USE OF THE IMMUNOFLUORESCENT METHOD FOR THE STUDY OF THE FORMATION OF ANTIBODIES TO POLLEN ANTIGENS

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N V. Medunitsyn and F. F. Lukmanova

Scientific Research Laboratory of the Study of Allergies (Head–Corresponding Member of the Academy of Medical Sciences of the USSR Professor A. D. Ado), Academy of Medical Sciences of the USSR, Moscow
(Presented by Active Member of the Academy of Medical Sciences of the USSR B. S. Preobrazhenskii)
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Plant pollen, which is responsible for numerous allergic conditions, possesses only weak antigenic properties. On the other hand, it represents a complicated conglomeration of different antigens. It includes individual antigens which are characteristic only for the given species of the plant as well as antigens common to the pollen of related species [1, 4, 5, 7]. The polyvalent nature of hypersensitivity in the allergic individuals may probably be explained by this nature of the pollen antigenicity.

In this work we have attempted to use the method of fluorescent antibodies, which permits the observation of immunological reactions at the cellular level, in the study of antibody formation to pollen antigens and that of isolation of common antigens in different species of pollen. The results of immunofluorescence were compared with those obtained in agar diffusion experiments.

EXPERIMENTAL

Antigen and Antibody. As antigen we have used an extract of pollen of timothy grass and for cross reactions extracts of pollen of dew grass, rye grass, meadow grass, fescue grass, foxtail, brome grass, ragweed, goose-foot, oak, ash, alder, hazel, birch, maple, and spruce. Extracts were prepared in Coca's fluid according to the customary methods in the Scientific Research Laboratory of the Study of Allergies of the Academy of Medical Sciences of the USSR.

The antiserum was obtained following immunization of rabbits with the extract of timothy grass pollen or with a 5% pollen suspension in complete Freund's adjuvant [2]. The antibody titers of the antisera corresponded to 1:3,200-1:5,120 in passive hemagglutination reaction with twice denitrified benzidine.

Conjugation of Immune Serum with Fluorochrome. The isolation of the globulin fraction of immune sera and labelling of proteins with fluorescin isothiocyanate was made by the general method [3], using 4 mg of the stain for 100 mg of protein. To avoid nonspecific fluorescence the labelled sera were passed through G-50 sephadex columns, diluted 4 to 8 times, and treated with powdered guinea pig liver and powdered rabbit bone marrow, using 50 mg of the powder per ml of serum.

Immunization of Guinea Pigs. Eighteen male guinea pigs weighing 250-300g were immunized with an extract of the timothy grass pollen mixed with an incomplete adjuvant in equal volumes. The mixture was injected in 0.1 ml amounts into the foot pads of the hind legs of the animals. Reimmunization with 0.1 ml of a saline extract of the pollen was done after 21 and 42 days into the same site.

Detection of Antibody-Forming Cells and of Circulating Antibodies. For the demonstration of antibody-forming cells the indirect method of Coons was used [6]. Guinea pigs were bled from the heart and killed after different periods of time following immunization; popliteal, inguinal, and axillary lymph nodes and pieces of spleen were fixed and embedded in paraffin according to the method of Sainte-Marie [8]. Undiluted

pollen extract was placed for 40 min on sections 4 μ in thickness, the preparations were washed with a phosphate buffer solution at pH 7.4 and then treated with the fluorescent antiserum. The sections were again washed and covered with buffered glycerin. The preparations were examined and photographed through a fluorescence microscope ML-2. To detect the circulating antibodies and common antigens in different species of pollen, Ouchterlony reactions in petri dishes with 1% agar were used.

RESULTS

Examination of organs for their content of antibody-forming cells was made 5, 14, and 21 days following each injection of the extract of pollen of timothy grass. On the fifth day after the first injection fluorescent cells were absent from lymphoid organs, but were present in all subsequent examinations. Remote lymph nodes, and especially the spleen, as a rule contained fewer fluorescent cells than did regional lymph nodes. After repeated injections of the pollen extract, antibody-containing cells appeared after shorter periods of time. Thousands of brightly luminescent cells were present in all lymph nodes as early as 5 days after reimmunization. Fluorescent cells were contained in the peripheral and medullar sinuses of lymph nodes. Embryonic centers did not contain these cells. According to their fluorescence the antibody-forming cells resembled plasma cells at different stages of maturity (Fig. 1a). Intensive antibody-formation took place also in the spleen in which large numbers of groups of fluorescent cells were found (Fig. 1b).

