Demonstration of Skin Antibodies in Sera of Pemphigus Vulgaris Patients by Indirect Immunofluorescent Staining.* (29622)

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Pemphigus vulgaris is a chronic, bullous skin disease characterized by the histologic picture showing suprabasal acantholysis and degeneration(1). The disease is nearly always fatal unless treated with corticosteroids. The etiology of pemphigus vulgaris remains unknown. Abnormalities in serum globulin levels have been reported(2,3) though no specific immune response had been demonstrated. This report sets forth our initial observations on the immunofluorescent demonstrations of antibodies to an intercellular substance of stratified squamous epithelium in the sera of some patients diagnosed as having pemphigus vulgaris and on the apparent *in vivo* reactions of these antibodies with the patient's own skin.

Methods. Indirect immunofluorescent (I.I.F.) staining as well as direct staining for gamma globulin was carried out by the established procedures(4,5,6) as previously described(7,8). Tissues for I.I.F. staining were quick frozen in liquid nitrogen, sectioned at 2-4 mμ in a cryostat and used for I.I.F. staining without fixation. Human sera were stored at —10°C until ready for use. Specificity controls were carried out with all experiments as described previously(9). Histologic examination of biopsies and diagnoses of pemphigus vulgaris were made by the physicians whose patients were included in this study, Dr. James Jordon, Dr. Joseph Aquilina, Dr. Parviz Lalezari, and Dr. Walter Lever.

Results. Sera of 8 of the 13 patients with diagnoses of pemphigus vulgaris yielded characteristic reaction patterns when tested by the I.I.F. staining method on sections of human skin. These I.I.F. staining reactions revealed the presence of antibodies in these sera to a substance on the surface of cells in stratified squamous epithelia. Fig. 1 illustrates the appearance of a positive reaction obtained with the serum of a fatal case of pemphigus vulgaris (All.) while Fig. 2 is a comparable photomicrograph of a negative control reaction obtained with a similar section of skin treated with a serum of a patient with bullous pemphigoid. The I.I.F. staining of an intercellular substance on the surface of the epithelial cells, particularly in the stratum spinosum or prickel cell layer of the epidermis, is evident in Fig. 1. Similar reactions occurred with other stratified epithelial tissues of human and Rhesus monkey origin. Fig. 3 illustrates the appearance of the positive reaction obtained with the same pemphigus vulgaris serum (All.) on a section of monkey esophagus. Fig. 4 depicts a similar field of another section of monkey esophagus treated with the serum of a psoriasis patient which contained antinuclear antibodies. Such antinuclear antibodies occur commonly (5 to 10%) in a variety of human diseases in low titers (in this case 1:30) and appear to have no etiologic relation to the diseases other than systemic lupus (see back reference in 11). While the antibodies in the pemphigus vulgaris sera that yield I.I.F. staining reaction appear to bind consistently to an intercellular substance in the stratum spinosum, the more superficial stratum granulosum appears to contain variable amounts of the antigen, *i.e.* it sometimes yields positive reactions and sometimes doubtful or negative reactions. The intercellular substance of the germinal layer adjacent to the corium yielded reactions with some (*e.g.* All.) but not with other (*e.g.* Bor.) sera. Some of these differences in staining intensities of different layers of the epithelium are illustrated in Fig. 5, a photomicrograph taken at a low magnification of a section of monkey esophagus stained by the I.I.F. method with the serum of the pemphigus vulgaris patient Bor. The negative reaction of the serum of a tubercular patient

