ANTIGEN-ANTIBODY REACTIONS IN ALLERGIC HUMAN TISSUES*

I. Preparation and Use of Fluorescein-Conjugated Reagins for Staining the Reaction Sites

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Plates 1 and 2

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When Coons, Creech, and Jones in 1941 (1) described the conjugation of antibodies with fluorescent dye they introduced a research tool which has become increasingly important in immunologic studies. The original technique they described, now frequently termed the "direct method," involves the staining of antigen with its specific fluorescent antibody. The "indirect method," described in 1954 by Weller and Coons (2) and by Gitlin and coworkers (3) is based on the use of three layers: the antigen, its specific antibody, and a conjugated fluorescent antiglobulin prepared against the middle layer as a stain. The middle layer thus acts as antibody in its relation to the antigen and as antigen in its combination with the conjugated antiglobulin. The advantage of the indirect method is the availability of one fluorescent antiglobulin for staining a variety of homologous antisera against different antigens. With this method, using convalescent human globulin as the middle layer, labeled anti-human globulin, has been used to study the localization of viruses in tissues (2, 4).

Fluorescent studies with human antibodies have heretofore been made only with the indirect technique. The purpose of this paper is to describe the conjugation of skin-sensitizing antibodies (reagins) with fluorescent dye and the use of this preparation for localizing with the "direct method" the antigen-antibody complex in atopic human skin. Visualization of the reaction depends on the assumption that specific antigen, when combined with reagin in tissue still has reactive sites free to combine with the conjugated reagin used as a stain.

Materials and Methods

Tissues.—Small skin biopsies (3 mm. in diameter) were removed by a motor-driven punch equipped for depth regulation. Despite the absence of local anesthesia the pain was sufficiently

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minimal and fleeting to permit three biopsies at one visit in each of the three patients. Two of the patients volunteered for a second set of biopsies required for other studies. Each had a history of clinical sensitivity to hen's egg albumin and showed marked skin reactions by the cutaneous method with 1–1000 solution of crystallized egg albumin (Armour).

The biopsies were done in the following order: (*a*) an unchallenged skin site; (*b*) the wheal site at the height of the reaction produced by a scratch test with 1–1000 histamine solution; (*c*) the wheal site at the height of the reaction produced by a scratch test with a 1–1000 solution of crystallized egg albumin (Armour).

Each biopsy was immediately frozen in isopentane cooled by liquid nitrogen to −165°C. and the tissue was dried *in vacuo* at −30°C. according to the Altmann-Gersh method (5). The dried tissue was then impregnated with diethylene glycol distearate (6) which, because of its greater firmness, was preferable to paraffin for cutting thin sections. One micron sections were cut with a diamond knife and 4 micron sections with a glass knife.

Conjugation of Reagins.—The reaginic serum was obtained from two of the three patients who volunteered for skin biopsies. The crude globulins were precipitated with 20 per cent alcohol at −5°—7°C(7). These precipitates were redissolved in buffered salt solution (0.15 M NaCl in M/100 phosphates, pH 7.4). The protein content was estimated by the microKjeldahl technique and the volume adjusted so that each milliliter of solution contained 20 mg. of globulin.

The crude globulins were conjugated according to the method described by Coons and Kaplan (8) with a solution of fluorescein isocyanate in acetone, obtained from Sylvania Chemical Co., Orange, New Jersey. The conjugation was carried out for 18 hours at −5°—7°C. Uncombined dye was removed by continuous flowing dialysis for 5 to 7 days at 4°C. against buffered M/100 saline, pH 7.8. Dialysis was discontinued when the dialysate showed no fluorescence under an HBO-200 mercury vapor lamp.

Despite such prolonged dialysis the globulin conjugate still stained histologic sections of normal human skin prepared by freeze-drying. Removal of the remaining non-specific staining materials was accomplished by Marshall's method (9) of repeated precipitation with cold alchohol.[1] Precipitation at −10°C was carried out with 30 per cent alcohol, cooled by an ice-salt mixture to −20°C. After each precipitation the preparation was centrifuged for about 2 minutes in a Servall centrifuge maintained at −4°C. and the supernatant alcohol decanted. The globulins were washed repeatedly with fresh 30 per cent alcohol at −20°C. until the washings were clear. The precipitate was then redissolved and subjected to three additional cycles of precipitation and washing. The final alcohol supernatant showed no fluorescence under an HBO-200 mercury vapor lamp. To assure removal of all non-specific stain, two additional alcohol precipitations were performed. When normal skin sections were treated with such conjugate preparations they showed no stain or a negligible trace of color primarily in the epidermis.

