

# Demonstration of gamma-2 globulin in human skin

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**A**lthough serum antibody activity has been well studied, much remains to be learned about the localization and role of reactive antibodies in the "shock organs." The skin is the shock organ for several immunologically based processes such as hives and angioneurotic edema, Rhus dermatitis, and atopic eczema. Skin also serves as the site for scratch, intradermal, and passive transfer testing in the diagnosis of allergies and in their experimental study.

Demonstration of specific antibody activity or antibody globulin at a site of testing or disease process can only be meaningful by comparison with uninvolved areas and uninvolved individuals. This paper describes the demonstration of  $\gamma_2$ -globulin in human skin by the fluorescent antibody technique.

## **MATERIALS AND METHODS**

### **Patients**

Twelve skin specimens were obtained, 10 at operation from 7 females and 3 males, and 2 from male laboratory workers by punch biopsy under local anesthesia (Table I). All patients were free of atopic allergies (asthma, hay fever, perennial allergic rhinitis, eczema, reactions to foods, and recurrent hives) and were skin-test negative to the intradermal injection of 0.025 ml. of a 1:1,000 dilution of mixed grass\* antigen. Further information on the patients and reasons for surgery are given in Table I.

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\*Mixture of equal parts of aqueous extracts from 10 pollens causing spring hay fever.

**Table I.** *Origin of skin specimens*

| <i>Patient No.</i> | <i>Sex</i> | <i>Age</i> | <i>Surgical procedure and diagnosis</i>                       |
|--------------------|------------|------------|---------------------------------------------------------------|
| 1                  | F          | 16         | Mammoplasty, enlarged breasts                                 |
| 2                  | F          | 35         | Mammoplasty, enlarged breasts                                 |
| 3                  | F          | 32         | Vein ligation, varicosities                                   |
| 4                  | F          | 70         | Cholecystectomy, recurrent cholecystitis                      |
| 5                  | F          | 60         | Cholecystectomy, recurrent cholecystitis                      |
| 6                  | F          | 75         | Radical mastectomy, carcinoma of the breast                   |
| 7                  | F          | 58         | Reconstruction of common duct, postoperative duct obstruction |
| 8                  | F          | 52         | Simple mastectomy, metastatic carcinoma of the breast         |
| 9                  | M          | 64         | Esophageal resection, carcinoma of esophagus                  |
| 10                 | M          | 68         | Exploratory laparotomy, metastatic carcinoma of colon         |
| 11                 | M          | 26         | Upper arm biopsy, healthy                                     |
| 12                 | M          | 24         | Upper arm biopsy, healthy                                     |

**Tissues**

Within 30 minutes of being obtained, the skin was grossly cleansed of blood, the fat was trimmed away, and 2 to 3 mm. pieces were cut with a sharp razor blade. These were frozen in isopentane cooled with liquid nitrogen, then dropped into celluloid tubes filled with liquid nitrogen. The tube tops were closed by insertion of 2 by 2 gauze into the opening, and the tubes with the tissue were transferred to a glass container inserted into the side of a  $-40^{\circ}$  C. refrigerator.\* Potassium pentoxide was placed in the container to absorb moisture. The container was evacuated and kept so until the tissues were dry (in about one week). The dry tissues were imbedded in degassed, low-melting-point ( $52.5^{\circ}$  C.) paraffin, and the blocks thus obtained were stored in a desiccator at  $5^{\circ}$  C. until use. Four micron sections were cut on an ordinary rotary microtome, floated in ethylene glycol monomethyl ether,† placed on albuminized microscope slides, and dried at  $37^{\circ}$  overnight.

