

(From London and the Department of Pathology, University of Cambridge)

THE SEROLOGICAL EXAMINATION OF SERA  
FROM NORMAL PERSONS AND FROM HAYFEVER  
PATIENTS BOTH BEFORE AND AFTER SPECIFIC  
HYPOSENSITIZATION FOR HEATSTABLE ANTI-  
BODIES TO THE PROTEINS OF TIMOTHY  
GRASS POLLEN

By

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INTRODUCTION

The introduction of the "coated tanned red cell technique" (Boyden, 1951) offered a new method for the serological detection of antibodies to pollen in hayfever patients undergoing specific hyposensitization.

Orlans, Rubinstein and Marrack (1953) using Boyden's (1951) tanned cell method demonstrated the rise in serum antibodies which occurred in persons receiving injections of the pollen extract. These authors mentioned that they were unable to show any relationship between the strength of the antibody detected and the clinical results of treatment. This suggested to them that the antibody had no relation to the offending allergen. Their finding is perhaps not surprising, for much has to be done before it can be hoped to be able to correlate the serological findings with the clinical picture.

Before serological methods can be of help as an objective measure paralleling the clinical relief arising from specific hyposensitization or have anything of practical value to offer

the clinical allergist, three aspects of the problem require attention.

1. First, new and more competent serological methods must be investigated for their capacity to detect serum antibodies of any variety to pollen antigens.

2. (a) The full specific mosaic of the patient's sensitization must be able to be determined by biological test and (b) if possible, by *in vitro* serological tests as well.

3. Finally, the full spectrum of serum antibodies produced by hyposensitization must be able to be detected serologically.

The present study is a small contribution to the first section only and reports the application of the "red cell linked antigen test" (Coombs, Howard and Mynors 1953) to the detection of heat stable antibodies to timothy grass pollen proteins in human sera. Theoretically this test should be capable of detecting incomplete antibodies to the pollen antigens should they occur as well as complete antibodies. The sera were also examined by the "coated tanned red cell technique" (Boyden, 1951) so that the two results could be compared.

The serological findings are purely in relation to Timothy grass pollen. This must be borne in mind in attempting to correlate the findings with the clinical condition.

#### MATERIALS AND METHODS

##### *Timothy grass pollen antigen.*

##### (a) *For skin tests.*

This was the standard glycerine extract of defatted pollen as put out by C. L. Bencard, Ltd. for skin testing.

##### (b) *For the "coated tanned red cell test" of Boyden.*

For this test the standard concentrated glycerine extract as prepared by C. L. Bencard, Ltd. was used.

##### (c) *For the "red cell linked antigen test".*

The pollen antigen in this case was prepared by

making an aqueous extract of the whole defatted pollen. The extract was concentrated by freeze-drying and dialysed against water to remove all dialysable material and subsequently freeze-dried again. It was reconstituted as a 5 % protein solution for conjugation with the proteins of a rabbit anti-ox red cell serum.

#### *Human sera.*

Blood was obtained by venepuncture. Serum was removed with aseptic precautions from the clotted blood—sent through the post from London to Cambridge and then stored at  $-20^{\circ}\text{C}$  till required for testing. The sera were inactivated at  $56^{\circ}\text{C}$  for half an hour and absorbed with ox red cells. Some sera as described later were specially absorbed with ox cells previously sensitized with rabbit antibody in order to absorb any non-specific agglutination enhancing factor that might have been present in the serum.

#### *Skin tests.*

The first 23 persons recorded in the table had scratch tests performed on them. The rest had prick tests.

#### *Coated tanned red cell test of Boyden.*

The treatment and coating of the red cell was carried out as described in our previous work (Hoet, Mynors, Coombs, Bell and Milner, 1952). Human cells of blood group O were used. The same serum sample was used for this test and the red cell linked antigen test and for this reason was inactivated and absorbed with ox red cells.

#### *Red cell linked antigen test.*

The method has been described in detail in a previous paper (Coombs, Howard and Mynors, 1953).

