

## The IgG subclasses of antibodies to castor bean allergen in patients with allergic asthma: detection of a high incidence of antibodies of the IgG4 subclass

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### Summary

Sera from patients with allergic asthma to castor bean dust were tested for specific antibodies in the red cell linked antigen–antiglobulin reaction (RCLAAR) using antiglobulin sera specific for IgE and the four subclasses of IgG. Approximately one-third of the patients with specific IgG antibodies had an antibody response that was predominantly in the IgG4 subclass. Two patients who were thought not to be sensitive to castor bean allergen, despite long exposure to the dust, did not have detectable IgE antibodies, but had high titres of IgG antibodies that were predominantly IgG4. Specific IgG4 antibodies to castor bean allergen were heat stable, did not appear to passively sensitize monkey skin in the PCA reaction and did not passively sensitize human basophils for the rosette reaction with castor bean-coated red cells.

### Introduction

The castor bean (*Ricinus communis*) contains some of the most potent allergenic proteins known. Hypersensitivity, which is mainly of the respiratory type, is common in people who are in contact with castor bean dust and is often associated with hypersensitivity to other inhalants such as house dust, moulds and grass pollen. Outbreaks of allergic asthma to the castor bean allergen have been described, not only in castor-oil mill workers, but also in people living in the vicinity of castor-oil mills (Panzani, 1957). Specific hyposensitization to castor bean allergen is rarely performed as commercial extracts are not usually free from ricin. Attempts to use *Ricinus* pollen extracts for desensitization have not proved successful (Panzani & Layton, 1963).

The red cell linked antigen–antiglobulin reaction (RCLAAR) was first described by Coombs, Howard & Mynors (1953) and has since proved a useful *in vitro* technique for the measurement of specific antibodies to a variety of different antigens (Coombs *et al.*, 1965; Devey *et al.*, 1970; Wheeler, 1971; Wilson *et al.*, 1972). Despite the development of the more sensitive radioallergosorbent test (RAST) for the measurement of

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specific IgE antibodies (Wide, Bennich & Johansson, 1967) the RCLAAR remains the only method in which antibodies of all the immunoglobulin classes (and subclasses) can be measured in a single test. Coombs *et al.* (1968), using the RCLAAR, demonstrated specific IgE antibodies to castor bean allergen in the sera of patients with allergic asthma living near to, or working in, castor-oil mills near Marseilles, France. These antibodies were heat sensitive and conferred passive cutaneous anaphylaxis (PCA) to baboon skin. High titres of IgG antibodies to castor bean allergen were also found in many of these patients.

Recently there has been much evidence for the existence of heat stable 'anaphylactic' antibodies of the IgG class in human sera, similar to those found in other animal species (Terr & Bentz, 1965; Augustin, 1967; Reid, 1970; Parish, 1970, 1971). These antibodies sensitize monkey skin for the PCA reaction for 1–4 hr, weakly sensitize human skin for 4–6 hr in the Prausnitz–Küstner test, passively sensitize human lung tissue for the release of histamine and appear to compete for a short time with IgE for the same, or closely adjacent, receptor sites on cells (Parish, 1974). In the present study sera from patients with allergic asthma to castor bean dust were re-examined in the RCLAAR in order to measure the IgG subclasses of specific antibodies and to determine if any of these antibodies were of the 'short-term' anaphylactic type.

## Materials and methods

### *Human sera*

Sera were obtained from patients mostly with allergic asthma living near to, or working in, castor-oil mills near Marseilles. These sera had been freeze-dried and were re-constituted with distilled water and stored at  $-20^{\circ}\text{C}$  before testing. Control sera were obtained from hay-fever patients and normal non-allergic individuals.

### *Castor bean allergen*

Purified *Ricinus communis* allergen CB-WU was a gift from Dr L. L. Layton, United States Department of Agriculture. It was dialysed against PBS and extensively absorbed with washed human red cells to remove the last traces of ricin.

### *Antiglobulin sera*

Antiglobulin sera specific for total IgG, IgG1, IgG2, IgG3 and IgG4 were raised and absorbed as described by Devey & Voak (1974). The specific antiglobulin serum to IgE was a generous gift from Dr S. G. O. Johansson, Uppsala, Sweden. All the antiglobulin sera were heat inactivated and absorbed with washed human red blood cells. Specificity was tested in haemagglutination tests using human red blood cells linked to purified myeloma proteins by the chromic chloride method (Gold & Fudenberg, 1967). The linked proteins were always different from those to which the sera had been raised to avoid reaction with idiotype.