Sections were deparaffined by progression through the following 7 baths: xylene, 2 minutes twice; 100 per cent ethyl alcohol, 30 seconds twice; 95 per cent ethyl alcohol, 30 minutes; 70 per cent alcohol, 1 minute, and 0.01M phosphate-buffered saline at pH 7.4, 1 minute. The sections were placed in moist chambers (large Petri dishes with moist filter paper over the bottoms, on which applicator sticks were placed to hold slides) and stained for 30 minutes with fluorescent antisera, washed 10 minutes with 2 changes of the above buffer, and mounted in buffered glycerol (90 per cent glycerol to 10 per cent above buffer). Slides were stored in the cold room until examined, usually within a few hours of preparation, occasionally the next day. It was found that satisfactory observations could be made after a week of cold storage with only a little loss of specific fluorescence. In blocking experiments or other controls, the sections were first covered with the nonlabeled (blocking) antiserum for 30 minutes, washed 10 minutes with 2 changes of buffer, and stained with the labeled serum, washed, and mounted as described above.

\*Lyophilizing apparatus courtesy of Department of Pathology, Stanford University School of Medicine.

†Certified, Fisher Scientific Company, Fair Lawn, N. J.

### Antisera

The following antisera were obtained from Antibodies Incorporated\*: nonfluorescent rabbit antihuman  $\gamma_2$ -globulin, fluorescent rabbit anti-BSA (bovine serum albumin), and fluorescent goat antirabbit gamma globulin. For a few experiments fluorescently labeled rabbit antihuman  $\gamma_2$ -globulin was obtained from the same company. For most experiments rabbit antihuman  $\gamma_2$ -globulin serum was labeled in our laboratory.

Fluorescein-conjugated sera were prepared by the method of Riggs<sup>1</sup> as modified by Rauch.<sup>2</sup> All precipitation, conjugation, and globulin separation procedures were performed at 5° C. The serum globulins were precipitated in 50 per cent ammonium sulfate by the following method: Five milliliters of anti-serum plus an equal volume of 0.03M phosphate buffer of pH 6.3 was placed in a 3/4 in. dialysis bag and suspended from a rotor in 10 ml. of saturated ammonium sulfate solution. The bag was slowly rotated for 2 hours. An equilibrium of 50 per cent saturation was achieved with resultant precipitation of the globulins. The precipitate was centrifuged, washed once with 50 per cent saturated ammonium sulfate, recentrifuged, and redissolved in 1 to 2 ml. distilled water. Following overnight dialysis against 0.15M NaCl to remove the ammonium sulfate, the protein content was determined by the Biuret method.<sup>3</sup> The protein solution was buffered to a pH near 9 by adding one-tenth volume of 0.5M sodium carbonate buffer,<sup>†</sup> pH 9. Fluorescein isothiocyanate (FITC) adsorbed in 10 per cent concentration on Celite was added to give a ratio of 1 mg. FITC per 100 mg. protein. Conjugation was carried out for 1 hour in a corked serum bottle using a small magnetic stirrer.

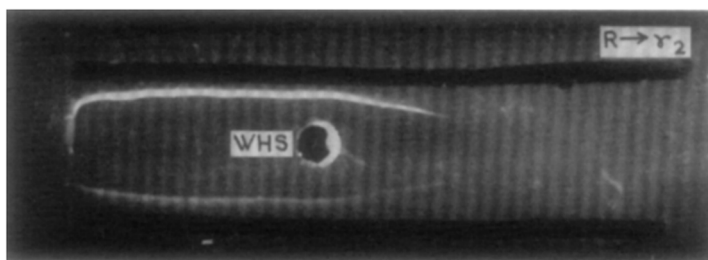
Free FITC was removed by passage of the crude conjugate through a 2 cm. diameter by 8 cm. high column of Sephadex G-25‡ equilibrated with 0.018M phosphate buffer, pH 6.3. The labeled proteins passed quickly through the Sephadex and constituted the first eluted colored band, while the free fluorescein was retarded by the Sephadex matrix. The conjugated globulins were separated by passage through a DEAE cellulose ion exchange column\* 1.4 cm. diameter by 10 cm. high (0.87 mEq. per gram) by using the following buffers: (1) 0.018M PO<sub>4</sub>, pH 6.3; (2) 0.1M PO<sub>4</sub>, pH 6.3; (3) 0.01M PO<sub>4</sub> in 1.25M NaCl, pH 6.3. The eluted proteins were concentrated by precipitation with 50 per cent saturated ammonium sulfate and reconstituted to a protein concentration of about 5 mg. per milliliter.