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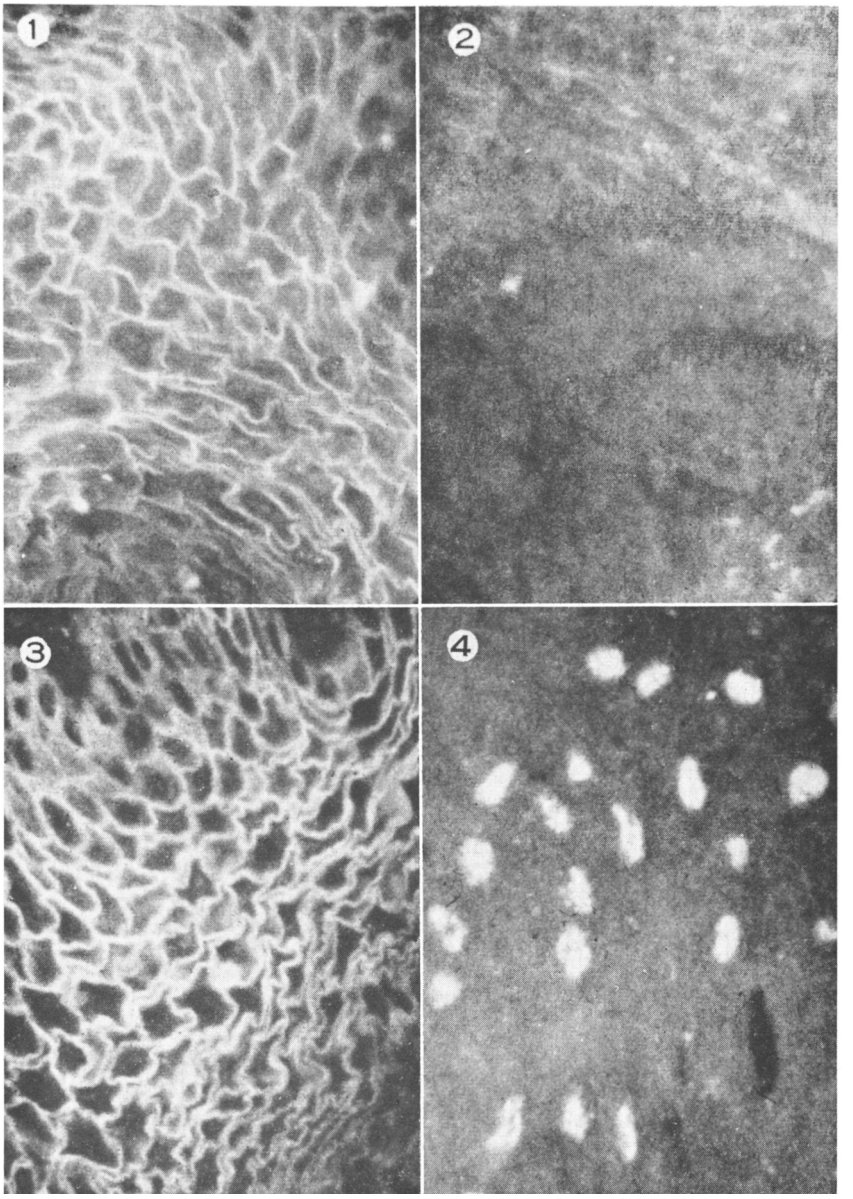

Figures 1 to 6 are photomicrographs of indirect immunofluorescent (I.I.F.) staining reactions on sections of human or Rhesus monkey tissues with human sera (see Methods).

FIG. 1. I.I.F. stain of normal human skin with serum (1:10 dilution) of a pemphigus vulgaris patient (All.). The corium lies above the picture. I.I.F. staining in intercellular spaces occurs predominantly in prickel cell layer. × 500.

FIG. 2. A field comparable to the one shown in Fig. 1. I.I.F. stain with serum (1:10) of a bullous pemphigoid patient. This is a negative reaction. × 500.

FIG. 3. I.I.F. stain of a section of monkey esophagus with the same pemphigus vulgaris patient serum (All., 1:10). Projections of the corium appear at top left corner and top right center. Specific I.I.F. staining appears again in intercellular spaces in stratum spinosum. × 500.

FIG. 4. A field comparable to the one shown in Fig. 3. I.I.F. stain with a 1:10 dilution of serum from a psoriasis patient which happened to contain nuclear antibodies. These antibodies appear to bear no etiologic relation to the disease of the patient. Nuclear staining is evident. × 700.