The ox red cell—pollen protein antigen unit was prepared

TABLE

*Pollen antibody levels as demonstrated by two serological techniques in the healed sera of hayfever patients undergoing specific hyposensitization.*

| Serum                                        | Clinical history                                   | Skin test to timothy pollen extract | Serum antibody titre to timothy grass pollen protein found by:- |                                                    |
|----------------------------------------------|----------------------------------------------------|-------------------------------------|-----------------------------------------------------------------|----------------------------------------------------|
|                                              |                                                    |                                     | Antigen coated tanned red cell method (Boydén 1951)             | Red cell linked antigen method (Coomps et al 1953) |
| Persons with no clinical history of hayfever |                                                    |                                     |                                                                 |                                                    |
| Co. 22.2.54                                  |                                                    | 0                                   | 0                                                               | 0                                                  |
| He. "                                        |                                                    | 0                                   | 0                                                               | (1)                                                |
| Mat. "                                       |                                                    | 0                                   | 0                                                               | 0                                                  |
| Wi. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| Ba. 1.3.54                                   |                                                    | 0                                   | 0                                                               | (1)                                                |
| Jo. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| Na. "                                        |                                                    | 0                                   | 0                                                               | 1                                                  |
| Mar. "                                       |                                                    | 0                                   | 0                                                               | 0                                                  |
| Fi.L. 8.3.54                                 | No clinical sensitivity .....                      | ++                                  | 0                                                               | 0                                                  |
| Fi.P. "                                      |                                                    | 0                                   | 0                                                               | 0                                                  |
| Ba. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| Sa. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| Ma. 15.3.54                                  |                                                    | 0                                   | 0                                                               | 0                                                  |
| As. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| Mo. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| Wi. 22.3.54                                  |                                                    | 0                                   | 0                                                               | 0                                                  |
| Ta. "                                        |                                                    | 0                                   | 0                                                               | (1)                                                |
| Sk. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| Ca. "                                        |                                                    | 0                                   | 0                                                               | 0                                                  |
| An. 29.3.54                                  |                                                    | 0                                   | 0                                                               | 0                                                  |
| Be. "                                        | No clinical sensitivity but allergic history ..... | ++                                  | 0                                                               | 0                                                  |

| A.W.B. |         |  | Hayfever. No pollen injections. Blood taken 10 min. |                  |                  |                  |
|--------|---------|--|-----------------------------------------------------|------------------|------------------|------------------|
|        |         |  | before skin tests                                   | after skin tests | after skin tests | after skin tests |
| D.A.C. | 5.9.51  |  | +                                                   | +                | 0                | (1)              |
| S.C.S. | 7.9.51  |  | +                                                   | +                | 0                | 2                |
| J.S.   | "       |  | +                                                   | +                | 0                | 0                |
| P.K.A. | 21.5.51 |  | +                                                   | +                | 0                | 2                |
| J.P.B. | 28.6.51 |  | +                                                   | +                | 0                | 1                |
| H.N.C. | 1.11.50 |  | +                                                   | +                | 0                | (1)              |
| J.M.H. | 3.7.51  |  | +                                                   | +                | 0                | 2                |
| A.O.   | 18.4.51 |  | +                                                   | +                | 0                | 1                |
| H.J.R. | 24.4.51 |  | +                                                   | +                | 0                | (1)              |
|        |         |  | +                                                   | +                | 0                | 1                |

The same persons after hyposensitizing injections\*  
of mixed pollen extracts

|        |         |                                                                            |   |           |     |
|--------|---------|----------------------------------------------------------------------------|---|-----------|-----|
| D.A.C. | 6.9.52  | 13 weeks after end of 1st course. Clinically much improved                 | + | 0         | 8   |
| J.S.   | 2.7.52  | 4 weeks after end of 1st course. Clinically much improved                  | + | zone 8-64 | 128 |
| P.K.A. | 10.7.52 | 5 weeks after end of 1st course. Clinically very much improved             | ± | 128       | 64  |
| H.N.C. | 5.11.51 | 21 weeks after end of 1st course. Clinically very much improved. No asthma | ± | 0         | 16  |
| "      | 18.6.52 | 2 weeks after end of 2nd course. Hayfever almost clear                     | + | 512       | 128 |

\* B<sub>4</sub> mixture of C. L. Bencard, Ltd.

