 −DTT/+DTT	Day 78 −DTT/+DTT
1	32	32	16	8192/512	4096/256	512/256
2	8	<4	<8	512/64	256/64	64/<64
3	32	32	64	4096/2048	4096/2048	1024/1024
4	16	16	16	2048/1024	2048/1024	1024/512
5	16	16	32	8192/512	8192/512	512/128
6	<4	<4	<4	128/64	64/64	64/<64
7	64	64	128	4096/512	8192/1024	1024/256
8	16	16	32	4096/1024	2048/512	512/256
9	32	64	64	8192/ND	4096/ND	4096/ND
10	32	16	<8	4096/ND	2048/1024	1024/256
Serum pool[d]	32	ND	32			
Serum pool-heated[e]	<2	ND	<4			

[a] Mice received 10 μg soluble OVA, ip, on Days 0, 2, 4, 7, 9, 11, and 14 (Day 0 = day of first injection—mice were approximately 3 months old when immunization was started).
[b] Passive cutaneous anaphylaxis titers performed in rats.
[c] Passive hemagglutination antibody titers to OVA-coated sheep erythrocytes measured in the absence or presence of dithiothreitol (DTT).
[d] Equal amounts of serum from all mice were pooled and then tested.
[e] Undiluted serum placed at 56° for 1 hr and then tested.

These data show that persistent IgE antibody responses can be induced without adjuvants, although the amount of antibody produced by this protocol is lower and more variable than in animals immunized by one injection of $Al(OH)_3$-adsorbed OVA.

Previous reports have shown that IgE antibody responses to antigen plus adjuvant can be obtained in neonatal rabbits (17) or very young rats (18). We therefore initiated a series of immunizations of OVA without adjuvant in neonatal mice. Mice were given progressive amounts of OVA (0.02−10 μg) ip, at approximately weekly intervals starting on the day of birth. A serum pool from seven mice obtained 48 days after birth and 7 days after the last injection displayed an anti-OVA PCA titer of 64 (data not tabulated).

IgE responses to NRS. IgE responses were also obtained when NRS was used as an antigen. The use of NRS was prompted by ongoing studies concerning the effect of a rabbit anti-μ antiserum on IgE responses in mice (19). Typical responses in adult mice given a series of injections of NRS without adjuvant are shown in Table II. PCA titers to NRS ranged from 16 to 128 in group I and from 8 to 64 in group II.

When immunizations were started on the day of birth, IgE responses to NRS were also induced. Starting on the day of birth, mice were given progressive amounts of NRS (0.05−0.10 ml) ip every 2−3 days until

TABLE II. IgE RESPONSES OF MICE IMMUNIZED BY MULTIPLE INJECTIONS[a] OF NORMAL RABBIT SERUM (NRS) WITHOUT ADJUVANT

		Anti-NRS PCA responses[b]
Group I	1	128
	2	128
	3	16
	4	64
Group II[c]	1	32
	2	8
	3	32
	4	64

[a] Mice in both groups received 0.25 ml NRS, ip, on Days 0, 2, 4, 7, 9, 11, and 14 (Day 0 = day of first injection—mice were approximately 3 months old when immunization was started).
[b] Passive cutaneous anaphylaxis performed in rats. Results are from sera collected on Day 18.
[c] Mice in group II received, in addition, one ip injection of 10 μg OVA + 1 mg alum on Day 9.

Day 42. On Day 46, 4 days after the last injection of NRS, serum obtained from these mice showed a heat labile, PCA reactive antibody against NRS. The titers ranged from 16 to 128 and six out of six mice responded (data not tabulated).

Antibodies other than PCA antibodies. With this immunization protocol, antibodies other than PCA active antibodies are produced by mice. When the OVA immunization regimen is started in adult animals, antibodies against OVA, detected by a passive hemagglutination assay, are present (see Table I). Both DTT-sensitive, presumably IgM, and DTT-resistant, presumably IgG, antibodies are present. Two mice (Nos. 2 and 6) did show HA activity at a time when they did not display detectable PCA antibody. The antibody titers are similar on Days 18 and 32 but by Day 78 appear to be declining. Preliminary studies indicate that one ip injection of 10 μg OVA does not induce either an HA or a PCA response.

Studies in the rat. The same protocols of immunization were carried out in outbred rats. We failed to detect any IgE antibodies to either OVA or NRS in these animals.

Endotoxin content of antigen preparations. We considered the possibility that endotoxin might be present in our antigen preparations and may be serving as an adjuvant. Endotoxin has been shown to enhance IgE responses in the mouse (20, 21). The OVA preparation contained approximately 13–26 pg/ml and this was primarily in the saline diluent. The NRS preparation contained 325–390 pg/ml; however, quantitations of endotoxin in serum must be interpreted cautiously because of the many other substances present in serum that may be active in this assay and therefore this value represents a maximum value.

From these results it appears that endotoxin is present in our antigen preparations but in very minute amounts. Each mouse received 0.25 ml of these preparations for each injection. Previous work (20, 21) has determined that endotoxin enhances IgE responses at 1- to 10-μg levels. Therefore the amount of endotoxin given was approximately 10^{-4} to 10^{-6} less than the amount shown to have an adjuvant effect on IgE antibody formation. Therefore, we feel that the amount of endotoxin in our preparations is probably not acting as an adjuvant for the production of IgE antibody.

Discussion. We show here that IgE antibody responses can be induced in mice simply by repeated administration of low doses of antigen. These findings point to a new model system for inducing IgE antibody in mice. We suggest that such multiple immunization without adjuvant presents a model for IgE induction that may be more similar to the manner in which individuals naturally acquire immediate hypersensitivity than is the common model (22) for allergic sensitization which utilizes a single injection of $Al(OH)_3$ adsorbed antigen. Also, the importance of a model for induction of IgE antibodies to NRS is indicated by the finding (23) that humans receiving rabbit human anti-thymocyte serum as a pre-bone marrow transplant preparative regimen make IgE antibody to NRS.

The amount of circulating IgE antibody probably represents the summation of several cellular systems, particularly the amount of IgE producing cells, the nature and quantity of regulatory cells, and the "mast cell sink." The effect of adjuvants, such as $Al(OH)_3$ gel, on these systems is not known. The method of inducing IgE responses presented here would eliminate the effect of adjuvant in animal studies of IgE. For example, a frequent question concerning IgE formation is whether there are certain properties of the eliciting antigen that might enhance its ability to induce an IgE antibody response. Our system of immunization would allow a more direct study of this question.

The fact that IgE antibody production can be induced by the method presented here may reflect the necessity for repeated stimulation of the cells involved in IgE antibody formation. The repeated stimulation might simply be necessary for the formation of enough IgE-secreting cells to produce a detectable quantity of IgE in the serum. Perhaps IgE is induced in most im-

munizations. Other host-related factors may also be involved in this kind of stimulation such as a genetic capability to respond to low doses of antigen (22) or plasma cortisol levels at the time of immunization (24).

Similar findings have been published recently using OVA as well as other antigens (25, 26). There was no mention of the possibility of the presence of endotoxin in the antigen preparations. As emphasized by Fumarola (27) recently, endotoxin is a "ubiquitous contaminant of most biological materials and has many biological effects." One of these effects has been to have an adjuvant effect on IgE antibody formation (20, 21). We feel, therefore, that the judgment that a response is adjuvant independent should be made only if the antigen preparation does not contain significant levels of endotoxin.

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