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Biological Characterization of a Formolated Allergoid from *Dermatophagoides pteronyssinus**

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A comparative study between the biological properties of the allergen *Dermatophagoides pteronyssinus* (Dp) and its formolated allergoid (DpHCHO) was performed. After immunizing rats with Dp or DpHCHO the serum level time course of IgE and IgG was determined by passive cutaneous anaphylaxis (PCA) and indirect-ELISA, respectively. The avidity for IgE (PCA and PCA-inhibition) or IgG (indirect ELISA and ELISA inhibition) and the ability to induce anaphylactic shock were also determined. Serum IgE pattern differs between Dp and DpHCHO while IgG levels were greater after DpHCHO. The allergoid has lost the ability to bind IgE while its avidity for IgG falls 5-6 times. The allergoid, but not the allergen, fails to induce anaphylactic shock. A schedule for assaying the biological characteristics of allergoids is presented.

Key words: *Dermatophagoides pteronyssinus*, Allergoids, IgE, IgG, ELISA, Passive cutaneous anaphylaxis.

Hyposensitization therapy has been assumed to yield benefits as a result of influences on the immune status of the individual (7, 8).

In particular, induction studies of the IgG class protective antibodies in the serum (3, 4, 17) and IgA in the secretions (3, 9) have been used to assess the relationship of changes in such parameters to efficacy.

Allergens used for hyposensitization therapy can be chemically modified so that undesirable properties such as reactivity with mast cell-bound IgE antibody are reduced but those properties thought

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to be required for hyposensitization are retained (12).

The potential of allergoids in the immunotherapy of allergic diseases is therefore based on the premise that they could be administered in high dosages with negligible risk of systemic reactions and with concomitant immunological protection against allergic symptoms (9).

In this study we have developed several tests *in vitro* and *in vivo* for biological preclinical assay of allergens and allergoids.

As allergen the extract of the mite *Dermatophagoides pteronyssinus*, one of the most frequent inhalant allergens in respiratory allergic diseases such as asthma and allergic rhinitis (2, 10, 16, 17) have been used. This allergen was chosen because an experimental model of Dp allergy had been developed in previous studies. As allergoid a formaldehyde-modified Dp extract was used.

Materials and Methods

Immunogenic agents. Allergen and formolated allergoid from the mite Dp were obtained from commercial sources (Allergopharma Joachin Ganzer, Reinbets). The allergenic extract was supplied in 1 % W/V solution with a protein content of 6000 pnu/ml. The allergoid was provided as lyophilized powder with a protein content of 100.400 pnu/ml. The units of protein are related as follows: 1 µg nitrogen = 100 pnu = 6.25 µg protein (15).

Animals. — Female Sprague-Dawley rats and Dunking-Harley guinea-pigs were used in this study. Rats and guinea-pigs were fed on standard diets (Biosure RMM and FD1 respectively) and kept in controlled climatic conditions. Food and drinking water were available *ad libitum*.

Immunization. — Rat anti-Dp reaginic antiserum was obtained from female rats weighing between 180 and 220 g. Blood was withdrawn by cardiac puncture 12 days after subcutaneous immunization with 0.5 ml/100 g of a suspension containing 350 pnu of Dp and 200 mg of alum per ml of saline (1750 pnu/kg).

IgE and IgG antibody responses were determined after Dp or DpHCHO immunization of rats. Four groups of rats were used in this experiment. Two groups were sensitized with Dp and the other two with DpHCHO. After twelve days one group of each (Dp or DpHCHO) was boosted s.c. with 0.5 ml/100 g of a suspension containing 70 pnu/ml of Dp or DpHCHO and 200 mg of alum in 1 ml of saline (350 pnu/ml). At 0, 12, 15, 21, 28 and 32 days 700 µl blood samples were obtained from each animal by retroorbital puncture. Serum samples were obtained and stored at -20 °C until used for antibody titration.

Titration of IgE and IgG antibodies. — The time-course of IgE and IgG anti-Dp serum levels of rats immunized with Dp or DpHCHO was determined by PCA (6) and indirect-ELISA respectively (5, 6).

