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Gene-Antigen Register

Immune Response Gene Control of the Murine Antibody Response to the Phospholipase A_2 of Honey Bee Venom

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Allergic reactions to honey bee venom (HBV) are mediated by immunoglobulin E (IgE) antibodies specific for venom components. Although several allergens have been identified in HBV to date (Shepherd et al. 1974, Hoffman and Shipman 1976, King et al. 1976), it is the phospholipase A_2 component (PLA), a well-defined enzyme with a molecular mass of approximately 15 000 which appears to be the primary allergen (Sobotka et al. 1976, Shipolini et al. 1974).

Little is known about the genetic factors which predispose individuals to venom hypersensitivity, but studies with other allergens and antigens would suggest that the major histocompatibility complex (MHC) probably plays an important role (Vaz and Levine 1970, Dorf et al. 1974, Marsh et al. 1982). This possibility seems particularly relevant in those situations where specific IgE rather than total IgE appears to be disregulated, i. e., where individuals are extremely sensitive to HBV but not to other venoms or allergens.

We thus undertook a study of the role of the *H-2* complex in influencing responsiveness to PLA. Here, we report that the total serum anti-PLA response is under *H-2*-linked gene control. Mice with *b* or *f* alleles in the *A* region of the H-2 are low responders, whereas those with *d*, *k*, *q*, or *s* alleles in the same region are high responders. Both specific IgE and IgG are similarly influenced.

Serum PLA-specific antibodies were measured using an enzyme immunoassay. Wells of polyvinyl microtiter plates were coated for 3 h at 37°C with PLA (Sigma Chemical Co., St. Louis, Missouri) at a concentration of 50 μg/ml in borate buffer, pH 8.4. The wells were then washed with 1% bovine serum albumin-phosphate buffered saline (BSA-PBS) and with PBS-0.1% Tween-20, followed by the addition of either serum (20 μl) diluted in BSA-PBS or BSA-PBS alone. After overnight incubation at 4°C, the wells were washed three times with PBS-Tween-20. In order to detect total PLA-specific antibodies, wells were reacted with a polyvalent rabbit anti-mouse Ig reagent (1 μg/ml) for 2 h at 37°C, washed as before, then reacted with goat rabbit IgG-specific antibodies conjugated with alkaline phosphatase (Sigma Chemical Co.). The goat antirabbit reagent was absorbed with mouse Ig Sepharose 4B beads to remove any cross-reactive antibodies. After 2 h at 37°C, the wells were washed, and 50 μl of p-nitrophenylphosphate (1 mg/ml in 0.1 *M* glycine, 0.001 *M* $MgCl_2$, 0.001 *M* $ZnCl_2$, pH 10.4) was added. Color development proceeded for approximately 30 min at room temperature, after which the reaction was stopped by the addition of 4 N NaOH. Absorbance was read at 405 nm using a Dynatech Microelisa Analyzer (Dynatech Laboratories, Inc., Alexandria, Virginia). Background optical density (OD) values generated in PLA-coated wells which received BSA-PBS in the first step of the assay instead of serum but which received all other subsequent reagents were subtracted from the OD values obtained with test or positive control sera. This background was usually less than 0.05 OD units.

Table 1. Total serum anti-PLA response of various strains*

n	Strain	*H-2*	Anti-PLA response (μg/ml) serum)†
13	BALB.B	*b*	$< 1; < 1; < 1; < 1; 8_4; 6_5$
11	C57BL/6	*b*	$< 1; < 1; < 1; < 1; < 1; < 1_4; 2_3$
9	BALB/c	*d*	4; 8; 26; 30; 40; 42; 48; 228; 3420
7	B6.*H-2*^{k}	*k*	61; 80; 80; 327; 494; 1482; 1710
3	BALB.K	*k*	58; 988; 1216
4	CBA *(M523)*	*km*^{1}	236; 650; 1976; 3230
5	DBA/1	*q*	361; 1121; 1159; 1482; 1482
6	A.SW	*s*	11; 18; 27; 106; 179; 213