Optimally labeled antibody was usually obtained in the eluate using buffer (2). Fraction I, obtained with buffer (1), contained lightly labeled antibody, while fraction III contained heavily labeled non-gamma globulin serum proteins. Fraction I gave weak specific fluorescent staining, and fraction III, nonspecific staining. Fraction II from a rabbit antihuman  $\gamma_2$ -globulin serum gave a single precipitin line when used to develop an immunoelectrophoretic pattern against whole human serum (Fig. 1). After eluting nonprecipitated labeled protein from the immunoelectrophoretic plate by soaking in 0.15M

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†1 part 0.5M Na<sub>2</sub>CO<sub>3</sub> + 4 parts 0.5M NaHCO<sub>3</sub>.

‡Pharmacia Fine Chemicals, Uppsala, Sweden.

**Fig. 1**

Agar electrophoresis of whole human serum (WHS) developed with rabbit antiserum against human  $\gamma_2$ -globulin ( $R \rightarrow \gamma_2$ ). Note single precipitin line indicating purity of antiserum. Lower trough is filled with a weaker  $R \rightarrow \gamma_2$  antiserum.

saline for 48 hours, the single precipitin line formed by the rabbit antihuman  $\gamma_2$ -globulin with the serum gamma globulin was found to be brightly fluorescent under an ultraviolet lamp. All antihuman sera were checked for antibody specificity and content by immunoelectrophoresis before conjugation or use on tissue. The conjugated antisera at a 1/5 dilution gave precipitin lines by the Ouchterlony<sup>4</sup> technique with dilutions of normal human serum up to 1:500. Weight per volume fluorescein/protein ratios of 2 to  $7 \times 10^{-3}$  in the labeled antisera were found to give the most satisfactory staining.

#### Staining procedure

Every set of slides made from each subject was submitted to the following stainings:

1. Fluorescent rabbit antihuman  $\gamma_2$ -globulin (diluted 1/5) for 30 minutes.
2. Nonfluorescent rabbit antihuman  $\gamma_2$  (undiluted) for 30 minutes followed by fluorescent rabbit antihuman  $\gamma_2$  1/5 for 30 minutes (blocking control).
3. Fluorescent rabbit anti-BSA 1/5, (30 minutes) (nonspecific staining control).
4. Buffer (autofluorescence control).

Only set-up 1 showed staining. Observations on each skin were performed many times, since in a given set at least two slides were submitted to the same procedure, several fluorescent antiserum batches were used at the same time, and each set (18 to 20 slides) was done at least twice.

In addition, the tissues of three patients were stained by the indirect method using rabbit antihuman  $\gamma_2$ -globulin followed by fluorescently labeled goat antirabbit globulin. This gave the same result as the direct method, but the specific fluorescence was brighter. The controls of normal rabbit serum followed by fluorescent goat antirabbit globulin were negative.

A control of specific absorption of antibody from the test fluorescent serum was performed as follows: Purified human  $\gamma_2$ -globulin (0.4 mg.) was added to 1 ml. of the 1:5 diluted, labeled rabbit anti- $\gamma_2$  serum to give an antigen

\*Carl Schleicher and Schuell Co., Keene, N. H.

undiluted serum ratio of 2 mg. per milliliter. This was incubated at 37° C. for 30 minutes, then at 5° for 48 hours, and centrifuged. The supernatant fluid was used to stain selected sections in the manner described. Sections so stained were free of specific fluorescence (Fig. 5), indicating that the anti- $\gamma_2$  antibodies had been removed. Antiserum similarly treated with human albumin retained ability to specifically stain skin sections.

The sections were observed with a Zeiss fluorescence microscope using an Osram HB-200 burner, BG-12 exciter filter, dark field condenser, and Sp orange I/Sp yellow (II/I) for barrier filters. Specificity of green fluorescence was checked with the UG-2 exciter filter and no barrier or only a Sp yellow filter. Fluorescence was not considered specific unless it remained apple green under light passed through the UG-2 exciter filter,<sup>5</sup> and all controls were negative.