In order to determine the specificity of fluorescence the following control experiments were performed: (1) staining of sections only with homologous or heterologous fluorescent antiserum, (2) treatment of preparations with a heterologous antigen (egg albumin), (3) preliminary treatment of sections with an unlabelled immune serum, prior to layering with the fluorescent serum, and (4) preliminary depletion of the fluorescent immune serum with the corresponding antigen. In the first 3 controls there was no specific fluorescence and in the fourth it appeared only in those cases when diluted extract was used for the depletion of the immune serum.

Agar diffusion tests were made with the sera of all the guinea pigs. When comparing the results obtained by these tests and by the method of Coons it was determined that at early stages of immunization the method of immunofluorescence was more sensitive than the Ouchterlony reaction. On the fourteenth and the twenty-first days following the first injection of the antigen, precipitation reactions with the animal sera were weaker or absent, while the lymphoid organs contained large numbers of fluorescing cells.

It must be noted that the intensity of fluorescence of cells which contained antibodies to the pollen antigen was not lower than the fluorescence of cells such as we had noted in experiments in which we used human γ-globulin as antigen, and consequently the method of Coons can be successfully used for the study of antibody formation to pollen antigens.

Two methods were used for the study of possible cross-reactions between the different species of pollen. In the first method a number of sections were made from the same lymph node. These were treated with extracts of pollen grasses and trees (one preparation for each extract), washed with a buffer solution, and then treated with the fluorescent serum immune to the timothy grass pollen. After the sections were embedded in buffered glycerin the intensity of the fluorescence and the number of fluorescent cells in different preparations were estimated and the corresponding areas in the different sections were photographed.

When the sections were treated with the extract of pollen of timothy grass a large number of brightly fluorescing cells was noted (Fig. 1c). In preparations treated with extracts of pollen of related plants (dew grass, rye grass, fescue grass, foxtail, brome grass, and meadow grass), the number of fluorescing cells and the degree of fluorescence were decreased (Fig. 1d). Very weak fluorescence was seen with extracts of pollen of rye, and it was totally absent with extracts of ragweed, goose foot, and all the trees.

The results obtained indicate that similar antigenic structure may exist in related species of pollen. Cells which contained antibodies to the pollen of timothy grass were also able to fix antigens of pollen of closely related plants.

The second method used by us consisted of a repeated treatment of sections according to the following scheme: extract of a species of pollen–timothy grass fluorescent antiserum–extract of timothy grass pollen–timothy grass fluorescent antiserum. One drop of buffered glycerin was placed on each section treated by the first method and they were not covered with cover slips. The most easily recognizable identical areas in these sections were photographed.

