Immunofluorescent Studies of Autoantibodies to Intercellular Areas of Epithelia in Brazilian Pemphigus Foliaceus* (32626)

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Previous indirect IF (immunofluorescent) staining studies with sera of patients suffering from pemphigus vulgaris and other forms of true pemphigus in North America and Europe (1) revealed the presence of autoantibodies to the intercellular areas of stratified squamous epithelium (Literature is reviewed in Refs. 2 and 3). Several observations on these autoantibodies suggest that they may be pathogenic, i.e., they may possibly be an etiologic factor in the disease process. Yet, one form of the disease, namely, Brazilian pemphigus foliaceus (BPF) or "fogo selvagem," has an epidemiology which is entirely different from that of nonendemic pemphigus, as it occurs in other parts of the world. "Fogo selvagem" (which means "wild fire" in Portuguese) occurs primarily in the south and central areas of Brazil and to a lesser extent in the neighboring areas of Bolivia, Paraguay, and Argentina.

Since very little is found in the English language literature on "fogo selvagem", a few basic observations are set forth in this introduction. The disease occurs mostly in the states of Sao Paulo, Parana, Minas Gerais, Mato Grosso and Goias. Each of these states, except Parana, have a special hospital or clinic for the management of severe cases. The disease occurs mostly in females (at about a 2:1 ratio). All ethnic and socioeconomic groups appear to be affected. About 10% of the cases come from homes in which other family members are similarly affected (4). The distribution of the disease is suggestive of an arthropod borne infection. No cases of direct contagion in the hospital for "fogo selvagem" have thus far been verified. Obviously, more precise and extensive epidemiologic studies of the disease are needed.

Since pemphigus foliaceus appears to be clinically and histologically the same as nonendemic pemphigus foliaceus the question arose as to whether sera of patients with "fogo selvagem" would also yield immunofluorescent staining reactions comparable to those observed with the nonendemic form of the disease. The observations summarized in this report indicate that intercellular antibodies characteristic of pemphigus do indeed occur in Brazilian pemphigus foliaceus.

Materials and Methods. A group of 30 patients at the Sao Paulo hospital for fogo selvagem were subjected to serologic studies. Twenty-eight of these patients were clinically diagnosed as Brazilian pemphigus foliaceus (BPF), one as pemphigus vulgaris and one as Hailey and Hailey's disease. In 28 of the 29 cases of pemphigus the diagnoses were confirmed by IF demonstration of intercellular antibodies. The remaining patient was in remission; his serum yielded a doubtful reaction. The details of these IF studies are set forth in the "Results" section of this report. No histopathologic examinations were performed.

The sera of the 30 patients were divided into two aliquots for testing in each of the two laboratories participating in the research, namely the Department of Bacteriology and Immunology, School of Medicine, SUNY at Buffalo, Buffalo, N.Y., and the Department of Microbiology and Parasitology, Escola Paulista de Medicina, Sao Paulo.

Separate groups of control sera were tested in each of the two laboratories. Titrations

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of intercellular antibodies were performed (by the methods described below) without knowledge of the diagnosis or clinical observations, results of titrations performed in the two laboratories having been reported to a central clearing office without knowedge of results obtained in the other laboratory.

Immunofluorescent staining was performed on sections of rabbit and (or) monkey (*Macaca ira*) esophagus by the methods described elsewhere (5).

Briefly, unfixed cryostat sections were successively incubated with patient serum dilutions, washed and stained with fluorescein conjugates of antiglobulin, washed for 1 hour and examined. The conjugates employed were prepared from antisera to DEAE-cellulose purified human gamma-2 globulin (IgG). They gave one single line in the IgG region in immunoelectrophoretic tests with whole human serum. The fluorescein-protein ratios (F:P) of conjugates were in the range of 4.5:5.2. (These are calculated as molar ratios.)

The undiluted conjugates (about 10 mg protein per ml) contained at least 4 units/ml or more in Ouchterlony tests, and were used at dilutions containing either ¼ or ⅛ units/ml. All titrations were performed using a known positive pemphigus vulgaris serum as a reference control. Only titrations in which the positive control serum yielded the expected titer of 1:320 with monkey esophagus sections were deemed valid. Normal serum and saline controls were also included in all experiments.