* Mice used in these experiments were bred and maintained in the mouse colony of Dr. Richard Dutton, Department of Biology, University of California, San Diego. Male and female mice were used between the ages of 8 and 16 weeks. Mice received 10 μg PLA in 2 mg alum i.p.; they were rested for 2–3 weeks and then boosted with the same dose using the same route

† Responses of individual mice or pools, which are indicated by a subscript designating the number of mice contributing to the pool. Blood was taken 5–12 days after the second injection

IgE PLA-specific antibodies were measured using purified rabbit IgE-specific antibodies (2 μg/ml) which were generously provided by Drs. L. Smith and M. Koshland of the University of California, Berkeley, California. This reagent, which was prepared against the epsilon heavy chain isolated from monoclonal IgE, binds specifically to IgE and not to μ,κ; μ,λ; γ_1,κ; γ_{2a},κ; γ_{2b},κ; or γ_3,κ. Furthermore, binding to the homologous IgE in solid phase can be completely inhibited by two unrelated monoclonal IgE antibodies. Bound rabbit IgE-specific antibody was detected with the goat antirabbit reagent as described above.

IgG PLA-specific antibodies were measured with a pool of alkaline phosphatase-conjugated goat antibodies (4 μg/ml) specific for the γ_1, γ_{2a}, γ_{2b}, and γ_3 subclasses (Southern Biotechnology Associates, Birmingham, Alabama).

In the assay for total PLA-specific antibodies, a titration of affinity-purified antibodies provided the standard curve. PLA-specific antibodies were isolated from a pool of hyperimmune sera by adsorption to PLA-Sepharose 4B and, after extensive washing, elution with 3.5 *M* NaSCN. The concentration of antibody in a test serum was calculated from duplicate titrations that included at least two dilutions giving linear increases in absorbance. The absorbance values were corrected for dilution, averaged, and converted to μg/ml of serum by comparison to the standard curve. This assay shows a sensitivity of approximately 10 ng of specific antibody per milliliter of serum. However, since 1 : 100 was the lowest dilution tested, we place the lower limit of the assay in the order of 1 μg/ml. The assay is specific for PLA-specific antibodies, since the binding of ~1 μg of antibody to PLA-coated wells is completely inhibited by preincubation with PLA (0.3 μg/ml) or HBV (1.0 μg/ml), but not by other venoms (yellow hornet, white-faced hornet, wasp, and yellow jacket) or by irrelevant antigens such as hen egg-white lysozyme, BSA-azobenzenearsonate, BSA-dinitrophenyl, and keyhole limpet hemocyanin. Furthermore, binding activity is removed by absorption of antisera with PLA-Sepharose 4B. No nonspecific binding of mouse Ig was detectable when antisera were tested on BSA-coated wells.

Table 1 shows the results of screening several strains of mice for responsiveness to PLA. We chose to concentrate on the response resulting after two injections because initial experiments indicated only a weak primary response. It is evident that mice with the *H-2*^{b} haplotype responded poorly, if at all, while mice with the H-2^d, *H-2*^{k}, *H-2*^{q}, and *H-2*^{s} haplotypes responded with the production of specific antibody. Despite some inter and intrastrain variation among the responders, the distinction between high and low responder strain titers is striking and can extend over three orders of magnitude. The response to PLA is specifically determined by the *H-2* complex, since strains which differ at H-2 but which are otherwise genetically identical, show profound differences in responsiveness, e. g., BALB/c and BALB.K versus BALB.B and B6.*H-2*^{k} versus C57BL/6. While we show only titers of sera obtained 5–12 days after the second injection, we observed that the high/low responder phenotype continues to be present for at least 40 days after the second injection.