The purified conjugate was redissolved in buffered saline (0.9 per cent at pH 7.4) so that

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[1] Purification by alcohol precipitation resulted in considerable loss of precipitin titer of the rabbit conjugated globulins. Other methods for purification were therefore tried. Ethyl acetate extraction described by Dineen and Ada (10) failed to remove the non-specific staining properties of both reagin and rabbit antibody conjugates. Absorption with tissue powder and with liver-spleen homogenate likewise resulted in rapid decrease of antibody titer of the preparations.

The strong affinity for fluorescent dye shown by cornified epidermis in our histological preparations suggested the use of finely ground human skin calluses for absorbing non-specific staining properties from thoroughly dialyzed conjugates. The advantages of this procedure will be described in a later study.

each milliliter of solution contained 10 mg. of globulin. The globulin solution was sterilized by passage through a Seitz filter and 1 ml. aliquots stored at −28°C. Later preparations were lyophilized for storage. No preservative was used, since addition of 1–10,000 merthiolate to the conjugated globulin was found to decrease the intensity of fluorescence of specifically stained tissue.

The frozen conjugate was found to be stable. Even 8 months after preparation the solution was clear on melting. Occasionally, after storage at 4°C. for several weeks between periods of staining, a fine precipitate would appear; this was removed by filtration through a Seitz or millipore filter, without demonstrable loss of staining capacity of the filtrate.

A rough determination was made to test the change in reagin titer after conjugation and purification. Passive sensitization of a non-allergic individual was accomplished by intracutaneous injection of one-twentieth milliliter of conjugated and non-conjugated homologous globulin, each in 1–10 and 1–100 dilutions. Two days later each of the four sites was tested by the cutaneous method with a 1–100 solution of crystallized egg albumin (Armour). No difference in the size or time of onset of the reaction was observed in the two preparations. Evidence will be cited later, however, that diminution of reaginic activity does result from the procedure used to precipitate the globulins.

In addition to the anti-egg reagins, anti-cottonseed reaginic globulins were similarly prepared. The serum was obtained from a patient acutely sensitive to cottonseed proteins. The preparation provided a heterologous antibody conjugate for control purpose.

The procedures described were used also for the conjugation of reaginic globulins with lissamine rhodamine B 200 (11) and with fluorescein isothiocyanate (12).[2]

Preparation of Rabbit Conjugated Anti-Egg Globulins.—An anti-serum against egg albumin (Armour) was obtained by pooling the sera of three hyperimmunized rabbits. The globulins were precipitated as described, conjugated with fluorescein isothiocyanate, and purified in the manner described for reagins.

Immunologic Staining.—The staining of the frozen dried sections was done by the method of Coons and Kaplan (8) with modifications described by Marshall (9). Sections of 1 and 4 micron thickness were cut and floated into a boat attached to the knife carrier which was filled with 1 per cent formaldehyde in 50 per cent dioxane for postfixation of the frozen dried tissues (13). The sections were exposed to this solution for about a minute before being transferred to a drop of water on a scrupulously clean glass slide which was then placed on a hot plate (40°C.) to dry. The tissue spread evenly and remained firmly attached to the clean glass without the need of adhesives.

After exposure to xylene for removal of wax and hydration with decreasing ethanol concentrations, each slide was quickly dipped in saline buffered at pH 7.4. The excess saline was wiped off, a drop of conjugated globulin was placed over each section, and the slide was placed at room temperature for 20 minutes in a covered Petri dish containing moist filter paper for humidification. The stained section was then washed in buffered saline (pH 7.4–7.6), using ten changes and constant gentle shaking on a mechanical shaker for 20 minutes. Dehydration was accomplished in progressive alcohol concentrations up to 95 per cent, in which concentration it was left for 30 minutes for fixation. The section was then mounted in non-fluorescent immersion oil (Zeiss), covered with a coverslip (0 thickness) and sealed with permount. Because this sealer is slightly soluble in oil it was allowed to dry to a thick consistency before being spread along the edges of the coverslip. The sealed preparations were kept in the refrigerator until used.