The haemagglutination reactions of the antiglobulin sera with red cells coated with purified myeloma proteins of the different classes and IgG subclasses are shown in Table 1. The optimal dilution selected for each antiglobulin reagent was the one at which no cross reactions occurred with these strongly coated red cells but which also gave strong haemagglutination reactions with cells at much lower levels of sensi-

tization. In addition, an attempt was made to use the IgG subclass-specific antiglobulin sera at dilutions that were approximately equipotent. The dilutions selected for these sera were ones that, on further dilution, gave similar end points with the appropriately coated red cells. These test red cells were shown to be coated with similar amounts of protein as they gave the same end point with a polyvalent antiserum. The anti- $\epsilon$  serum did not cross react with any of the IgG subclasses, IgA or IgM but had been shown to be specific for the IgE myeloma ND in precipitation tests.

Table 1. Specificity of the class- and IgG subclass-specific antiglobulin sera

| Antiglobulin sera | Haemagglutination titres of human red blood cells linked to purified myeloma proteins by the chromic chloride method |        |        |        |     |     |
|-------------------|----------------------------------------------------------------------------------------------------------------------|--------|--------|--------|-----|-----|
|                   | IgG1                                                                                                                 | IgG2   | IgG3   | IgG4   | IgA | IgM |
| Anti- $\gamma$    | > 2560                                                                                                               | > 2560 | > 2560 | > 2560 | 0*  | 0   |
| Anti- $\gamma$ 1† | 32                                                                                                                   | 0      | 0      | 0      | 0   | 0   |
| Anti- $\gamma$ 2‡ | 0                                                                                                                    | 64     | 0      | 0      | 0   | 0   |
| Anti- $\gamma$ 3† | 0                                                                                                                    | 0      | > 2560 | 0      | 0   | 0   |
| Anti- $\gamma$ 4¶ | 0                                                                                                                    | 0      | 0      | 2560   | 0   | 0   |
| Anti- $\epsilon$  | 0                                                                                                                    | 0      | 0      | 0      | 0   | 0   |

\* 0 = <2.

† Sera raised in baboons.

‡ Serum raised in a rabbit.

¶ Serum raised in mice by Dr H. G. Kunkel.

#### *Preparation and testing of the antibody-coupled castor bean reagent*

A 7S fraction of a rabbit antiserum to human group 0 red blood cells was coupled to the castor bean allergen by photo-oxidization using rose bengal, as described by Steele & Coombs (1964). Serial dilutions of the antibody-coupled castor reagent were incubated with equal volumes of washed 2% human group 0 red blood cells. After washing in PBS the red cells were resuspended and single drops added to a microtitre plate containing normal rabbit serum in row 1, a goat antiserum to rabbit IgG in row 2, and a rabbit antiserum to castor bean allergen in row 3. The optimal dilution of the coupled reagent for coating red cells was the one that gave strong agglutination in row 3 without direct agglutination in row 1.

#### *The red cell linked antigen-antiglobulin reaction*

This was performed as described by Coombs *et al.* (1968). Equal volumes of 2% washed human group 0 red blood cells and the antibody-coupled castor bean reagent were mixed at room temperature. After 30 min the cells were washed in PBS to remove uncoupled allergen and resuspended to 2%. Equal volumes of these castor bean-coated red cells were then added to serial dilutions of the test serum. After 30 min at room temperature the cells were washed in PBS containing 0.1% gelatin (PBS/G) to remove unabsorbed serum proteins, and resuspended to 2% in PBS/G. The uptake of specific antibodies to castor bean allergen was measured by the addition of the red cells to microtitre plates containing class and subclass-specific antiglobulin sera. Haemagglutination patterns were read after 1 hr at room temperature.

*Inhibition tests*

It was essential to show that the antibodies reacting in the RCLAAR were specifically directed to castor bean allergen and were not non-specifically absorbed by the red cells. This was demonstrated by the addition of castor bean allergen to the dilutions of test sera which blocked the subsequent uptake of antibody onto the castor bean-coated red cells in the RCLAAR. The specificity of the antiglobulin sera was checked in the last stage of the RCLAAR by addition of purified myeloma proteins to the antiglobulin sera before addition of the red cells. Haemagglutination reactions of the antiglobulin sera were only inhibited by their corresponding myeloma protein and not significantly by those of any other class or IgG subclass.

*Passive cutaneous anaphylaxis*

PCA tests were performed in Cynomolgous monkeys sedated with Sernylan (Parke-Davis). Undiluted test sera (0.1 ml) were injected intradermally into shaved sites in the abdomen, chest, arms or legs, 2 or 24 hr before intravenous challenge with 2 ml 1% Evans' Blue dye followed after 10 min by 2 ml castor bean allergen. Reactions were read after 20 min.