Allergenicity testing. — Allergenicity of Dp and DpHCHO were determined using two methods.

A direct measure of the avidity *in vivo* of IgE anti Dp for Dp or DpHCHO was performed using the PCA test. In summary, 3500 pnu/kg of Dp or DpHCHO were administered i.v. in rats with four dorsal skin sites previously sensitized with anti-Dp antiserum diluted 1/4. The amount of the dye in each site after challenge with allergen or allergoid was measured.

An indirect measurement of allergenicity was the PCA-inhibition test (PCA-I). The ability of Dp or DpHCHO to neutralize the specific IgE anti-Dp was determined. For PCA-inhibition, 200 µl of several saline concentrations of Dp or DpHCHO were mixed with equal vol-

umes of 1/4 serum anti-Dp dilution. The mixtures were left overnight at 4 °C and 0.1 ml of each injected into the skin of recipient rats. Two days later an i.v. dose of Dp and Evans blue was administered and the concentration of dye in the flare was determined. This method is based on that described by BLACK and FRANCIS (1).

Titration of immunogenicity. — The relative avidity of IgG anti-Dp for Dp or DpHCHO was evaluated by indirect ELISA. The procedure was similar to that described in the previous paragraph. Thus, 100 µl of increasing Dp (from 15 to 35 pnu/ml) or DpHCHO (from 15 to 140 pnu/ml) concentrations were seeded to different wells of microELISA plates. In the next step different dilutions of anti-Dp serum were added for each immunogen concentration.

To exclude the possibility that a different affinity of Dp or DpHCHO for the plate surface "a" could give false avidity measurements, an indirect ELISA-inhibition test was developed. In short the procedure consists of incubating a standard rat anti-Dp overnight at -4 °C with 0, 15, 35, 70 and 140 pnu/ml of Dp or DpHCHO. The neutralized serum (100 µl) was added to a microELISA plate previously coated with 35 µl/ml of Dp and incubated for 3 h at room temperature. The plates were washed and incubated with 100 µl/well of rabbit anti-rat IgG-peroxidase. Subsequent steps were the same as outlined before.

Anaphylactic shock in guinea-pigs. — Two groups of guinea pigs were sensitized by injection of a primary dose of 1750 pnu/ml of Dp in alum (day 0) and an identical secondary dose 12 days later. Twenty two days after the first dose, half the animals received an i.v. challenge with Dp and the other half with DpHCHO, both at doses of 3500 pnu/kg. Two additional groups were immunized with DpHCHO, using the same protocol; half were challenged with Dp and the rest with DpHCHO. As a standard group seven guinea-pigs were sensitized with ovalbumin grade V (Fluka) 1 mg/kg, the same protocol was followed using ovoalbumin 1.5 mg/kg for i.v. challenge.

Statistical analysis of data was performed using Mann-Whitney U-test.

Results

After immunizing rats with a single dose of allergoid, serum anti-allergen antibodies were seen to appear. The pattern

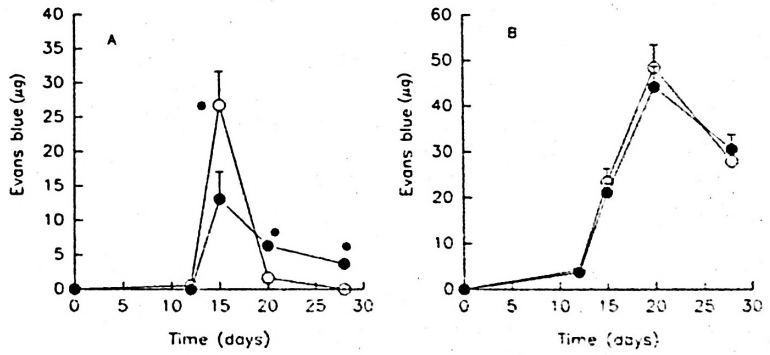

Fig. 1. *Time-course of anti-Dp IgE antibodies in serum of rats after immunization with a primary dose (A) of Dp (○) or DpHCHO (●) and after immunization with a primary and a secondary dose (B) of Dp (○) or DpHCHO (●).*
Each point represents the arithmetic mean ± SEM of 4 rats. * $p < 0.05$ Mann-Whitney U-test.