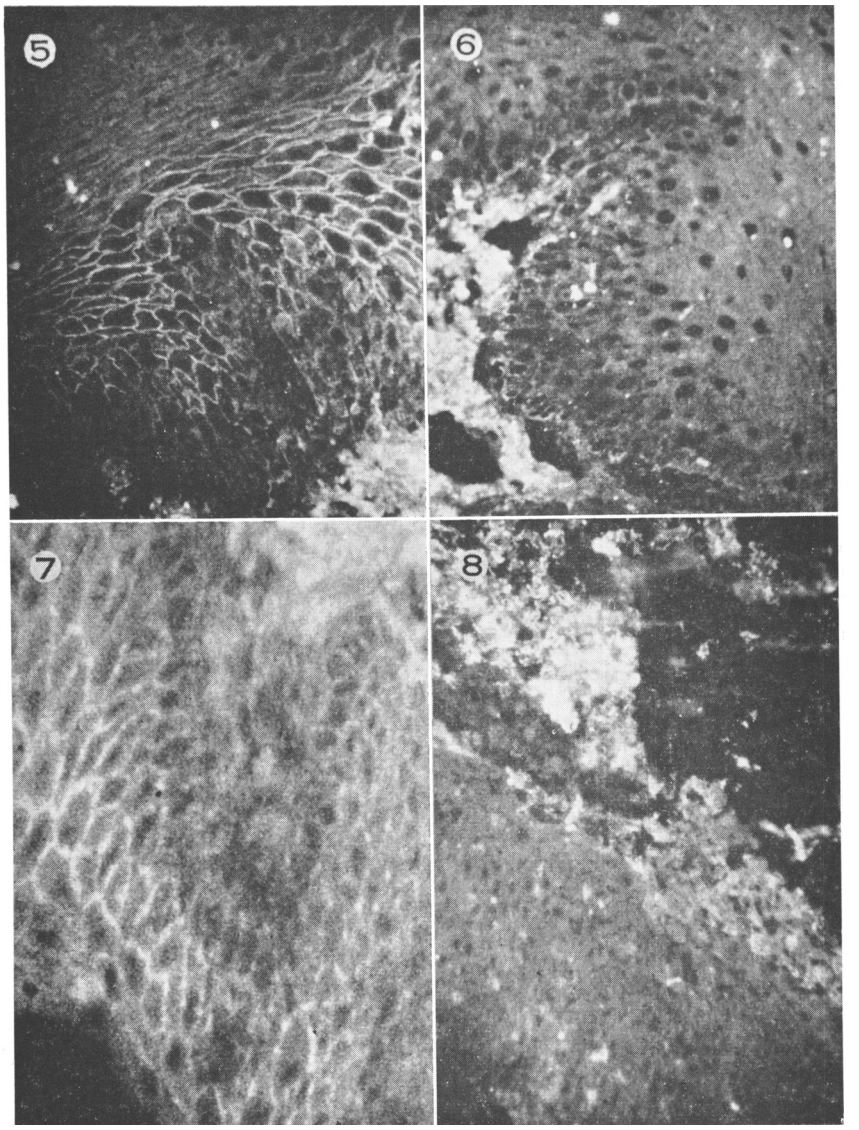

FIG. 5. I.I.F. stain of epithelium of monkey esophagus treated with a 1:10 dilution of serum of a pemphigus vulgaris (Bor.) patient. The lumen lies beyond upper left corner. A finger-like projection of the corium extends from lower right to center. Bright area in lower right corner is blue autofluorescence of the connective tissue. Stronger I.I.F. staining occurred in intercellular spaces primarily in stratum spinosum and to a lesser degree in stratum granulosum. × 200.

FIG. 6. I.I.F. stain of a field comparable to the one shown in Fig. 3 with serum of a tubercular patient (Per., 1:10). Bright area at lower left is blue autofluorescence of connective tissue. No specific I.I.F. staining appears in epithelial cell layer. × 200.

FIG. 7 and 8 are photomicrographs of sections of a skin biopsy from a patient with pemphigus vulgaris (Bor). stained by the direct fluorescent antibody method for localization of gamma globuliin.

FIG. 7. Direct stain for gamma globulin in a section of skin from patient Bor., about 1 cm from a vesicle. Grossly, the area of skin appeared normal. Surface of the skin is seen at lower left corner and blue autofluorescence of connective tissue appears in upper right corner. A broad finger-like projection of the corium extends from top past the center of picture. Specific localization of gamma globulin appears in intercellular spaces of prickel cell layer. This reaction pattern could not be distinguished from the I.I.F. staining with the patient's serum on comparable sections and on epithelium (e.g., Figure 5). × 500.

FIG. 8. Direct stain for gamma globulin in a section of a vesicle of pemphigus vulgaris patient Bor. Acantholytic cells in upper half of picture stained brightly while the underlying epithelium contained no bound gamma globulin. The intercellular antigen could not be demonstrated by I.I.F. staining of these epithelial cells. × 200.

TABLE I. I.I.F.* Staining Reaction Obtained with Normal and Pathologic Sera on Sections of Human and Monkey Epithelium.†

Diagnosis	No. cases	I.I.F.* staining No. pos	No. neg
Pemphigus vulgaris	13	8	5‡
Systemic lupus erythematosus	4	0	4‡
Other collagen diseases	3	0	3
" bullous skin diseases	5	0	5§
" skin diseases	60	0	60‡
Myasthenia gravis	11	0	11
Normal controls	5	0	5
Totals	101	8	88

* I.I.F.—indirect immunofluorescent staining of intercellular spaces in epithelium.

† Human skin or oral mucosa and monkey oral mucosa or esophagus served as test antigen.