To identify more precisely the genes governing responsiveness to PLA, we tested the responses of B10 recombinant mice (Table 2). Possession of *H-2*^{b} alleles at K, A_α, A_β, and E_β [B10.A(5R)] is sufficient for the low responder phenotype. Since B10.MBR mice are high responders, K^b is unlikely to influence responsiveness. Thus, low response maps to the A_α^b, A_β^b, and E_β^b genes.

Table 2. Total serum anti-PLA response of B10 recombinant mice*

n	Strain	*H-2*	*K*	A_β	A_α	E_β	*J*	E_α	*S*	*D*	Anti-PLA response (μg/ml serum)†
10	B10	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	<1; <1; <1; <1; <1; <1; <1; <1; <1; 3
7	B10.A(3R)	*i3*	*b*	*b*	*b*	*b*	*b*	*k*	*d*	*d*	<1; <1; <1; <1; <1; <1; <1
16	B10.A(5R)	*i5*	*b*	*b*	*b*	*b*	*k*	*k*	*d*	*d*	<1; <1; <1; <1; <1; <1; <1; <1; <1; <1; <1; <1; <1; <1; <1; 7
10	B10.M(11R)	*ap1*	*f*	*f*	*f*	*f*	*f*	*f*	*f*	*d*	<1; <1; <1; <1; <1; <1; <1; <1; <1; <1
11	B10.A(4R)	*h4*	*k*	*k*	*k*	*k*	*b*	*b*	*b*	*b*	<1; 46; 53; 212_8
7	B10.MBR	*bq1*	*b*	*k*	*k*	*k*	*k*	*k*	*k*	*q*	391_3; 912_4
13	B10.A(15R)	*h15*	*k*	*k*	*k*	*k*	*k*	*k*	*d*	*b*	<1; <1; <1; 17; 26; 44; 190; 209; 266; 418; 494; 1102; 1634
7	B10.BR	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	418; 418; 532; 684; 950; 950; 988
7	B10.AQR	*y1*	*q*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	13; 23; 32; 95; 110; 372; 912
5	B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	<1; 80; 209_3
4	B10.HTT	*t3*	*s*	*s*	*s*	*s*	*s*	*k*	*k*	*d*	720_4
10	B10.T(6R)	*y2*	*q*	*q*	*q*	*q*	*q*	*q*	*q*	*d*	<1; <1; <1; 437; 532; 771; 988; 1349; 2052; 2660