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[2] Lissamine rhodamine B 200 was obtained through the courtesy of Mr. Arnold Hoffman of Providence; fluorescein isothiocyanate from the Baltimore Biological Laboratory, Baltimore.

Control of Staining Specificity.—The pitfalls of interpreting the specificity of the staining reactions were recognized by Coons and later workers. In addition to the use of conjugated preparations which gave only slight or no staining of normal skin, the following control procedures recommended by Coons and Kaplan (8) with minor modifications to be described, were used on the sections from the challenged tissue site:

1. Inhibition of the reaction by unconjugated antibodies. As a modification of the recommendations by Coons and Kaplan (8) unfractionated human anti-egg reaginic serum, rather than the crude globulins, was coated over the unstained section for 1 hour at room temperature, rather than for 20 minutes, and then stained for 20 minutes, rather than 10 minutes, with the conjugated human anti-egg globulins. The choice of the unfractionated reaginic serum was made when it was determined that the alcohol precipitated globulins were less effective than the unfractionated serum in inhibiting staining with the conjugated globulins. This indicates that treatment with 20 per cent alcohol either partially denatures the precipitated globulins or fails to precipitate all reagins. This question requires clarification, since a parallel observation was not observed with the rabbit antibodies. The precipitated rabbit anti-egg albumin globulins were more effective than the unfractionated antiserum in inhibiting staining with conjugated rabbit antibodies.

2. Treatment of the unstained section with normal human serum for 1 hour followed by the specific conjugated globulin for 20 minutes.

3. Staining with conjugated heterologous (cottonseed) reaginic human globulins for 20 minutes.

4. Controls with the other two tissue sites. The unchallenged and histamine-tested sites stained with the human anti-egg conjugated globulins served as further controls for the specificity of the reaction. It will be recalled that non-allergic human skin had already been used during the preparation of the conjugate for determining the presence of non-specific staining properties.

Instruments and Photography.—A Zeiss microscope equipped with an HBO-200 mercury vapor burner and various filters were used in this work. The primary filter used for photography was the UG 5, 2 mm. thick (Schott), while a secondary filter was provided by a 50 per cent solution of $CuSO_4$ and an OG 5 (Schott). Eastman Kodak spectroscopic plates (3 ¼ × 4¼ G-103a) exposed for 1.5 minutes were found most suitable for sections stained with the isocyanate or isothiocyanate conjugate because of their sensitivity to the green and yellow parts of the spectrum.

RESULTS

1. Staining with Conjugated Reagins

Antigen-Challenged Skin.—The biopsy site tested with egg albumin (challenged tissue site) showed specific staining with conjugated anti-egg reaginic globulins. Surprisingly, all cells of epithelial origin were stained, including the epidermal cells, those of hair follicles, sebaceous and sweat glands.

Epidermis.—The stratum corneum stained intensely and non-specifically in the challenged tissue as in all skin preparations. Below this and including the basal layer there was within each cell an intensely fluorescent zone about 1 micron in width limited to or immediately within the cell border (Figs. 1 and 2). The remainder of the cytoplasm and the nucleus were unstained. The intercellular cement substance was likewise unstained.

Hair Follicles, Sweat and Sebaceous Glands.—The epithelial cells of these structures stained just inside their borders like those of the epidermis (Figs. 7 to 11). The sebaceous cells had a narrow ring of fluorescence surrounding the fat globule within the cell (Fig. 9). The sebaceous debris was likewise fluorescent. Within some of the glands intensely stained macrophages were present.

Dermis.—In the dermis the staining was most intense in macrophages and in large, ovoid cells (called pericapillary cells or pericytes) lining the lumina of some of the small blood vessels. Only a relatively small proportion of the total number of macrophages were stained, mostly those about the blood vessels. Two zones of differing staining intensity were present in the dermis. The zone adjacent to the epidermis, extending in depth about 0.5 mm., stained much less intensely than the layer below it (Fig. 1), because of the greater number of highly fluorescent macrophages and pericapillary cells in the deeper dermis (Figs. 3 *a* and 3 *b*).