‡ The 4 sera of patients with S.L.E., one of the negative pemphigus vulgaris sera, and one of the sera of patients with another skin disease contained antinuclear antibodies but gave no I.F.F. staining of the intercellular spaces.

§ Two of these sera of patients diagnosed as having dermatitis herpetiformis yield immunofluorescent staining reactions of basement membrane which separates the corium from the epidermis. Another patient with bullous pemphigoid yielded no such reaction.

with a comparable section is illustrated in Fig. 6. Technical difficulties in the form of weak, nonspecific staining of intercellular spaces were encountered with monkey epithelium (not with human materials). Such nonspecific reactions were sometimes observed in preparations treated with the conjugated goat antiserum to human gamma globulin alone or with known normal sera and this conjugate. Such nonspecific staining, unlike the specific I.I.F. staining, appeared primarily in the stratum germinativum. With proper selection of conjugated antisera to human gamma globulin, little or no nonspecific staining occurred. In those experiments in which nonspecific staining did occur it could readily be distinguished from specific I.I.F. staining by its distribution, low intensity and the immunologic specificity controls included with each experiment.

Table I summarizes the findings obtained with some 101 human sera that have been tested for their I.I.F. staining reactions on stratified epithelium of human and monkey origin. The 8 of the 13 sera of patients with pemphigus vulgaris yielded the observed reaction pattern. Of these 8 sera, 4 yielded titers in the range of 1:30 to 1:120 while the remaining 4 gave positive reactions only at the 1:10 dilution, the lowest dilution tested. These are regarded as weak positive or doubtful reactions. Two of the negative sera came from patients whose histologic diagnoses were not conclusive. The remaining 11 cases of pemphigus vulgaris were diagnosed histologically. Six of the 8 positive sera yielded reactions only in the stratum spinosum while the remaining 2 sera also yielded reactions in the stratum granulosum. Typical nuclear staining patterns were obtained with 4 sera of patients with systemic lupus erythematosus, one of the pemphigus vulgaris sera, and one of the 60 sera of patients with other skin diseases. This nuclear staining pattern contrasts sharply with the one obtained with the pemphigus vulgaris sera as seen in Fig. 3 and 4. It is noteworthy that 2 of the sera of patients with other bullous skin lesions contained what appeared to be an antibody to the basement membrane of the epidermis. The characteristics of this antibody-like reaction remain to be determined and its significance remains unknown.

Two of the strongly positive sera were utilized in a study of the organ specificity of the reaction. Table II summarizes results of these studies. The I.I.F. staining thus far has only been observed in the intercellular spaces of stratified squamous epithelium of the skin, the nasal and oral mucosa, esophagus, and rectum. The other types of epithelia, stromal elements, and parenchymal cells tested to date have failed to yield positive reactions.

The question arose as to whether the observed I.I.F. staining reaction would occur with sections of the skin from an antibody producing patient. One surgical skin biopsy of one of the antibody producing patients was subjected to detailed study. The biopsy included a newly formed vesicle and some adjacent, apparently normal skin. The vesicle and normal portions of the biopsy were processed separately. The sections of the normal portion of the skin biopsy yielded

TABLE II. Organ Specificity of Indirect Immunofluorescent Staining with Sera of Two Patients with Pemphigus Vulgaris.

Tissues	I.I.F. staining* Bor.	All.	Tissues	I.I.F. staining* Bor.	All.
Skin (H,M)†	+	+	Trachea & bronchi (M)	—	—
Lip mucosa (M)	+	+	Stomach (M)	—	—
Gingiva (H,M)	+	+	Duodenum & ilium (M)	—	—
Nasal mucosa (M)	+	+	Rectum (M)	—	—
Tongue (M)	+	+	Bladder (M)	—	—
Soft palate (M)	+	+	Submaxillary (M)	—	—
Uvula (M)	+	+	Thyroid (H,M)	—	—
Esophagus (M)	+	+	Liver (M)	—	—
Anus (M)	+	+	Kidney (M)	—	—
			Testis (M)	—	—

* Positive I.I.F. (indirect immunofluorescent) staining reactions occurred in the intercellular spaces of stratified epithelium.