Results. Sera of patients with Brazilian pemphigus foliaceus when tested by indirect IF staining on various stratified squamous epithelia yielded strongly positive staining of the intercellular areas. Figures 1 and 2 illustrate the appearance of this reaction on sections of monkey esophagus. Figure 1 is a positive reaction obtained at a 1:160 dilution of "fogo selvagem" serum, while Fig. 2 illustrates the appearance of the negative reaction obtained with a control serum at the same dilution.

The pattern of immunofluorescent staining observed with the "fogo selvagem" sera is indistinguishable from that obtained with sera of patients with pemphigus vulgaris and other

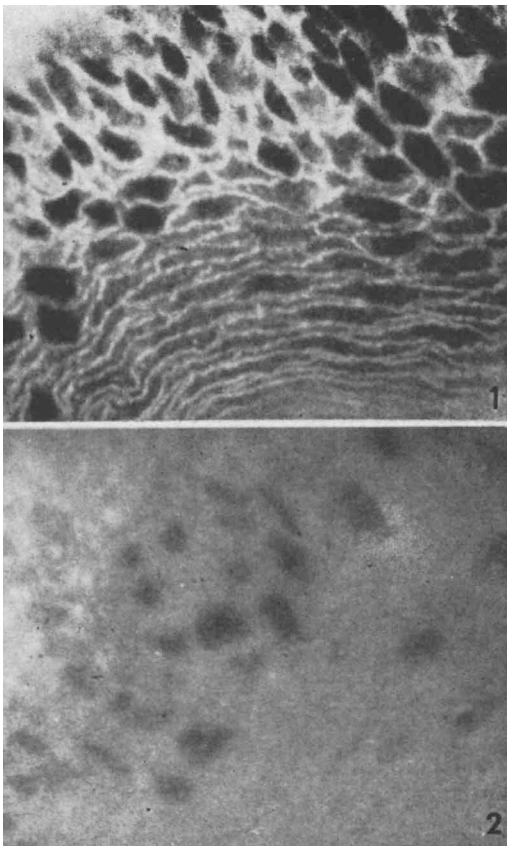

FIG. 1. and 2 are photomicrographs of indirect immunofluorescent (IF) staining reactions of human sera with epithelium of unfixed sections of normal monkey esophagus with a conjugate of a rabbit antiserum to chromatographically pure human IgG. (For further details and techniques see section on "Methods.") × 100.

FIG. 1. Indirect IF staining of intercellular areas with serum of a patient with Brazilian pemhigus foliaceus or fogo selvagem. The serum was diluted 1:160. The lumen of the esophagus is toward the bottom. FIG. 2. Negative reaction obtained with normal human serum under conditions identical to those used for the preparation shown in Fig. 1. The lumen of the esophagus is toward the lower right.

forms of nonendemic pemphigus. As a matter of fact, a pemphigus vulgaris serum served as a positive control in all experiments in one of the laboratories. Also the reaction pattern of a serum from a patient clinically diagnosed as pemphigus vulgaris which was included with the other unknown sera could not be distinguished from those of "fogo selvagem" in either of the two laboratories. A striking though not necessarily distinguishing

TABLE I. Example of the Titration[a] of a Serum from a Patient with Brazilian Pemphigus Foliaceus.

		Indirect immunofluorescent staining of intercellular areas on esophageal sections[a]			
		Monkey		Rabbit	
Sera	Dilution	Reaction	Titer	Reaction	Titer
Saline	—	—		—	
NHS[b]	1/10	—	Neg.	—	Neg.
(Control)	1/80	—		—	
PV[b]	1/80	+++		++	
(Control)	1/160	++		—	
	1/320	+	320	—	80
	1/640	±		—	
BPF[b]	1/10			±	
(Unknown)	1/40			++	
	1/60			+++	
	1/320	+++		++	
	1/640	++	1280	+	640
	1/1280	+		—	
	1/2560	±		—	

[a] Titrations were performed with a conjugate (C-229) with an F:P ratio of 5.2 containing 32 units/ml (in undiluted form) and used at a dilution corresponding to ⅛ unit/ml.

[b] N.H.S. = Normal human serum (negative control). P.V. = Serum of a pemphigus vulgaris patient which contained intercellular antibodies at a titer of 1/320 (positive control). B.P.F. = Serum of a case of Brazilian pemphigus foliaceus (patient C.D.O.).

feature of the IF staining with the "fogo selvagem" sera in this study was the high titer and intensity of the reactions. Thus, for example, the staining reactions illustrated in Fig. 1 were obtained at a serum dilution of 1:160.