TABLE (continued)

| Serum                                                                                                                  | Clinical history | Skin test to<br>timothy<br>pollen<br>extract                                                                  | Serum antibody titre to<br>timothy grass pollen<br>protein found by:- |                                                                  |
|------------------------------------------------------------------------------------------------------------------------|------------------|---------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|------------------------------------------------------------------|
|                                                                                                                        |                  |                                                                                                               | Antigen coa-<br>ted tanned<br>red cell me-<br>thod (Boy-<br>den 1951) | Red cell lin-<br>ked antigen<br>method<br>(Coomps et<br>al 1953) |
| The following hayfever patients had all received<br>hyposensitizing injections at some period before<br>first examined |                  |                                                                                                               |                                                                       |                                                                  |
| C.C.A.                                                                                                                 | 18.5.51          | 1 week after end of 1st course. Clinically much<br>better .....                                               | 0                                                                     | 64                                                               |
| "                                                                                                                      | 27.10.51         | 25 weeks after end of 1st course—almost free of<br>symptoms .....                                             | ++                                                                    | 16                                                               |
| "                                                                                                                      | 6.6.52           | 1 week after end of 2nd course. Still almost free,<br>but one or two bad days .....                           | ±                                                                     | 64                                                               |
| J.E.G.                                                                                                                 | 11.11.50         | 22 weeks after end of 1st course. Hayfever, asthma<br>+ V.R. Better year .....                                | +                                                                     | 1                                                                |
| "                                                                                                                      | 23.11.51         | 24 weeks after end of 2nd course. Better year .....                                                           | ±                                                                     | 4                                                                |
| "                                                                                                                      | 2.7.52           | 4 weeks after end of 3rd course .....                                                                         |                                                                       | 32                                                               |
| E.P.                                                                                                                   | 16.4.51          | Two courses before 1944. Third course in 1950<br>ended relapsing H.F. ....                                    | ++                                                                    | 0                                                                |
| "                                                                                                                      | 2.2.52           | 30 weeks after end of fourth course. 1951 was a<br>better year .....                                          | ±                                                                     | 16                                                               |
| "                                                                                                                      | 15.7.52          | 6 weeks after end of fifth course. No change .....                                                            | +                                                                     | 256                                                              |
| P.H.                                                                                                                   | 15.10.51         | 19 weeks after end of 1st course. Better year .....                                                           | ++                                                                    | 8                                                                |
| "                                                                                                                      | 6.10.52          | 18 weeks after end of 2nd course. Better year .....                                                           | ±                                                                     | 16                                                               |
| T.D.                                                                                                                   | 10.11.51         | Hayfever and asthma. Previously 3 courses of<br>injections with freedom but relapsed two years<br>later ..... | ++                                                                    | 0                                                                |
| "                                                                                                                      | 21.6.52          | 3 weeks after a further course. Excellent year ...                                                            |                                                                       | 128                                                              |
| J.R.                                                                                                                   | 6.9.51           | A previous course in 1946. Relapsed hayfever<br>1950 .....                                                    | ++                                                                    | 4                                                                |