The slides were observed without previous knowledge of the procedures by at least one of us.

Photographs of tissue sections were taken with a Polaroid camera using high speed film, B6-12 exciter filter and III/I barrier filters.

## RESULTS

### Autofluorescence

Slides treated only with buffer showed that human skin has considerable autofluorescence under UV microscopy. Although such autofluorescence is green under the BG-12 filter, it is ice blue when observed under the UG-2 exciter filter. Even under BG-12 optics autofluorescence is much less bright than the specific apple green positive staining achieved with appropriate fluorescent antisera.

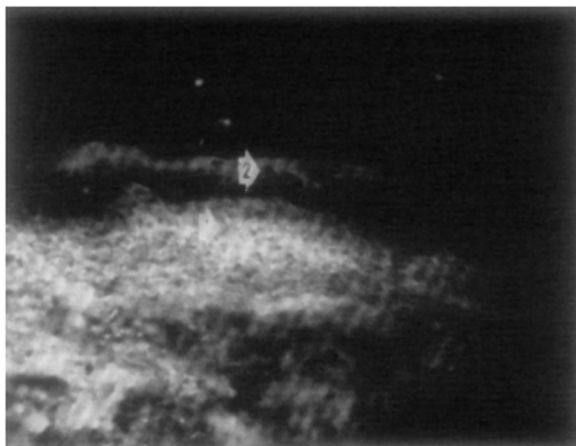
### Fluorescent staining

In all twelve cases the results were the same. In sections stained with fluorescent rabbit antihuman  $\gamma_2$ -globulin serum or with rabbit antihuman  $\gamma_2$ -globulin followed by goat antirabbit globulin serum, a band of striking green fluorescence was present beneath the epidermis occupying the papillary and upper reticular layers of the dermis (Figs. 2, 3, and control, Fig. 4). This band gradually disappeared into the deep dermis and was clearly limited above by the basement membrane of the epidermis. The band of fluorescence in the papillary dermis remained apple green under UG-2 optics and was therefore considered to represent antihuman gamma globulin in this area of the skin. Patients with cancers appeared to possess the same amount and distribution of  $\gamma_2$ -globulin as non-cancerous individuals.

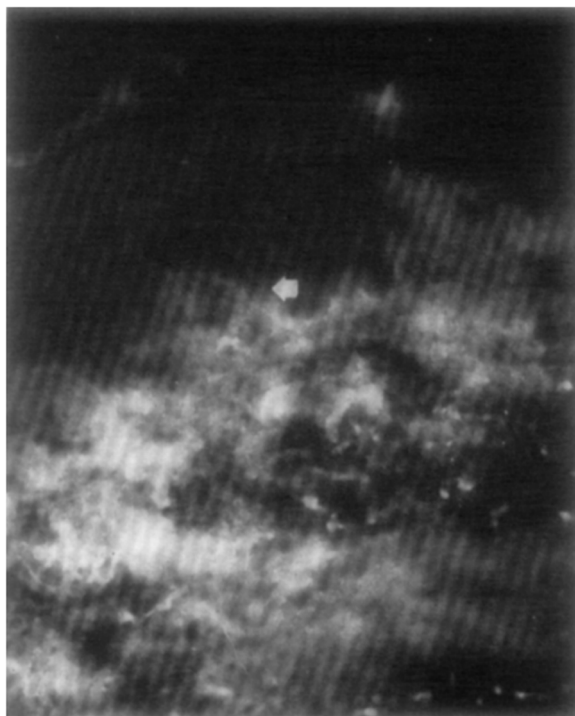
There were no specific structures or cells associated intimately with the specific fluorescence. It seems to be rather evenly distributed on the ground substance of the upper dermis.

Sections stained with fluorescent anti-BSA did not show fluorescence under UG-2 optics and were similar to slides treated with buffer only.