All positive sera were titrated by indirect IF staining together with a standard positive pemphigus vulgaris serum and negative controls. The following Table I affords an example of two such titrations, the first on rabbit and the second on monkey esophagus sections.

Normal serum dilutions as well as the saline control yielded the usual negative reactions on both monkey and rabbit esophagus as shown in Table I. The standard positive control serum yielded the expected titer of 320 on the monkey tissue but only a titer of 80 on the rabbit tissue. Such low titers were obtained in all titrations of this positive control serum on rabbit esophagus. The BPF serum titration shown in Table I gave a prozone and a titer of 640 on rabbit esophagus. It was found that BPF serum had a titer of 1280 on monkey esophagus sections. For experimental economy, only higher dilutions were tested.

In general rabbit esophagus was less satisfactory than monkey esophagus. Several rabbit esophagi proved to be unusable because they yielded unsatisfactory results. Most positive sera (16/28) gave higher titers on the monkey tissue. Two BPF sera yielded variable titers on the rabbit tissue and the positive control serum had titers of only 40–80 on sections of rabbit esophagus. For these various reasons the results described in the remainder of this report deal exclusively with the indirect IF staining reaction obtained on sections of monkey esophagus.

Replicate titrations of intercellular antibodies were performed in the two laboratories on the 29 pemphigus sera and on the one serum from a patient with familial benign pemphigus (Hailey and Hailey). Results were reported to one individual without knowledge of the findings in the other laboratory and without knowedge of the clinical observations on the patients. The following correlations were obtained between the titers observed with 30 sera tested on monkey esophagus sections in the two laboratories: (1) Identical titers with 12 sera, (2) One dilution difference with 16 sera, (3) Two dilutions difference with 1 serum, and (4) Variable results with 1 serum.

This latter case was subjected to further studies, some of which are reported below. In view of the correlation between the results obtained in the two laboratories, most of the analyses given in this paper are based on the data from only one of the two laboratories. Comparable analyses made with the titers obtained in the other laboratory yielded completely comparable correlations.

A group of 56 control sera as well as the 30 unknown sera referred to above were sub-

TABLE II. Results of Indirect Immunofluorescent Staining for Intercellular Antibodies with Sera of Brazilian Pemphigus Foliaceus and Control Sera.

Clinical diagnosis	No. of cases	Results of immunofluorescent staining	
		Positive	Negative
Brazilian pemphigus foliaceus	28	27[a]	1
Pemphigus vulgaris	1	1	
Hailey and Hailey	1		1
Ocular pemphigus	2		2
Bullous pemphigoid	10		10[b]
Burns	2		2
Dermatitis herpetiformis	3		3
Erythema multiforme	4		4
Other bullous diseases	3		3
Other dermatoses	17		17
Systemic lupus	9		9[c]
Other collagen diseases	3		3
Other diseases	2		2

[a] Included here is one serum which yielded variable titers.

[b] Three of these sera had antibodies to the basement membrane. No effort was made here to relate these antibody levels to the disease activity of the patient.

[c] Of these 9 sera, 7 yielded positive immunofluorescent staining of the nuclei on esophageal sections; also 5 of 10 bullous pemphigoid sera, 1 of 3 dermatitis herpetiformis sera and 2 of 3 sera from patients with other collagen diseases have some antinuclear antibody activity.

jected to indirect IF tests with monkey esophagus sections. Table II summarized the results obtained.

It is evident from the results summarized in Table II that most sera from patients with pemphigus yielded clearly positive reactions for intercellular antibodies while none of the control sera gave such reactions. However, some of the control sera particularly those of patients with systemic lupus erythematosus, showed antinuclear antibody reaction on the esophageal sections notably in the epithelial portions. Interestingly enough, none of the "fogo selvagem" sera included in this study contained antinuclear antibodies. In a comparable number of sera from patients with active nonendemic pemphigus at least one or more would be expected to contain antinuclear factor. In general the observations summarized in Table II are comparable to those reported in other studies of the disease in regard to the specificity of the intercellular antibodies (2,3).

The diagnoses and the assessment of the evolutions or condition of the patients were made without knowledge of the titers of the intercelluar antibodies and viceversa. Table III affords a tabulation of the titers of intercellular antibodies versus the clinical evaluations of the condition of the 28 patients with Brazilian pemphigus foliaceus.