|          |          |  |                                                                             |
|----------|----------|--|-----------------------------------------------------------------------------|
| "        | 18.6.52  |  | 2 weeks after end of 2nd course. Clinically better still                    |
| P.J.     | 4.12.51  |  | 26 weeks after end of 1st course. H.F. and asthma. Clinically improved      |
| "        | 9.6.52   |  | 1 week after end of 2nd course. Very much better                            |
| M.C.     | 6.9.51   |  | H.F. and asthma. Had received injections 1943-1945. Fresh course ended      |
| "        | 17.6.52  |  | 2 weeks after a further course. Still bad hay-fever                         |
| G.W.     | 6.3.52   |  | 35 weeks after end of 1st course. Hayfever and asthma improved              |
| "        | 4.7.52   |  | 4 weeks after end of second course. Clinically much better                  |
| D.O'N.   | 16.10.51 |  | Asthma and slight hayfever. 19 weeks after end of 1st course. Asthma better |
| "        | 13.6.52  |  | 2 weeks after end of 2nd course. About the same                             |
| "        | 24.10.52 |  | 18 weeks after end of 2nd course                                            |
| F.R.     | 3.10.51  |  | 20 weeks after end of 1st course. Asthma and hay-fever better               |
| "        | 29.9.52  |  | 19 weeks after end of 2nd course. No asthma, still little hayfever          |
| A.A.     | 14.11.51 |  | 23 weeks after end of 1st course. Hayfever much better                      |
| "        | 14.7.52  |  | 6 weeks after end of 2nd course. Very much better                           |
| G.B.     | 27.9.51  |  | Two previous courses, the last ending 16 weeks earlier. Clinically better   |
| P.B.     | 16.4.51  |  | 40 weeks after end of 1st course. Much better                               |
| "        | 10.6.52  |  | 1 week after end of 2nd course. Better still                                |
| N.J.W.B. | 20.11.50 |  | Asthma and hayfever. Had only had an incomplete course                      |
| "        | 12.12.51 |  | 26 weeks after second course. A little better                               |
| "        | 23.6.52  |  | 3 weeks after third course. Much better                                     |
| I.B.     | 30.10.51 |  | Various courses over 20 years. The last in 1947. Beginning asthma           |
| "        | 19.6.52  |  | 2 weeks after a further course. Clinically improved                         |
| J.B.     | 11.9.51  |  | 14 weeks after end of 1st course. A little better                           |
| "        | 16.7.52  |  | 6 weeks after end of 2nd course. Much better                                |

| Serum    | Clinical history                                                                                       | Skin test to<br>timothy<br>pollen<br>extract | Serum antibody titre to<br>timothy grass pollen<br>protein found by:- |                                                                  |
|----------|--------------------------------------------------------------------------------------------------------|----------------------------------------------|-----------------------------------------------------------------------|------------------------------------------------------------------|
|          |                                                                                                        |                                              | Antigen coa-<br>ted tanned<br>red cell me-<br>thod (Boy-<br>den 1951) | Red cell lin-<br>ked antigen<br>method<br>(Coombs et<br>al 1955) |
| M.D.     | 17.11.50                                                                                               | +                                            | 16                                                                    | 16                                                               |
| "        | 30.1.52                                                                                                | +                                            | 32                                                                    | 8                                                                |
| "        | 25.6.52                                                                                                | —                                            | 512                                                                   | 128                                                              |
| D.W.C.D. | 10.10.51                                                                                               |                                              |                                                                       |                                                                  |
|          | 27 weeks after end of 1st course. Hayfever better ...                                                  | ++                                           | 512                                                                   | 32                                                               |
|          | 30 weeks after end of 2nd course. Better still .....                                                   | +                                            | 512                                                                   | 128                                                              |
|          | 3 weeks after end of 3rd course. Very much better                                                      |                                              |                                                                       |                                                                  |
|          | 18 weeks after end of 1st course. Hayfever much better .....                                           |                                              |                                                                       |                                                                  |
|          | 5 weeks after end of 2nd course .....                                                                  |                                              |                                                                       |                                                                  |
| H-M.     | 4.7.52                                                                                                 |                                              |                                                                       |                                                                  |
|          | 24.9.51                                                                                                |                                              |                                                                       |                                                                  |
|          | 16 weeks after end of 1st course. Did not quite com-<br>plete course. Hayfever and asthma better ..... | ++                                           | 0                                                                     | 2                                                                |
| "        | 27.9.52                                                                                                | ±                                            | 32                                                                    | 16                                                               |
| B.H.     | 7.11.51                                                                                                |                                              |                                                                       |                                                                  |
|          | Various courses over last 10 years, with im-<br>provement but relapse on stopping .....                | ++                                           | 0                                                                     | 4                                                                |
| "        | 1.7.52                                                                                                 |                                              |                                                                       |                                                                  |
|          | 3 weeks after end of fresh course. Clinically much<br>better .....                                     | +                                            | 64                                                                    | 32                                                               |
| "        | 16.10.52                                                                                               |                                              | 4                                                                     | 8                                                                |
|          | 14 weeks after end of same course .....                                                                |                                              |                                                                       |                                                                  |