Specific fluorescence was absent in blocked sections, with the exception of an occasional thin layer of subepidermal brightness. This was easily distin-

**Fig. 2**

Skin section stained with rabbit antihuman  $\gamma_2$ globulin followed by fluorescent goat anti-rabbit serum. The band of bright fluorescence in the papillary dermis (arrow No. 1) is limited above by the basement membrane and gradually decreases in intensity downward into the reticular dermis. Note the fluorescing cells in the epidermis (arrow No. 2, see text). (Original magnification  $\times 100$ , exposure time, 15 sec.)

**Fig. 3**

Specific staining. Section of skin treated with rabbit antihuman  $\gamma_2$ globulin followed by fluorescent goat antirabbit serum. Note fluorescence in upper papillary dermis (arrow) and limitation of this fluorescence by the basal layer of the epidermis. (15 sec. exposure time, original magnification  $\times 250$ .)

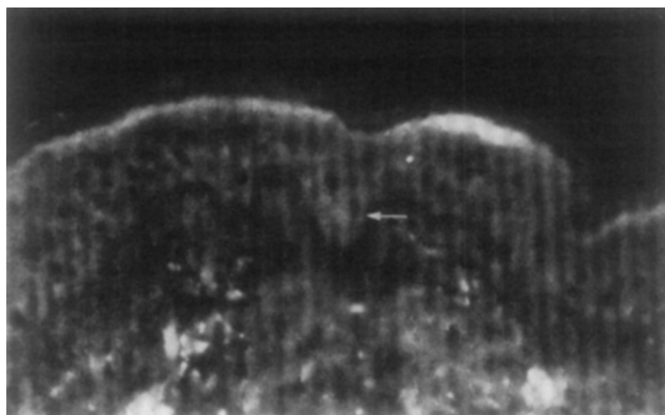


**Fig. 4**

Control. Section stained with fluorescently labeled rabbit antihuman  $\gamma_2$ -globulin serum previously treated with purified human  $\gamma_2$ -globulin. Exposure time lengthened to 30 sec. to show skin structures. Arrow indicates dermal-epidermal junction. (Original magnification  $\times 160$ .)

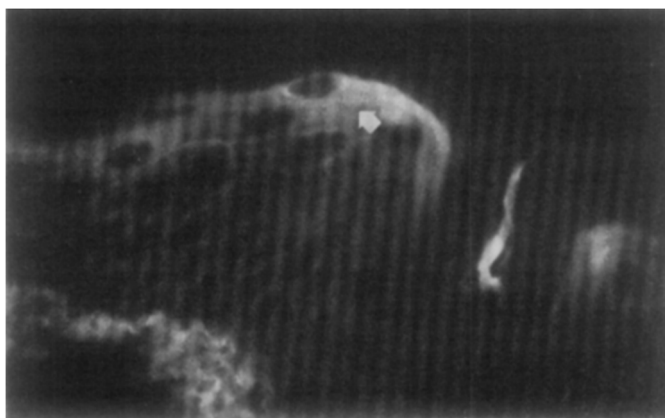


**Fig. 5**

High power view of epidermal cells in the human skin stained for  $\gamma_2$ -globulin. Note fluorescing cytoplasm and dark nuclei of cells in the stratum granulosum area (arrow). (Original magnification  $\times 400$ .)

guished from the bright and broad fluorescent band seen in the unblocked sections. It was also absent in sections stained with antisera from which the anti- $\gamma_2$  antibodies had been absorbed (Fig. 4). No specific fluorescence was seen in the indirect technique when normal rabbit serum was substituted for the rabbit antihuman  $\gamma_2$  serum.

In most sections the epidermis showed a dull green color under the BG-12 filter and a blue to bluish-purple color under the UG-2 filter. In some sections, however, "nests" of brightly fluorescing epidermal cells were seen (Figs. 2, 5). This fluorescence remained green under the UG-2 filter and appeared to be in the cytoplasm, leaving the nuclear areas dark (Fig. 5). These cells usually appeared singly or in nests of 2 to 3. They were found most often in the stratum

granulosum and not infrequently in the stratum mucosum, but they were not seen in the stratum germinativum. Some sections contained many "nest" cells, some contained a few, and many contained none. No correlation was noted between this finding and diagnosis (Table I) or staining technique. A similar phenomenon was observed in sections treated with fluorescent anti-BSA or unstained sections. In these the "nest" areas were not green but merely differed slightly in autofluorescent quality from surrounding epidermal cells.