* Source of mice and injections were as indicated in Table 1

† Responses of individual mice or pools as indicated in Table 1

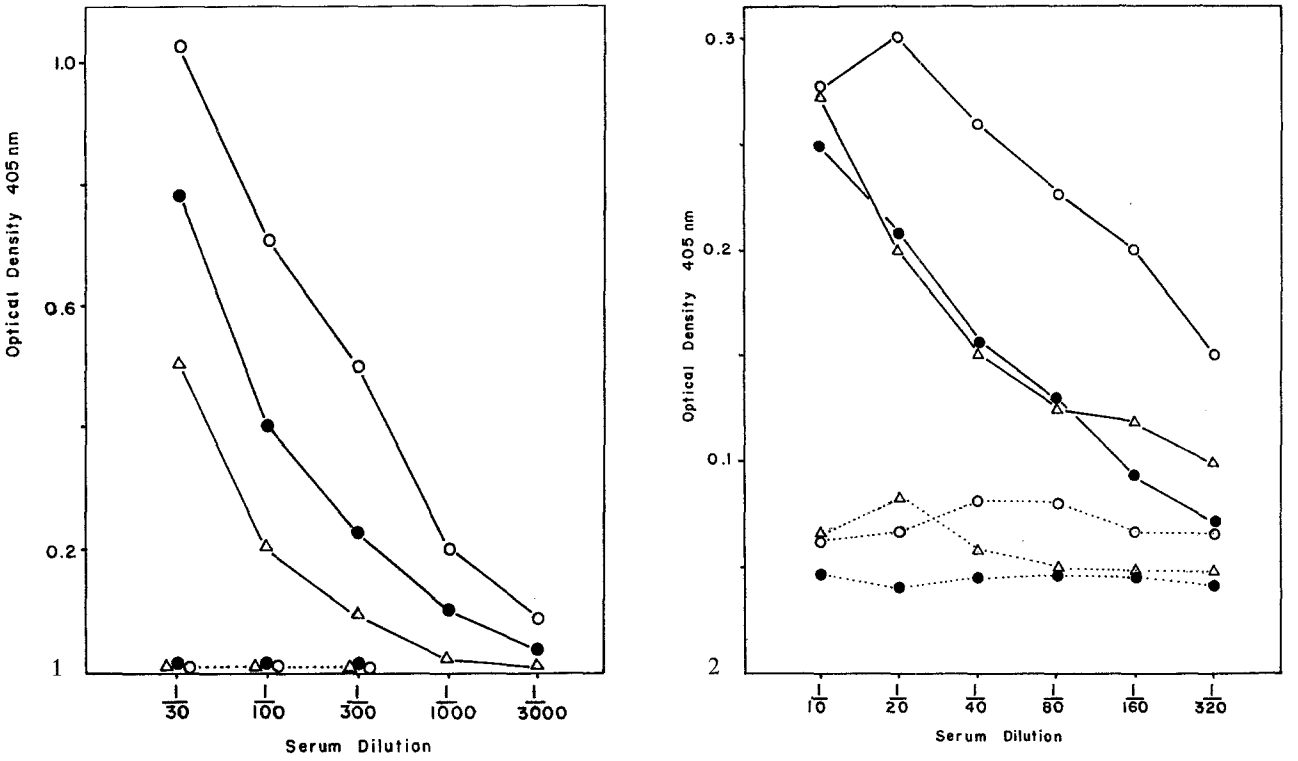

Fig. 1. Enzyme immunoassay of serum IgG PLA-specific antibodies. Mice received 2 μg PLA in 2 mg alum i.p.; they were rested for 2 weeks and were then boosted with the same dose using the same route. Blood was taken on day 10 following the second injection. B10, △ . . . △ (9); B10.D2, △——△ (5); BL/6, ● . . . ● (4); BL/6-*H-2*^{k}, ●——● (8); BALB.B, ○ . . . ○ (7); BALB.K, ○——○ (6). Numbers in parentheses refer to the number of mice in each group. Individual sera were pooled within each group

Fig. 2. Enzyme immunoassay of serum IgE PLA-specific antibodies. Mice and injections are the same as indicated in Figure 1

The responder status of the B10.A(4R) strain supports this conclusion. In addition to H-2^b, H-2^{ap1} mice are low responders to PLA.

Mice with *b*, *s*, *f*, and *q* haplotypes do not possess cell-surface E molecules due to defects in expression of E_α (Jones et al. 1981, Mathis et al. 1983). The failure of H-2^b and H-2^{ap1} mice to respond to PLA does not appear to be due to their inability to express E because: (1) E expression per se is not a prerequisite for the response to PLA, since H-2^q and H-2^s mice are responders; (2) despite their expression of $E_\beta{}^b E_\alpha{}^k$, B10.A(5R) and (3R) mice remain low responders; and (3) B10.A(4R) mice, which also do not express E, are most certainly responders due to their expression of A^k and not because of their possession of a phenotypically silent $E_\beta{}^k$ allele. Thus, while low response maps to the $A_\alpha{}^b$, $A_\beta{}^b$, and $E_\beta{}^b$ genes, we think it is likely that H-2^b-governed low responsiveness is actually mediated via A^b.