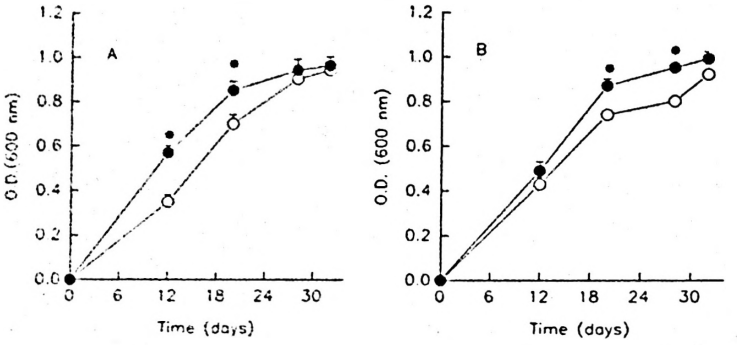

Fig. 2. *Time-course of anti-Dp IgG antibodies in serum of rats after immunization with a primary dose (A) of Dp (○) or DpHCHO (●) and after immunization with a primary and a secondary dose (B) of Dp (○) or DpHCHO (●).*
Each point represents the arithmetic mean ± SEM of 4 rats. * $p < 0.05$ Mann-Whitney U-test.

of IgE anti-Dp levels induced by allergoid was quite different from that seen after giving allergen. The IgE levels induced by the allergoid were lower but more sustained than those induced by Dp (fig. 1 A). When two separate doses of allergoid or allergen were given to rats, the IgE anti-Dp levels were similar (fig. 1 B).

The IgG anti Dp response was always greater after allergoid than after allergen irrespective of the immunization schedule (fig. 2).

The IgE anti-Dp did not show avidity for the allergoid bound to cutaneous mast cells as demonstrated by PCA. As shown in fig. 3 A, the i.v. DpHCHO administration failed to give a local anaphylactic reaction at the same dose as allergen. When the allergen was given i.v. after the allergoid, the same degree of degranula-

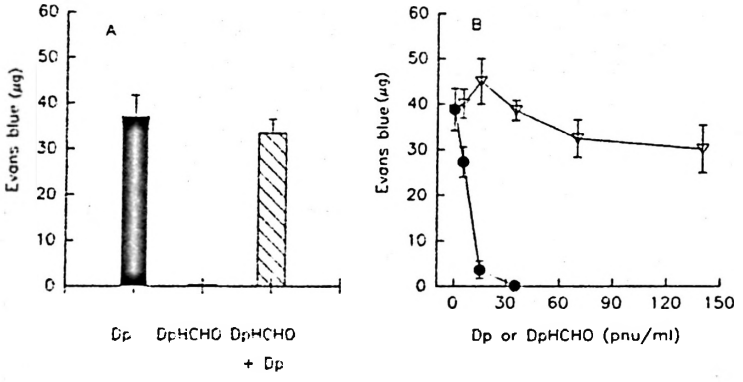

Fig. 3. *Relative avidity of i.v. Dp, DpHCHO, and Dp after giving DpHCHO i.v. for IgE fixed to cutaneous mast cells (A).*
Each bar represents the arithmetic mean ± SEM of 6 rats. Inhibition of the skin mast cell sensitization by neutralizing with Dp (●) or DpHCHO (▽) an anti-IgG antiserum (B). The µg of Evans blue in the skin spot indicates the degre of mast cell degranulation. Each point represents the arithmetic mean ± SEM of 4 experiments.