The macrophages stained as intensely as the epidermal cells. They were present in groups adjacent to small blood vessels or scattered throughout the dermis. In proportion to the entire number present in any field, the stained macrophages were few. Those that were unstained could be readily distinguished from the intensely yellow fluorescent cell by their clear auto-fluorescent turquoise color and their sharp angular outlines (Fig. 4). In black and white photography these differences are not as readily apparent.

The staining intensity of the capillaries depended on the presence or absence of pericapillary cells in their walls. The great majority of capillaries in a section at a given level did not show these cells. Such capillaries stained very lightly, and, because of their preponderance, produced a general fluorescence throughout the tissue (Fig. 1). The remaining capillaries, though fewer in number, stood out brilliantly because of the intense yellow fluorescence of the pericapillary cells within their walls (Figs. 3 *a* and 3 *b*). These pericytes, ovoid elongated cells with long thick processes which were flattened on cross-section, have been classified by histologists as phagocytes (14).

The fibrous tissue in 4 micron stained sections was definitely more fluorescent than in the unstained sections or in those pretreated with unfractionated antiserum before staining.

The Histamine-Tested and the Unchallenged Skin Sites.—Sections from these two sites did not stain with the conjugated reagins (Figs. 5 and 6), since no antigen was present.

2. Staining with Conjugated Rabbit Antibodies

The rabbit anti-egg albumin globulins conjugated with fluorescein isothiocyanate gave similar staining of the epithelial elements and macrophages in the antigen-challenged skin as the conjugated reagins. The intensity of staining was less with the conjugated rabbit antibodies than with the conjugated reagins.

Neither the histamine-tested nor the unchallenged skin sites stained with the rabbit conjugated antibodies.

3. Control of Immunologic Specificity of Conjugates.

The absence of non-specific staining properties of the fluorescein conjugated antibodies was demonstrated by their failure to stain the following skin sections: (*a*) normal (non-atopic); (*b*) the histamine-challenged site from an egg-sensitive patient (Fig. 6); (*c*) the unchallenged skin from the same patient (Fig. 5). The immunologic specificity of the staining reaction was demonstrated by the procedures recommended by Coons and Kaplan (8):

(*a*) Pretreatment for 1 hour with reaginic anti-egg serum followed by conjugated anti-egg reaginic globulins for 20 minutes showed complete or almost complete inhibition of staining (Figs. 2, 4, 6, 8).

(*b*) Coating for 1 hour with normal (non-reaginic) human serum did not inhibit staining with the conjugated reagins.

(*c*) No staining was present after coating for 20 minutes with heterologous (cottonseed) conjugated reaginic globulins (Figs. 10 and 11).

(*d*) One-tenth ml. of 1-1000 egg albumin was added to an equal amount of conjugated anti-egg reagins and allowed to stand at room temperature for 1 hour. The mixture was found to stain antigen-challenged sections as intensely as conjugated reagins which did not contain antigen.

Similar procedures, as noted in (*a*), (*b*), and (*c*), were carried out to assure the immunologic specificity of the stain with rabbit conjugated antibodies. As previously noted, the unconjugated globulins were a little more effective than the rabbit antiserum in inhibiting the specific stain.

DISCUSSION

In view of the role of reagin in the immediate wheal type allergic reaction, it is not surprising that its conjugation with fluorescent dyes should make it effective in localizing the antigen-antibody complex in atopic tissues. The present work demonstrates that reagin can be conjugated with fluorescent dyes without great reduction in its capacity to combine with specific antigen.

The specific staining by fluorescent antibody of epithelial cells should not be confused with the non-specific staining of normal tissue cells by fluorescein-conjugated globulins (15). The specificity of the staining reaction is indicated by the following facts: (*a*) the conjugated preparations used in this study were refined to a point that normal skin was not stained by them; (*b*) sections from the histamine-treated and unchallenged skin sites, when not coated by antigen, did not stain; (*c*) specific inhibition of staining with unconjugated antibody was demonstrable; and (*d*) no staining of the challenged tissue site occurred when heterologous (cottonseed) conjugated antibody was used.

While this study demonstrates the presence of specific antibodies in various

sites of the challenged tissue only, the author has also demonstrated that when sections from an unchallenged atopic skin site and from a histamine-treated site are coated *in vitro* with the specific antigen and then layered with the conjugated reagins, the distribution of antibodies is similar to that found in sections from the challenged wheal site. The significance of this finding, and especially of the presence of antibodies in cells of epithelial origin, will be more fully discussed in a subsequent report.