*Passive sensitization of human basophils with sera containing antibodies to castor bean allergen*

This was performed by the method of Wilson, Marchand & Coombs (1971). Briefly, a leucocyte suspension was prepared from the blood of a normal non-allergic donor with a relatively high basophil count. The cells were resuspended in a diluent containing EDTA and heparin at pH 7.4 (Levy & Osler, 1967) and incubated with the test serum for 1.5 hr at 37°C. After washing, the cells were briefly centrifuged with human red cells coated with castor bean allergen, resuspended and mounted in toluidine blue stain on siliconed microscope slides. Up to fifty basophils were counted in each preparation and the number forming rosettes with three or more castor bean-coated red cells was recorded.

**Results***Antibodies to castor bean allergen*

Forty-nine sera from patients living near to, or working in, castor-oil mills near Marseilles were tested for antibodies to castor bean allergen in the RCLAAR. Thirty-six of these sera (73%) contained IgG antibodies to castor bean allergen and thirty-two (65%) also contained specific IgE antibodies. Significant titres of antibody were not detected in the remaining sera, nor in control sera from five hay-fever patients and five non-allergic individuals.

The antibody distribution between the IgG subclasses in typical patients, together with their clinical status, is shown in Table 2. Twenty-two (61%) of the sera containing specific IgG antibodies showed an antibody response that was roughly proportional to the relative amounts of the IgG subclasses in normal serum, that is mainly IgG1 which constitutes about 72% of the total IgG. For example, the IgG antibodies to castor bean allergen in patients CE, ME and IM were predominantly IgG1, occasionally with quite high titres of IgG2. However, in fourteen sera (39%), for example BA, RA, MA, LA and NI, there appeared to be a disproportionate increase in antibodies of the IgG4 subclass, as this subclass represents only 3–4% of total IgG. Antibodies

of the IgG3 subclass were rarely detected and were usually of low titre with the exception of patient TA whose antibody response was predominantly IgG3 and IgG1.

**Table 2.** Antibodies to castor bean allergen measured by the RCLAAR in patients living near to, or working in, castor-oil mills

| Patient | Clinical history* | Specific antibody titre |      |      |      |      |     |
|---------|-------------------|-------------------------|------|------|------|------|-----|
|         |                   | Total IgG               | IgG1 | IgG2 | IgG3 | IgG4 | IgE |
| CE      | 1,2               | 512                     | 64   | 64   | x†   | x    | 256 |
| ME      | 1,2,3,4           | 32                      | 16   | 8    | 4    | 4    | 16  |
| IM      | 1,2               | 16                      | 8    | 0‡   | 0    | 0    | 8   |
| BA      | 4,5               | 512                     | 0    | 0    | 0    | 256  | 0   |
| RA      | (1,2) 6           | 512                     | 8    | 8    | 0    | 128  | 0   |
| MA      | 1,2 4             | 512                     | 64   | x    | x    | 64   | 128 |
| LA      | 1,2 4             | 32                      | 0    | 0    | 0    | 16   | 32  |
| NI      | 1,2               | 128                     | 16   | 4    | 0    | 32   | 32  |
| TA      | 1,2               | 128                     | 16   | 0    | 32   | 0    | 32  |
| UB      | 3,4,5             | 0                       | 0    | 0    | 0    | 0    | 0   |
| IC      | 4,5               | 0                       | 0    | 0    | 0    | 0    | 0   |

\* Clinical histories: 1 allergic asthma to castor bean dust, 2 positive skin test to castor bean allergen, 3 has received injections of castor pollen extract for hyposensitization, 4 allergic asthma to other inhalants, 5 negative skin test to castor bean allergen, 6 thought to have become 'naturally desensitized'.

† x, antiglobulin titres not greater than direct agglutination in 1/50 normal rabbit serum.

‡ 0 = <2.

Of particular interest were patients BA and RA. Neither appeared to have IgE antibodies to castor bean allergen, but in both, high titres of IgG antibodies were measured and these were, exclusively in BA and predominantly in RA, of the IgG4 subclass. It was thought possible that these IgG4 antibodies may have been mediating some form of clinical hypersensitivity in these patients, but their clinical histories made this unlikely. Patient BA had worked in the castor-oil mill for 31 years, he had clinical asthma to other inhalants (IgE antibodies could be demonstrated to grass pollen in the RCLAAR) but he had a negative skin test to castor bean allergen and was not thought to be allergic to it. Patient RA had also worked at the mill for a long period of time and he had, at one time, had allergic asthma to castor bean dust, but not to any other inhalant. He had not received hyposensitizing injections, but was thought to have become 'naturally desensitized' by continuous exposure to the allergen.