|        |          |                                                                         |    |                |         |
|--------|----------|-------------------------------------------------------------------------|----|----------------|---------|
| L.H.R. | 8.12.51  | 26 weeks after end of 1st course. Asthma a little better .....          | ++ | 2              | 16      |
| "      | 23.6.52  | 3 weeks after end of 2nd course. Clinically much better .....           | +  | 16             | 128     |
| D.J.R. | 1.1.52   | Various courses over 12 years. Asthma slightly improved .....           | ++ | 4              | 4       |
| "      | 2.7.52   | 4 weeks after end of fresh cours. Slight but definite improvement ..... |    | 8              | 8       |
| P.S.   | 6.3.52   | Various courses over 10 years. Hayfever better after last course .....  | +  | 16             | 16      |
| "      | 18.6.52  | 2 weeks after end of fresh course. Clinically much better .....         |    | 512            | 256     |
| R.V.   | 19.10.51 | Various courses over 10 years. Last course 4 years previously .....     | +  | 0              | 2       |
| "      | 27.6.52  | 3 weeks after new course. Asthma and hayfever almost clear .....        | ±  | zone 2-64<br>8 | 32<br>8 |
| "      | 8.10.52  | Later bleed 18 weeks after course finished .....                        |    |                |         |
| Wi.    | 19.9.51  | 15 weeks after end of 1st course. Hayfever and asthma much better ..... | ++ | 256            | 128     |
| "      | 22.12.52 | 26 weeks after end of 2nd course. Almost clear ...                      | +  | 512            | 256     |

The skin tests on the first 23 persons in the table were scratch tests—the remainder were prick tests.

0 = No agglutination.

1-512 = Titre of serum producing agglutination.

by allowing a 2 % suspension of washed ox cells (of the "inagglutinable type", Coombs, Gleeson-White and Hall, 1951) and a 1:20 dilution of the coupled rabbit anti-ox cell serum containing ox cell antibodies-azo-benzidine-azo-pollen protein to interact.

The ox cell-antigen unit as a washed suspension was then added to the titration of the human sera under investigation. Adsorption of human antibody to the pollen antigen was detected by an indicator system consisting of alternate applications of rabbit anti-human globulin serum, solution of human globulin and finally anti-human globulin serum again.

In the testing of undiluted human sera, adsorption of a non-specific agglutination enhancing factor on to the red cell-antigen unit could simulate adsorption of a specific antibody. However this non-specific factor can be removed from a serum by an absorption of the serum with a washed suspension of ox cells previously sensitized with rabbit antibody. This was, in fact, done with all sera which showed an apparent antibody titre of 1 or 2 in order to be certain that the reaction was due to a specific pollen antibody.

*Coupling the proteins in rabbit anti-ox red cell serum with Timothy grass pollen proteins.*

The appropriate amount of a solution of benzidine tetrazonium chloride was added in the cold to a mixture of 5 c.c. rabbit anti-ox cell serum and 2 c.c. of a 5 % solution of Timothy grass pollen proteins (molecular weight taken as 10-20,000) in order to have 8 moles of the chemical per 2 moles of protein (Coombs, Howard and Wild, 1952). After dialysis the serum containing ox cell antibody coupled with pollen protein was stored at  $-20^{\circ}\text{C}$  till required for testing.

#### EXPERIMENTAL STUDY

All sera were examined undiluted and in increasing dilutions for antibodies to the proteins of Timothy grass pollen.