While no staining of ground substance could be seen, this may have been due to insufficient intensity for visualization rather than absence of staining. This explanation is suggested by the fact that fibrous tissue bundles in the challenged sections were definitely fluorescent. It is more likely that the source of fluorescence was the antibodies in the ground substance coating the fibers rather than the collagen itself.

The greater effectiveness of unfractionated antiserum over the precipitated globulins in inhibiting specific staining with conjugated reagins indicated either that not all reagins are precipitated by 20 per cent alcohol or, more probably, that the precipitation process denatures some of the reagins. With rabbit antibodies, the precipitated globulins were more effective than the unfractionated rabbit antiserum for inhibition of specific staining.

The conjugation of reagin with fluorescent antibody offers a tool for immunohistologic investigations in allergy. Among the problems we are presently studying are the antigen-antibody reactions in human allergic nasal mucosa, the differences in localization of antigen-antibody complexes in active and passive sensitization, the role of the blocking antibody, and the effect of antihistamines and of histamine liberators on the localization of antigen-antibody complexes.

SUMMARY

Skin sensitizing human antibodies were conjugated with various fluorescent dyes without significant loss in their ability to combine with specific antigen *in vitro*.

A biopsy of the skin site challenged with egg albumin in a patient sensitive to this antigen could be stained specifically by the fluorescent reagins. The epithelial cells of the epidermis, sweat glands, hair follicles, and sebaceous glands in such a challenged site showed specific staining. In addition to the epithelial cells, the most intense staining was in macrophages and in pericapillary cells. The endothelium of the small blood vessels stained less intensely. Fibrous tissue bundles were specifically stained.

The immunologic staining with the conjugated reagins was similar to but more intense than that obtained with conjugated rabbit anti-egg albumin globulins.

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EXPLANATION OF PLATES

The tissues illustrated in Figs. 1 to 6 and Figs. 7 to 12 are from the skin of an egg-sensitive patient. The "challenged tissues" were from a site tested by scratch test with 1-1000 egg albumin; the "unchallenged" from an untested site; the "histamine-tested" from an area tested by scratch test with 1-1000 histamine.

"Staining" was either with homologous reagins (anti-egg) conjugated with fluorescein isothiocyanate or with heterologous reagin (anti-cottonseed) similarly conjugated.

Inhibition of specific "staining" was with unconjugated anti-egg reaginic serum followed by layering with conjugated anti-egg reagins.

PLATE 1

FIG. 1. The fluorescence of the epidermal cell is limited to the cell periphery. The capillary network (*c*) is faintly fluorescent. Intensely staining macrophages (*m*) are present. 1 micron × 400.