Heating sera at 56°C for 2 hr did not affect the titres of IgG antibodies of any of the subclasses. In sera with very high titres of IgE antibodies these were significantly reduced but not completely destroyed by this heat treatment. This is compatible with the relative resistance to heat of the combining site of IgE antibodies as compared with the lability of the tissue fixing site (Ishizaka, Ishizaka & Menzel, 1967). However, heating sera at 56°C for 4 hr completely destroyed all IgE antibody activity as measured by the RCLAAR, whereas the IgG titres were only slightly reduced.

#### *Passive cutaneous anaphylaxis in monkey skin*

The results from the PCA tests on five sera performed in a single monkey are shown

in Table 3. Undiluted sera were tested unheated and also after heating at 56°C for 2 hr. The 24 hr reactions correlated well with the specific IgE antibody titres measured by the RCLAAR. Sera containing IgE antibodies also gave weak positive reactions at 2 hr. However, as all reactions were abolished by pre-heating the sera, it was assumed that they were all mediated by IgE antibodies and not by a heat-stable IgG antibody. Neither of the sera containing high titres of IgG4 antibodies (BA and RA) gave positive PCA reactions at 2 or 24 hr. These sera were also heated at 56°C for 2 hr to destroy the possible blocking activity of IgE antibodies of other specificities, but still remained negative, suggesting that neither contained heat stable, short-term skin sensitizing antibodies.

**Table 3.** Short-term and long-term PCA reactions in a Cynomolgous monkey with undiluted sera containing antibodies to castor bean allergen

| Sera       | 2 hr<br>PCA* | 24 hr<br>PCA* | Specific antibody titre |      |      |      |     |
|------------|--------------|---------------|-------------------------|------|------|------|-----|
|            |              |               | IgG1                    | IgG2 | IgG3 | IgG4 | IgE |
| CE         | 15           | 50            | 64                      | 64   | x†   | x    | 256 |
| CE heated‡ | 0            | 0             |                         |      |      |      |     |
| FU         | 5            | 32.5          | x                       | 16   | x    | 32   | 64  |
| FU heated  | 0            | 0             |                         |      |      |      |     |
| TA         | 8            | 25            | 16                      | 0    | 32   | 0    | 32  |
| TA heated  | 0            | 0             |                         |      |      |      |     |
| BA         | 0            | 0             | 0                       | 0    | 0    | 256  | 0   |
| BA heated  | 0            | 0             |                         |      |      |      |     |
| RA         | 0            | 0             | 8                       | 8    | 0    | 128  | 0   |
| RA heated  | 0            | 0             |                         |      |      |      |     |

\* Mean diameter in mm.

† x = antiglobulin titres not greater than direct agglutination.

‡ Heated at 56°C for 2 hr.

#### *Passive sensitization of human basophils*

It has been shown by Wilson *et al.* (1971) that normal human basophils can be passively sensitized with IgE antibodies to castor bean allergen as shown by the rosette reaction with castor bean-coated red cells. These results were confirmed in the present study (Table 4). Passive sensitization of basophils occurred only with sera containing IgE antibodies to castor bean allergen (CE and MA) and the number of rosettes correlated well with the titres of IgE antibody. Pre-heating the sera at 56°C for 2 hr completely abolished their ability to passively sensitize basophils. No rosettes were obtained after incubation of the basophil suspension with either of the two sera containing IgG4 antibodies to castor bean allergen (BA and RA) before or after heating the sera at 56°C for 2 hr. These results suggested that the IgG4 antibodies in these patients, unlike the IgE antibodies, were not capable of passive sensitization of human basophils in a manner that allowed subsequent combination with allergen. However, the conditions necessary for the passive sensitization of basophils by IgG antibodies may be rather different from those required by IgE.

#### **Discussion**

Specific IgG and IgE antibodies were demonstrated by the RCLAAR in the sera of

patients with allergic asthma to castor bean dust. Approximately one-third of the patients with IgG antibodies showed a disproportionate increase in antibodies of the IgG4 subclass which constitutes only 3–4% of total IgG. In a few of these patients there was a particularly marked antibody restriction to the IgG4 subclass.