## DISCUSSION AND CONCLUSION

The presence and distribution of the plasma protein in the skin have been studied somewhat scantily. Boas,<sup>6</sup> studying hexosamines in rat connective tissue, found that in the subcutaneous tissue plasma proteins were present in greater amounts than could be accounted for by the blood content alone. He arrived at this conclusion by comparing the concentration of protein in the tissue with that of hemoglobin. Humphrey and his associates<sup>7</sup> found in rat and rabbit skin and tendon amounts of protein in excess of that accounted for by the blood content of the tissue. Their results, however, referred more specifically to albumin. Zimmer and co-workers<sup>8-11</sup> identified the soluble proteins of the dermis by their electrophoretic mobility in agar and then by immunoelectrophoresis. They found the dermis to contain alpha-1, beta-1 (three lines), and gamma globulin. No alpha-2 globulin was found. They reported finding a beta-2 globulin present in the dermis but not in the serum. Again, the amount of hemoglobulin was not sufficient to account for these proteins as constituents of the blood in the tissue. Bonnelli and Armuzzi<sup>12</sup> compared proteins extracted from the dermis and epidermis with serum proteins by the paper electrophoresis method and identified gamma globulin in the dermis. Using the fluorescent antibody technique, Gitlin<sup>13</sup> found gamma globulin as well as other serum proteins in the skin and other organs of children (necropsy material). However, Raskin<sup>14</sup> failed to find specific fluorescence of gamma globulin in the normal skin of patients with Rhus dermatitis. Rappaport<sup>15</sup> reported finding no gamma globulin in the skin of one normal individual, whereas he observed it in the skin of two egg-sensitive, atopic patients. This evidence needs further clarification in view of the recent findings associating atopic reaginic antibody with  $\gamma_{1A}$  ( $\beta_{2A}$ )-globulin rather than the more abundant  $\gamma_2$ -globulin.<sup>16-18</sup> If there is a difference between atopic and normal skin, one would expect to find an increase of  $\gamma_{1A}$ - rather than of  $\gamma_2$ -globulin.

We believe that the presence of specific fluorescence in the papillary and upper reticular layers of the dermis demonstrates the presence of  $\gamma_2$ -globulin in nonatopic skin, since: (1) The fluorescence differed from tissue autofluorescence; (2) it was not produced by staining with fluorescent anti-BSA sera; (3) it was inhibited by blocking with specific antiserum; (4) it was produced only by sera whose specificity had been demonstrated; and (5) the removal of the anti- $\gamma_2$  antibody from the labeled antiserum rendered it unable to stain skin sections.

No experiments were devised to investigate the origin of the globulin, but its distribution coincides with the progressive thinning of blood vessel walls

and with the zone of greater capillary-intercapillary substance exchange. Therefore, its origin from plasma seems probable. The significance of the inconstant epidermal fluorescence cannot presently be evaluated, and further inquiry is warranted. In general, the basement membrane seemed to be a very effective barrier to the gamma globulin.

The presence in the dermis of  $\gamma_2$ -globulin, of which conventional antibody is composed, would justify the assumption that at least 7S heat-stable, conventional antibody is present in this area. Whether or not the other two immunoglobulins,  $\gamma_{1A}$  and  $\gamma_{1M}$ , are present is being determined in further studies.

## SUMMARY

Gamma-2 globulin was found to be present in the skin of twelve nonatopic patients by the immunofluorescence technique. It appeared as a band in the papillary dermis, sharply limited from the epidermis by the basement membrane and gradually decreasing into the deep dermis. Occasionally, a fluorescing cell or group of cells were seen in the epidermis.

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