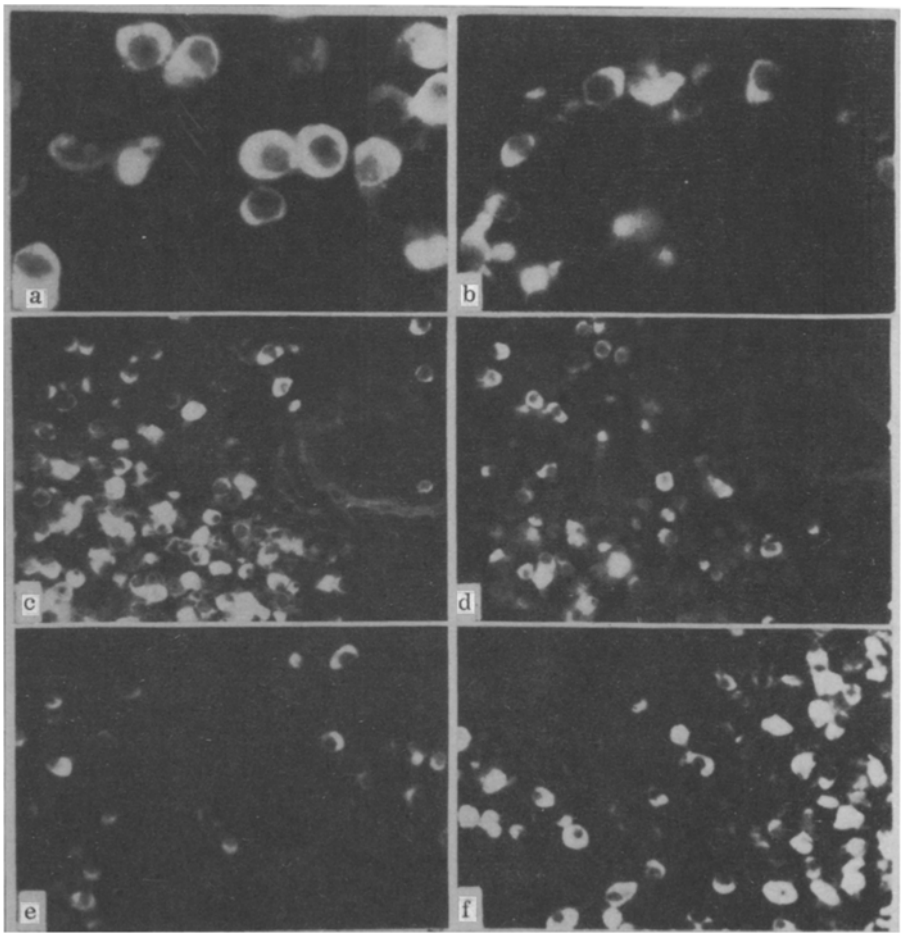

Fig. 1. Antibody producing cells in lymphoid organs of a guinea pig immunized with timothy grass pollen. a)Lymph node; b) spleen. Magnification 450 ×. c and d) Corresponding areas on sections of the same node treated with an extract of timothy grass (c) and dew grass (d) prior to layering with fluorescent antitimothy grass antiserum; e) section treated with extract of rye grass prior to layering with fluorescent antiserum to timothy grass; f) the same section after additional treatment with extract of timothy grass and the corresponding fluorescent antiserum. Magnification 200 ×.

The sections were washed for 2-3 min in the buffer solution, treated with extract of timothy grass pollen, and then with the corresponding fluorescent immune serum. Following this the same areas were photographed again.

After the supplementary staining there appeared bright specific fluorescence in those preparations which were previously treated with extracts of pollen of ragweed, goose foot, and trees, and which did not contain fluorescing cells. In preparations which were treated twice with extract of pollen of timothy grass the degree of fluorescence of cells was somewhat increased, although the number of fluorescing cells remained constant.

A sharp increase in the degree of fluorescence of cells took place in those preparations which were preliminarily treated with extracts of pollen of other related species of grasses. At the same time new fluorescing cells, which were not present prior to restaining, appeared in sections (Fig. 1e and f). It is quite probable that these cells contained antibodies to antigens present only in the timothy grass pollen. According to current beliefs a single cell is able to produce antibodies to not more than two types of antigens. This may possibly explain the appearance of new fluorescing cells following the restaining of sections. However, further experimentation is needed to answer this question fully.

The results obtained in immunefluorescence experiments corresponded to those obtained in agar diffusion experiments. In diffusion experiments using sera of guinea pigs sensitized with timothy grass pollen and extracts of different species of pollen it was determined that the sera reacted not only with the antigens of timothy grass pollen but with extracts of pollen of other grasses as well. On the other hand, the extract of timothy grass pollen produced a separate precipitation line. As was seen with immunofluorescence, the antiserum did not react with extracts of pollen of ragweed, goose foot, and the trees.

Thus, it was determined that the pollen of timothy grass, dew grass, rye grass, fescue grass, brome grass, foxtail, and meadow grass had common antigenic properties. Timothy grass pollen gave weak cross-reactions with the pollen of rye but not with that of ragweed, goose foot, and the trees. The immunofluorescence methods described above can be used for the study of antigenic properties in different species of antigens.

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