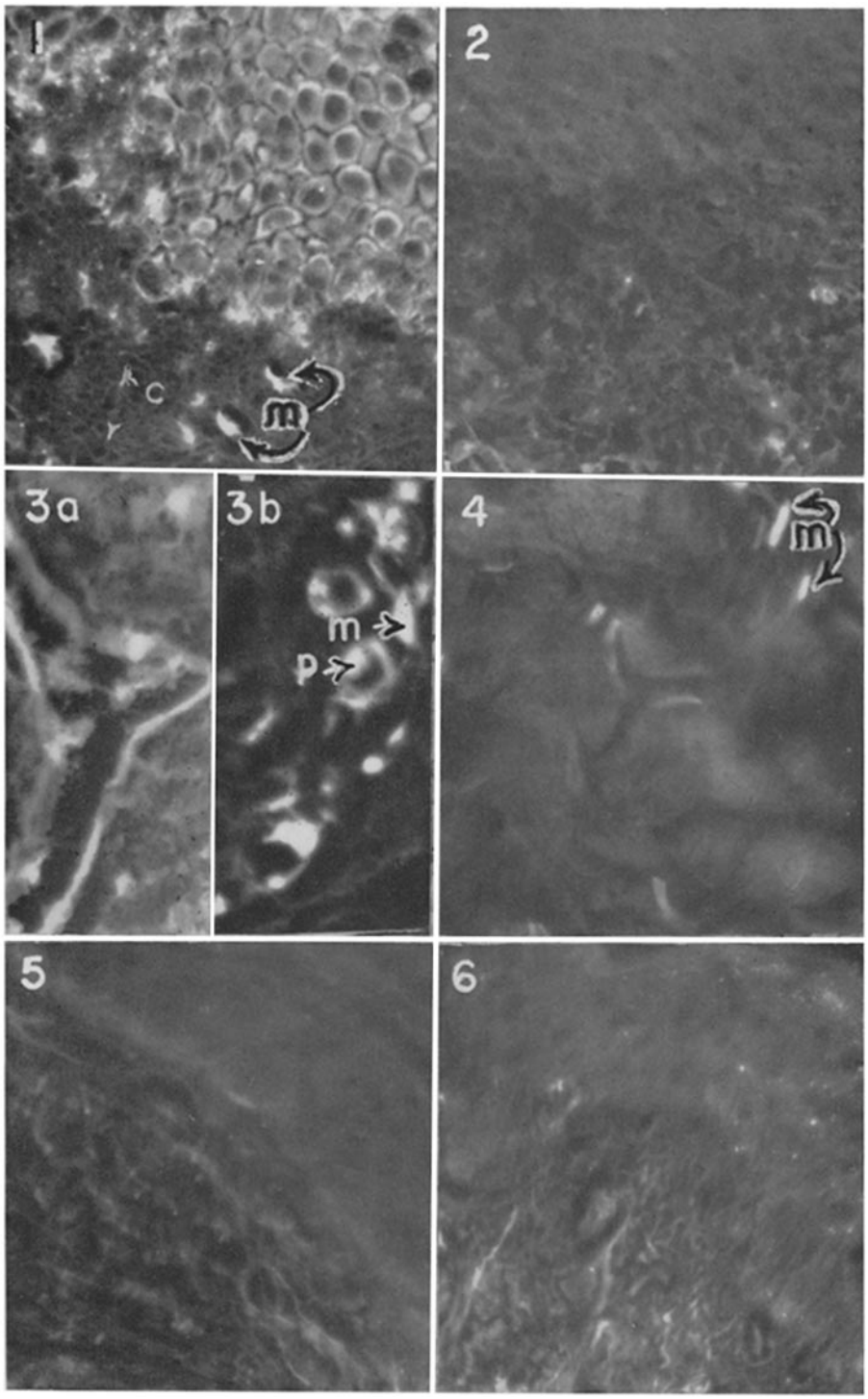
FIG. 2. Inhibition of reaction in challenged skin. No specific fluorescence can be observed. 1 micron × 400.

FIGS. 3 *a* and 3 *b*. Sections through deep dermis of challenged skin specifically stained. Fig. 3 *a* shows longitudinal section of a small blood vessel. Fig. 3 *b* shows a number of capillaries in cross-section. Intensely staining pericytes (*p*) line the vessel walls and equally fluorescent macrophages (*m*) are present in the adjacent dermis. 4 microns × 400.

FIG. 4. Inhibition of specific staining in deep dermis of challenged skin. Autofluorescent macrophages (*m*) are shown. No specific staining is present. 4 microns × 400.

FIG. 5. Unchallenged skin site specifically stained. No specific fluorescence is present. 4 microns × 400.

FIG. 6. Histamine-tested skin site specifically stained. No specific fluorescence is present. 4 microns × 400.



(Rappaport: Antigen-antibody reaction in allergic tissues. I)

PLATE 2

FIG. 7. Specific staining of hair follicle in challenged skin. Fluorescence is intense but limited to the periphery of the follicle cell. Stained macrophages (*m*) are present in the adjacent dermis. 4 microns × 400.