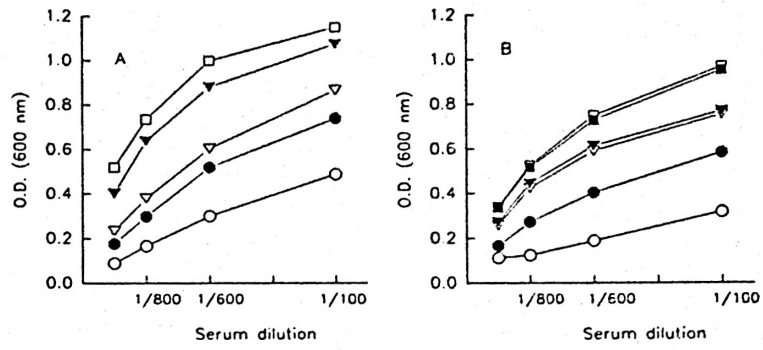

Fig. 4. *Relative avidity of Dp (A) and DpHCHO (B), fixed to the solid phase of an ELISA plate to different dilutions of IgG anti-Dp serum.*
Test performed by indirect ELISA. Concentrations (pnu/ml) for Dp are 15, 20, 25, 30, 35 (○, ●, ▽, ▼, □) and for DpHCHO 15, 35, 70, 140, 180, 240 (○, ●, ▽, ▼, □, ■).

tion and Evans blue extravasation was observed. Thus, allergoid did not antagonize the degranulating process induced by the allergen.

Reactivity of DpHCHO with specific IgG antibodies was assessed by direct and inhibition systems. DpHCHO concentrations between 180-240 pnu/ml attached to the solid phase of the ELISA plate had lower avidity for specific IgG antiserum than 35 pnu/ml of Dp, as evidenced by the lower colorimetric measurement obtained when the indirect ELISA was developed (fig. 4).

The allergoid failed to neutralize serum IgE anti-Dp as shown by the PCA-inhibition test. Thus, 15 pnu/ml of Dp inhibited the above mentioned reaction by 90 % while no inhibition was observed with 140 pnu/ml of allergoid (fig. 3 B).

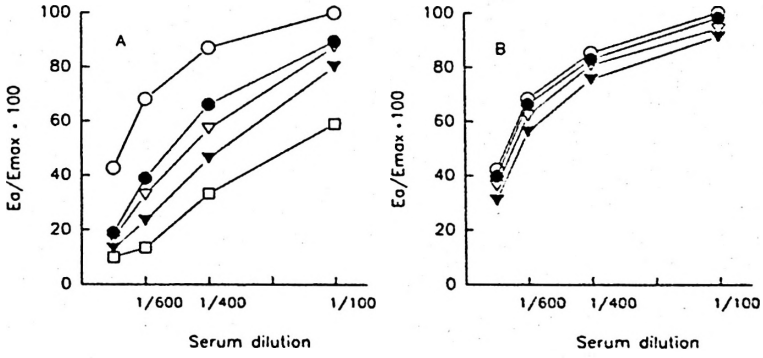

Fig. 5. *Dp (A) and DpHCHO (B) neutralizing capacity of IgG anti Dp present in different antiserum dilutions evaluated by ELISA-inhibition.*
Concentrations (pnu/ml) for Dp are 0, 15, 35, 70 and 140 (○, ●, ▽, ▼, □) and for DpHCHO 0, 35, 70 and 240 (○, ●, ▽, ▼). Individual results (Ea) were expressed as percent of the maximal O.D. obtained without antagonist.

Avidity of DpHCHO for IgG was partially retained while it was abolished for IgE.

Similarly, the allergoid lacked the ability to bind rat IgG specific antibodies of an antiserum as shown by ELISA-inhibition. Thus, 140 pnu/ml did not reduce the maximal colorimetric reaction obtained without neutralizing the antiserum while 50 % inhibition was obtained using only 35 pnu/ml of allergen (fig. 5).

In guinea pigs immunized with Dp, a mortal anaphylactic shock appeared in 66.6 % of the animals after i.v. challenge with Dp. The allergoid failed to induce anaphylactic shock in guinea-pigs immunized with Dp. When these were immunized with DpHCHO and challenged, either with the allergen or the allergoid, no sign of anaphylactic shock appeared. All the animals sensitized with the standard allergen ovoalbumin suffered mortal anaphylactic shock after challenge.