**Table 4.** Passive sensitization of human basophils by IgE antibodies to castor bean allergen

| Sera       | No. of<br>rosettes/50 basophils |            | Specific antibody titre |      |      |      |     |
|------------|---------------------------------|------------|-------------------------|------|------|------|-----|
|            | Castor bean-coated RBC          | Normal RBC | IgG1                    | IgG2 | IgG3 | IgG4 | IgE |
| CE         | 47                              | 0          | 64                      | 64   | x*   | x    | 256 |
| CE heated† | 0                               | 0          |                         |      |      |      |     |
| MA         | 33                              | 0          | 64                      | x    | x    | 64   | 128 |
| MA heated  | 0                               | 0          |                         |      |      |      |     |
| BA         | 0                               | 0          | 0                       | 0    | 0    | 256  | 0   |
| BA heated  | 0                               | 0          |                         |      |      |      |     |
| RA         | 0                               | 0          | 8                       | 8    | 0    | 128  | 0   |
| RA heated  | 0                               | 0          |                         |      |      |      |     |

\* x = antiglobulin titre not greater than direct agglutination.

† Heated at 56°C for 2 hr.

Although it is appreciated that the RCLAAR is only a semiquantitative test and that the antibody titres obtained may depend on the potency of the antiglobulin reagents used, it was hoped that some relationship between the four subclasses of IgG could be established by using antiglobulin sera at dilutions that were approximately equipotent. Despite this, the possibility remained that the enhanced titres of IgG4 antibodies detected here were merely due to a stronger antiglobulin serum to this subclass. However, this was considered unlikely as similarly high titres of IgG4 antibodies had not been obtained to a variety of other antigens, such as casein,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin and insulin (Devey, unpublished observations) or to the Rh antigen (Devey & Voak, 1974), using the same antiglobulin reagents in similar testing systems.

The finding of two sera with very high titres of IgG4 antibodies to castor bean allergen in the absence of detectable IgE antibodies provided an opportunity to study further the biological properties of this subclass; particularly in the light of the recent observations that prior injection of an IgG4 myeloma protein will block the subsequent binding 4 hr later of IgE pollen antibodies in the PCA reaction in baboon skin (Stanworth & Smith, 1973) and the reported association between IgG4 and heat-stable, short-term skin sensitizing antibodies (Parish, 1974). However, the findings in this study did not provide evidence that the IgG4 antibodies in these patients were of the short-term anaphylactic type perhaps mediating some form of immediate hypersensitivity. Neither patient appeared to have clinical sensitivity or positive skin tests to castor bean allergen. Indeed, one of the patients (RA) who had in the past had allergic asthma to castor bean dust was thought to have undergone 'natural desensitization' through continuous contact with the allergen. Unlike IgE antibodies to castor bean allergen the IgG4 antibodies did not mediate PCA reactions in monkey

skin either at 2 or 24 hr. However, it must be noted that a serum containing heat-stable short-term skin sensitizing antibodies was not available as a positive control in these tests. Similarly the sera containing IgG4 antibodies in the absence of IgE antibodies did not appear to passively sensitize human basophils as demonstrated by the rosette reaction with castor bean-coated red cells. It has, however, been shown that histamine release from basophils passively sensitized with IgG antibodies can only be mediated by anti-IgG and not by antigen (Parish, 1974). It is therefore possible that the combining site of such antibody may not be accessible to antigen and therefore rosettes would not be formed with antigen-coated red cells despite the presence of IgG on the basophils.

It was concluded that IgG4 antibodies to castor bean allergen in patients with allergic asthma did not have anaphylactic activity. This would suggest that if the short-term skin sensitizing IgG is associated with a particular IgG subclass it is not IgG4. However, this does not exclude the possibilities that perhaps only some IgG4 molecules are involved or that the activity is spread over several IgG subclasses. Enhanced production of IgG4 antibodies to castor bean allergen may be a selective response to the particular antigen or the route of antigen administration. The biological role of IgG4 antibodies to castor bean allergen may be as so-called 'blocking antibodies' which, presumably together with antibodies of other IgG subclasses combine with antigen thereby preventing its subsequent reaction with cell bound IgE. However, it may be possible that IgG4 antibodies have a more specific role, perhaps by blocking the uptake of IgE onto mast cells and basophils. This function would not be incompatible with the reported short-term inhibition of IgE sensitization of baboon skin described by Stanworth & Smith (1973). If IgG4 does function in this way it may combine with the same receptors as IgE or ones closely adjacent, but in a way that either does not allow combination with antigen or, if antigen combination occurs, does not cause release of pharmacological mediators. This might explain why IgG4 antibodies to castor bean allergen did not mediate PCA reactions in monkeys or passively sensitize human basophils for the rosette reaction.

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