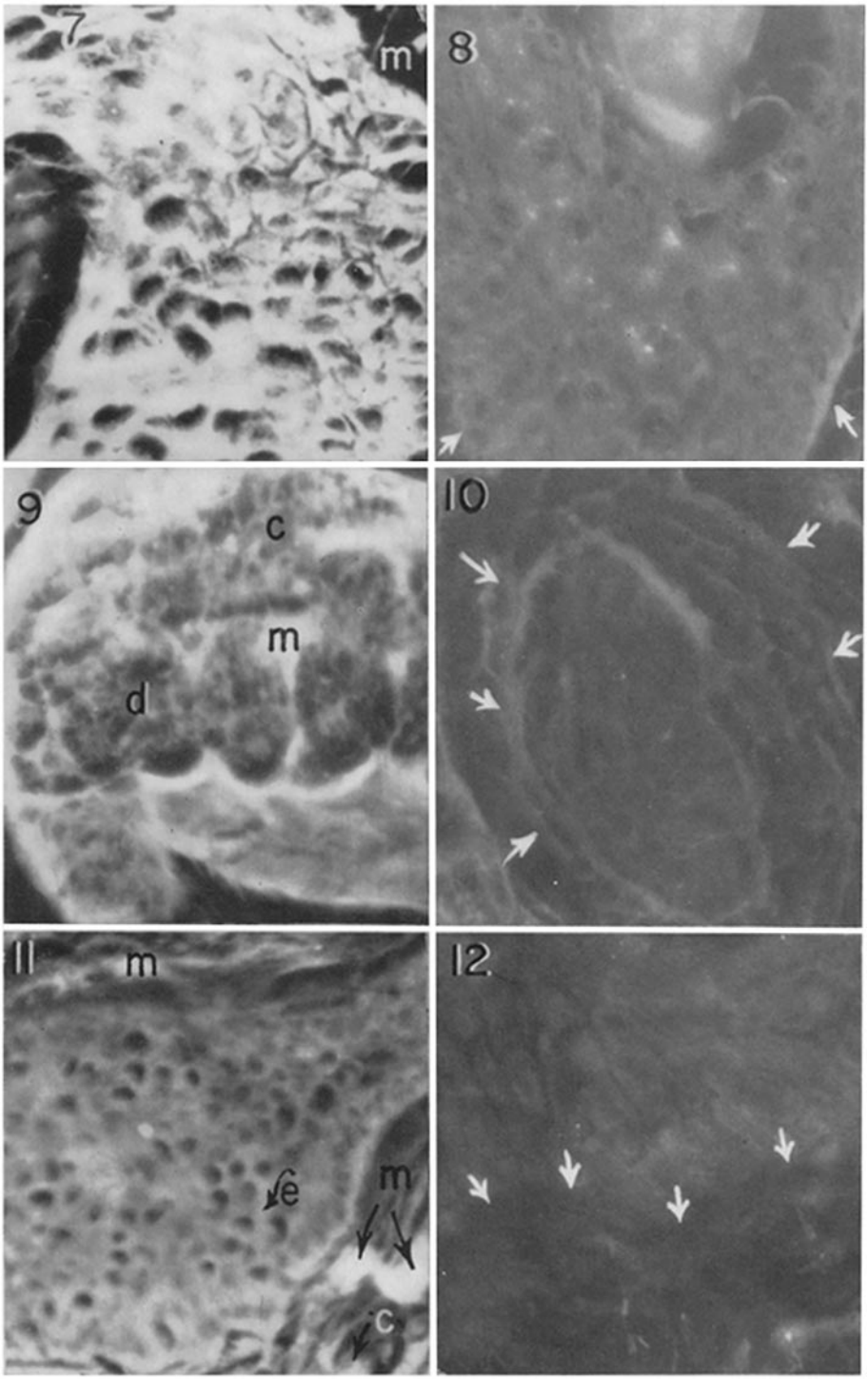
FIG. 8. Inhibition of specific staining of hair follicle in challenged tissue. No specific fluorescence is present. The arrows indicate the borders of the hair follicle. 4 microns × 400.

FIG. 9. Specific staining of sebaceous gland in challenged skin. Fluorescence is limited to the cell periphery (*c*). Stained macrophages (*m*) are present in the fluorescent debris (*d*). 4 microns × 400.

FIG. 10. Staining of a sebaceous gland in challenged tissue with heterologous (anti-cottonseed) conjugated reagins. The gland is indicated by arrows. No specific fluorescence is present. 4 microns × 400.

FIG. 11. Specific staining of sweat gland in challenged skin. The fluorescence is limited to the cell periphery (*e*). Fluorescent macrophages (*m*) and pericytes lining a capillary (*c*) are present. 4 microns × 250.

FIG. 12. Staining of epidermis and dermis of challenged skin with heterologous (anti-cottonseed) conjugated reagins. Arrows indicate the epidermal-dermal junction. No specific fluorescence is present. 4 microns × 400.



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