Each serum was examined by two techniques, the "red cell linked antigen test" (Coombs, Howard and Mynors, 1953) and the "antigen coated tanned red cell method" (Boyden, 1951).

Other than the 23 sera obtained for control purposes from apparently normal persons, the sera were from patients with histories of hayfever. The serum antibody levels obtained by the two techniques are given in the main table.

#### DISCUSSION

Injection of pollen extracts into normal persons or hayfever subjects stimulates the development of immune or "blocking" antibodies (Cooke, 1947). The word blocking in this connection applies to the behaviour of these antibodies in skin tests and must not be confused with the use of the term "blocking" in the serological sense (Wiener, 1944).

Two new serological techniques, the "red cell linked antigen test" and the "coated tanned red cell technique" have been used in this present work to demonstrate *in vitro* the development of these immune heat stable antibodies. It was decided to confine the present investigation to antibodies reactive with Timothy grass pollen. Sera were examined from hayfever patients receiving injections of mixed pollen extracts, (Bencard's B.4. mixture) for the purpose of hypersensitization.

With the "coated tanned technique" no antibodies were demonstrable in the sera of 23 normal persons or in the sera of 11 hayfever patients who had never been injected. Using the "red cell linked antigen test" five of the 23 normal sera showed a trace of antibody although only with the undiluted serum. In the sera of the 11 hayfever patients however this latter test picked up definite antibody in seven cases although only in the very low titres of 1-4. The significance of this finding is uncertain. It is thought unlikely to be the antibody responsible for the hayfever sensitization such as is demonstrable by the Prausnitz-Küstner technique, because of its very

low titre. It had originally been hoped that this test would pick up the antibody in a serum responsible for the Prausnitz-Küstner test, for although this antibody loses its capacity to passively sensitise skin after being heated to  $56^{\circ}\text{C}$  it does not necessarily mean that it is totally destroyed and unable to combine with antigen. It is obvious that these sera must now be examined unheated. This requires a slight modification in the technique to avoid haemolysis and will be done on the later development of this "red cell linked antigen test" which uses sheep red cells instead of those of the ox (Coombs and Fiset, 1954).

An immune heat stable antibody reacting with Timothy pollen protein could be detected by the red cell linked antigen test in the sera of all the hayfever patients injected with mixed pollen extract. It is presumed that this antibody corresponds with Cooke's (1947) skin sensitization blocking antibody. The antibody level rose as the hyposensitization procedure progressed. Theoretically, as already stated, this "red cell linked antigen test" should be capable of detecting both complete and incomplete types of antibody. The "coated tanned red cell method" while putting in evidence the antibody in the majority of these sera, and in some cases to a higher titre than with the "red cell linked antigen test", failed with many sera to demonstrate the antibody. Two interpretations are possible to explain this—either the antibody in these sera was of the incomplete type or the "coated cell method" is not sensitive in demonstrating a small amount of antibody in the greater concentration of proteins occurring in the lower serum dilutions. We rather favour this latter explanation, although in one or two cases, such as sera M.W. and C.C.A., the antibody may have been of the incomplete type. It must also be remembered that the antigens used in the two tests were not identical.

In summary the results definitely show that the injection of pollen extracts stimulates the development of antibodies in hayfever patients which can be detected serologically *in vitro*. The measurement of these antibodies is only likely to

be of value to the clinician in cases where the exact specificity of the sensitivity is known and where the antigens concerned are available for the serological testing.

#### SUMMARY

1. Heat stable antibodies to Timothy grass pollen antigens have been demonstrated serologically in the sera of hay-fever patients receiving injections of mixed pollen extract (containing Timothy grass pollen extract) for the purpose of hyposensitization.
2. Two serological techniques have been used; the "red cell linked antigen test" and the "coated tanned red cell method".

The authors wish to express their thanks to Dr. C. H. Whittle who carried out the skin scratch tests on the 23 control normal patients. Also they wish to thank C. L. Bencard, Ltd., for generously supplying them with pollen extracts and whole pollen.

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