The inheritance of responsiveness to PLA was studied in two (responder × nonresponder) F_1 crosses, BALB/c × BALB.B and B10.D2 × B10. Mice were injected and bled as indicated in Table 1. Of ten BALB/c × BALB.B mice tested, nine responded with serum titers of 1 μg/ml or more, the average of the responders being 130 μg/ml (range = 1–350). Of 15 B10.D2 × B10 mice tested, 11 responded with an average titer of 169 μg/ml (range = 2–600). We therefore conclude that responsiveness to PLA is inherited as a dominant trait.

Previous studies of other immune response (Ir) gene-controlled responses have shown that the distinction between high and low responders becomes less evident when higher doses of antigen are used (Dorf et al. 1974, Vaz and Levine 1970, Levine and Vaz 1970). We attempted to demonstrate a similar phenomenon in the response to PLA. H-2^k and H-2^b mice were immunized with varying doses of PLA (2–50 μg) on alum, and the sera were tested for total PLA-specific antibody after the second injection. While H-2^k mice responded at all the doses tested, no antibody (less than 1 μg/ml) was detected in the sera of H-2^b mice (data not shown). However, when H-2^b and H-2^k mice were primed with 50 μg PLA in complete Freund's adjuvant i.p. and boosted with 50 μg soluble PLA i.p. two weeks later, a significant antibody response was detected in the sera 5 days after the second injection. B10 and C57BL/6 (*H-2*^{b}) mice responded with average serum titers of 33 μg/ml (n = 6; range = 6–63) and 98 μg/ml (n = 4; range = 10–323), respectively, whereas B10.BR mice (*H-2*^{k}) responded with an average titer of

1780 μg/ml (n = 4; range = 1026–3800). Thus, while the data indicate that so-called nonresponder mice can indeed synthesize significant antibody with appropriate immunization, the distinction between high and low responders remains striking nevertheless.

In order to directly establish that the observed *Ir* gene applied to both IgE and IgG isotypes, we examined the secondary responses of three different congenic pairs using isotype-specific developing reagents. A dose of 2 μg PLA in alum was used in this experiment, because we had observed that this dose induced slightly higher specific IgE responses than the 10 μg dose (data not shown). Figure 1 shows the titration of the IgG anti-PLA responses. As expected, H-2^d mice responded, whereas H-2^b mice did not. The specific IgE response follows the same pattern (Fig. 2). H-2^d mice possessed specific IgE, while no IgE anti-PLA was detectable in the sera of H-2^b mice. Because specific IgG antibodies could interfere with the detection of specific IgE, it remains possible that we have underestimated the IgE titers in the responder mice. Attempts to remove IgG by absorption of sera with *Staphylococcus aureus* bacteria failed to diminish the IgG anti-PLA titer sufficiently to alter the titration of specific IgE (data not shown).

To determine whether the response to PLA was T cell-dependent, we examined the total serum anti-PLA response of BALB/c-*nu/nu* and -*nu*/+ mice. Eight days after the second injection of 10 μg PLA in alum, the *nu*/+ mice responded with an average serum titer of 349 μg/ml (n = 8; range = 1–1672), whereas the *nu/nu* mice responded with an average titer of 10 μg/ml (n = 9; range = < 1–38). Thus, we conclude that the response to PLA is, for the most part, T cell-dependent.

In summary, we have shown that the antibody response to PLA is under *Ir* gene control. In keeping with earlier studies (Vaz and Levine 1970, Dorf et al. 1974), the magnitude of both the specific IgE and IgG response is influenced by genes of the *H-2* complex. This effect is presumably manifest via the interactions of antigen-specific, MHC-restricted T cells, antigen, and MHC molecules. While different T-cell populations might be involved in the regulation of the IgE and IgG responses (Kishimoto and Ishizaka 1973), these results would indicate that the PLA-specific T cells, which control the expression of both isotypes, probably share similar MHC restriction specificity. The induction and regulation of IgE responses are complex and multifactorial. The genetic determinants are likewise complex and are thought to involve MHC-linked and non-MHC-linked loci (Revoltella and Ovary 1969, Levine and Vas 1970, Bazaral et al. 1974, Marsh and Bias 1978). Nonetheless, the data presented here suggest to us that it would be worthwhile to examine the human response to PLA to determine whether particular class II alleles are associated with high IgE responses or with high IgG 'blocking' antibody responses induced during the course of venom desensitization therapy.