† Tissues tested were of human (H) and/or Rhesus monkey (M) origin.

staining of the intercellular substance when treated with the fluorescein labeled goat antiserum to human gamma globulin. Fig. 5 illustrates the appearance of the staining pattern. This reaction was indistinguishable from the I.I.F. staining pattern obtained on comparable sections of the patient's skin using either the patient's own serum or another strongly positive pemphigus serum. Thus, the *in vivo* binding of gamma globulin to the intercellular spaces of the prickel cell layer made it impossible to demonstrate the autoreactivity of the patient's serum in *in vitro* tests method on the single biopsy of a pemphigus vulgaris patient that was studied. All of the 24 specimens of skin or oral mucosa or esophagus tested to date reacted by I.I.F. staining with the pemphigus sera and none of the tissue specimens yielded such a staining reaction for gamma globulin. The type of staining reaction illustrated in Fig. 5 appeared to be specific for gamma globulin as revealed by absorption and blocking experiments. A fluorescein labeled globulin preparation of normal goat serum yielded no reaction.

Examination of sections of the vesicle of the antibody producing patient by direct staining for gamma globulin revealed an intense reaction on the surface of the acantholytic cells. However, the subjacent epithelium was completely negative for gamma globulin localization. Fig. 6 illustrates the appearance of this reaction. Areas distal from the vesicle contained some bound gamma globulin in the intercellular spaces of the prickel cell layer comparable to that illustrated in Fig. 7. The I.I.F. staining of sections of the vesicle with the patient's own serum or another positive pemphigus serum also failed to yield positive reactions of the intercellular substance near the vesicle. Thus, it appears that the stratum spinosum subjacent to this vesicle contained neither bound gamma globulin nor the antigen reactive with the antibodies in the patient's serum.

Discussion. As indicated in the preliminary abstract of these studies(10), the sera of at least some patients with pemphigus vulgaris contain an antibody reactive with an intercellular substance of the epidermis. Only stratified squamous epithelium appears to contain this antigen. Thus far such antibody activity has not been found in sera of patients with other skin diseases. While the observations by I.I.F. staining are unequivocal, other serologic methods need to be developed to elucidate further the nature of antigen-antibody reaction. The preliminary studies on one skin biopsy of an antibody producing patient with pemphigus vulgaris suggest that the antibodies reacted with their homologous antigen in the living patient, *i.e.* the antigenic sites in the skin of the antibody producing patient appear to contain bound gamma globulin. While this evidence strongly suggests that the observed antibodies in the sera of some patients with pemphigus vulgaris are indeed autoantibodies, proof of this is lacking since it has not been possible to

demonstrate a reaction of the patient's serum with his own skin by the *in vitro* I.I.F. staining technique. Further studies are needed to determine whether the apparent *in vivo* binding of antibody occurs uniformly, only in some parts of the patient's epithelium, or possibly only in some pemphigus patients. Also the reason why only 8 of 13 sera appear to be reactive remains to be determined. A partial explanation may lie in the histopathologic diagnosis; the interpretation of finding in 2 of our 5 negative cases have been disputed.

It must be kept in mind that not all autoantibodies appear to react with their homologous antigen in the living individual, *e.g.* nuclear antibodies(11) and antibodies to the cells of thyroids(12) fail to do so, muscle autoantibodies associated with myasthenia gravis do so to only a limited extent(13) while red cell autoantibodies(14) appear to react fully in the patient. The evidence set forth here suggests that the latter situation may prevail in pemphigus vulgaris. If, indeed, the observed immune response in pemphigus vulgaris is found to trigger the pathologic changes of the skin, the question still remains what mechanism(s) are responsible for stimulating the observed autoimmune response to an intercellular substance of the skin. The observation that most cases occur in persons of Jewish extraction(1) or in persons from the Mediterranean area, suggests that some hereditary factors may be operative.

Summary. 1. Eight of 13 sera from patients with pemphigus vulgaris were found to contain antibodies to a substance at the surface of the cells of stratified squamous epithelium, particularly in the stratum spinosum, as demonstrated by indirect immunofluorescent (I.I.F.) staining. The reactions of 4 of these 8 sera were deemed to be weakly positive or doubtful while the remaining 4 sera yielded titers of 1:30 to 1:120 by I.I.F. staining. The reactive antigen was found only in stratified squamous epithelium. Other types of epithelial tissue studied to date do not appear to contain the antigen. None of the 88 sera of normal individuals, patients with other skin diseases, or patients with other immunologic disturbances that have been tested to date appear to contain such antibody activity. However, 2 of the sera of patients with other chronic bullous skin diseases appeared to contain an antibody to the basement membrane of the skin and other stratified squamous epithelia. 2. A skin biopsy of one of the antibody producing patients with pemphigus vulgaris revealed the presence of bound gamma globulin on the surface of the epithelial cells in an apparently normal portion of the skin adjacent to a vesicle. This localization was identical to that obtained by I.I.F. staining with the patient's serum.

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