Discussion

One of the claims for allergoids is their retained ability to induce blocking antibodies against the native allergen and to reduce the IgE formation. One of the main objectives in this field is to create more effective and safer products for immunotherapy (9).

The relative immunogenic and allergenic response of native and formaldehyde-modified allergens was investigated by their capacity to induce anti-allergen IgG or IgE antibodies in the rat.

As previously seen, DpHCHO can induce the production of IgE anti-Dp, the risk of anaphylactic reactions thus persisting. It is noteworthy that IgE anti-Dp production rose more slowly after a single dose of DpHCHO than after the same dose of Dp. In contrast, IgG anti-Dp antibodies were always higher after administration of the allergoid than after allergen. This may be related with the higher molecular weight of the formolated allergoid.

The IgG anti Dp antibodies may be regarded as IgG blocking antibodies, and thus they may be responsible for the efficacy of immunotherapy. Despite the doubt regarding the functional significance of IgG antibody induction in hyposensitization therapy, a rise in the specific antibody of this isotype or in secretory antibodies is still regarded as a useful indicator of a potentially successful treatment (13, 14). However, although induced antibodies may produce blocking effects, they may produce beneficial actions. Thus they may induce antiidiotypic responses which may suppress allergen specific IgE antibody. Alternatively, they may produce desensitizing effects on mast cells via reactivity with cell-bound IgE (11).

Data presented in this study show that the allergoid was essentially non-reactive with IgE antibody. The reduction in the ability of DpHCHO to give positive skin test is correlated with the loss reactivity with IgE anti-Dp seen by PCA-inhibition. Thus, allergoid lacks avidity for IgE.

Allergoid doses which are seven-fold those of the allergen do not achieve the same degree of reaction with the allergen as evidenced by indirect ELISA. Those results should be considered with some reserve, as the changes introduced in the allergen to give an allergoid may have altered its affinity to plate binding sites and thus when incubating the same dose of either Dp or DpHCHO a different degree of binding may occur. To clarify this doubt an ELISA-inhibition test was developed in which Dp binds to solid phase and afterwards the antiserum, previously neutralized *in vitro* with the allergen or allergoid, is added before developing the plate. Using this procedure none of the allergoid doses assayed have been shown to be able to neutralize the IgG anti-Dp of a standard antiserum. Thus, in this case both procedures give the same result.

In guinea pigs Dp appears to have higher sensitizing properties than the allergoid. Animals sensitized with Dp died as a consequence of challenge with Dp, but those immunized with DpHCHO survived a shock dose of this allergoid given by i.v.

On the other hand, neither the Dp nor the DpHCHO have the ability to induce anaphylactic shock in animals immunized with DpHCHO or Dp, respectively.

This phenomenon was probably either a reflection of a different spectra for allergen or allergoid epitopes.

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Resumen

Se estudian las propiedades biológicas del alergeno *Dermatophagoides pteronyssinus* (Dp) y de su alergoide formolado (DpHCHO). En ratas inmunizadas con Dp o DpHCHO se valora la evolución de los niveles séricos de IgE y de IgG anti-Dp por anafilaxia cutánea pasiva y ELISA-indirecto, respectivamente. Se estudia la avidez de Dp o DpHCHO por la IgE (PCA y PCA-inhibición) y por la IgG (ELISA indirecto y ELISA-inhibición) y la capacidad para inducir choque anafiláctico en cobayas. Se aprecia una evolución diferente entre los niveles de IgE inducidos por Dp o DpHCHO. El alergoide produce niveles superiores de IgG y pierde la capacidad para unirse a la IgE, mientras que la avidez por la IgG se reduce 5-6 veces. A diferencia del alergeno, el alergoide no produce choque anafiláctico en cobayas sensibilizados con Dp. Se desarrolla una pauta para la evaluación preclínica de las características biológicas de los alergoides.

Palabras clave: *Dermatophagoides pteronyssinus*, Alergoides, IgE, IgG, ELISA, Anafilaxia cutánea pasiva.

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