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References

Bazaral, M., Orgel, A. H., and Hamburger, R. N.: Genetics of IgE and allergy: Serum IgE levels in twins. *J. Allergy Clin. Immunol. 54:* 288-304, 1974

Dorf, M. E., Newburger, P. E., Hamaoka, T., Katz, D. H., and Benacerraf, B.: Characterization of an immune response gene in mice controlling IgG and IgE antibody responses to ragweed pollen extract and its 2,4-dinitrophenylated derivative. *Eur. J. Immunol. 4:* 346-349, 1974

Hoffman, D. E. and Shipman, W. H.: Allergens in bee venom. I. Separation and identification of the major allergens. *J. Allergy Clin. Immunol. 58:* 551-562, 1976

Jones, P. P., Murphy, D. B., and McDevitt, H. O.: Variable synthesis and expression of E_α and A_e (E_β) Ia polypeptide chains in mice of different *H-2* haplotypes. *Immunogenetics 12:* 321-337, 1981

King, T. P., Sobotka, A. K., Konchoumion, L., and Lichtenstein, L. M.: Allergens of honey bee venom. *Arch. Biochem. Biophys. 172:* 661-671, 1976

Kishimoto, T. and Ishizaka, K.: Regulation of antibody response in vitro. VI. Carrier specific helper cells for IgG and IgE antibody response. *J. Immunol. 111:* 720-732, 1973

Levine, B. B. and Vaz, N. M.: Effect of combinations of inbred strain antigen, and antigen dose on immune responsiveness and reagin production in the mouse. *Int. Arch. Allergy 39:* 156-171, 1970

Marsh, D. G. and Bias, V. W.: The genetics of atopic allergy. *In* M. Samter (ed.): *Immunological Diseases*, p. 819-831, Little, Brown and Company, Boston, 1978

Marsh, D. G., Hsu, S. H., Roebber, M., Ehrlich-Kautzky, E., Friedhoff, L. R., Meyers, D. A., Pollard, M. K., and Bias, W. B.: HLA-DW2: A genetic marker for human immune response to short ragweed pollen allergen Ra5. I. Response resulting primarily from natural antigenic exposure. *J. Exp. Med. 155:* 1439–1451, 1982

Mathis, D. J., Benoist, C., Williams, V. E., Kanter, M., and McDevitt, H. O.: Several mechanisms can account for defective E_α gene expression in different mouse haplotypes. *Proc. Natl. Acad. Sci. U.S.A. 80:* 273-277, 1983

Revoltella, R. and Ovary, Z.: Reaginic antibody production in different mouse strains. *Immunology 17:* 45-54, 1969

Shepherd, G. W., Elliot, W. B., and Arbesman, C. E.: Fractionation of bee venom. I. Preparation and characterization of four antigenic components. *Prep. Biochem. 4:* 71-88, 1974

Shipolini, R. A., Callewaert, G. L., Cottrell, R. C., and Vernon, C. A.: The amino acid sequence and carbohydrate content of phospholipase A_2 from bee venom. *Eur. J. Biochem. 48:* 465-474, 1974

Sobotka, A. K., Franklin, R. M., Adkinson, N. F., Valentine, M., Baer, H., and Lichtenstein, L. M.: Allergy to insect stings. II. Phospholipase A: The major allergen in honey bee venom. *J. Allergy Clin. Immunol. 57:* 29-40, 1976

Vaz, N. M. and Levine, B. B.: Immune responses of inbred mice to repeated low doses of antigen: Relationship to histocompatibility (H-2) type. *Science 168:* 852-854, 1970

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