Table III shows that there is at least a rough correlation between the titer of intercellular antibodies and the severity of the disease process. A more detailed examination reveals several additional facts: (a) The range of titers observed with these sera was higher than any groups previously reported in the literature. This may be a characteristic of Brazilian pemphigus foliaceus. Interestingly, the serum of one fatal case of pemphigus vulgaris (included with the "double blind" group) had a titer of 320 (see *a*); this did not fall in line with the rest of the group of "Deteriorating" patients. However, titers in the range of 320 (i.e., 160–640) are characteristic of severe cases of pemphigus vulgaris. (b) One serum in the "stationary" group yielded variable titers, ranging from completely negative reaction (< 10) to 160 in replicate experiments. This variation may be ascribable to an inhibitor. Also striking prozones have been observed with almost all of the fogo selvagem sera tested, being most pronounced when the sera were fresh or kept frozen and thawed only once. While the cause of the prozones remains obscure, it may be that the same or a similar inhibitor are involved in both phenomena. Somewhat comparable inhibition of the IF staining reactions of intercellular antibodies has been produced by mixing a serum containing rheumatoid factor with an active pemphigus serum. Most pemphigus sera that have been tested appear to contain rheumatoid factor (6). These observations suggest that rheumatoid factor may be the inhibitor responsible for the prozones and the variable titers obtained with one serum. It must be

TABLE III. Relationship between Severity of Lesions in 28 Cases of Brazilian Pemphigus Foliaceus[a] and Titers of Indirect Immunofluorescent Staining of Intercellular Antibodies.

Severity of disease	No. of cases	Titers of intercellular antibodies						
		2560	1280	640	320	160	80–10	<10
Deteriorating	2	2			—[a]			
Stationary	14	3[c]	6	4			(1)[b]	
Improving	10	3	2	3	1	1		
Remission	2						1	1

[a] 1 fatal case of pemphigus vulgaris was examined together with the sera of Brazilian pemphigus foliaceus and gave a positive reaction at a dilution of 1/320; it is not included in this table.

[b] This serum gave variable titers ranging from <10 to 160 in numerous tests on various tissues (see text for further details on this case); three other specimens of this patient yielded titers of 640, 640, and 2560.

[c] The serum titer of one of these 3 cases differed by 2 dilutions in the double blind titrations; in one of the 2 series of laboratory studies this serum yielded a titer of 10,240.

added that three further serum specimens from this patient (taken and tested after decoding the results from the double blind study) yielded titers of 2560, 640, and 640, respectively. As is evident from Table III these latter titers fall in the expected range for patients whose disease is "stationary."

Discussion. Considering the fact that clinical evaluations and IF titrations of intercellular antibodies were performed in a "double blind" study, the correlation between the two is striking, particularly if we isolate the serum of pemphigus vulgaris from the remaining cases of "fogo selvagem." The fact that one serum yielded such variable results by the test methods empoyed clearly indicates that these methods must be improved. This problem is being studied at the present time. Since the titers of intercellular antibodies of the patients with BPF are significantly higher than those of patients with other forms of pemphigus that have been studied so far, we may speculate that slightly pathogenic mechanisms are operative in different varieties of pemphigus.

An increasing body of circumstantial evidence now suggests that the observed circulating antibodies in pemphigus may indeed play an etiologic role in the disease process (cf. Refs. 2 and 3). This body of evidence may be summarized briefly as follows:

(1) The observed antibodies to an intercellular component of stratified squamous epithelia are autoantibodies.

(2) The histologic site of antibody binding corresponds to the site of the first observable pathologic changes.

(3) These antibodies have been found in virtually all active cases of all forms of true pemphigus. So far these antibodies have not been found in sera of patients with any other disease, including Hailey and Hailey's disease.

(4) The titers of the intercellular antibodies are approximately proportional to and fluctuate with the severity of the disease process. The significant deviation from this pattern that have been found may be considered as false negatives and due to the presence of inhibitors.

(5) The antibodies bind to their homologous antigen *in vivo* when antibody containing sera are injected intradermally in the monkey.

(6) Both IgG and complement are fixed *in vivo* to the intercellular areas in skin biopsies of patients with active pemphigus, particularly at or near the sites of bullae.

(7) In at least one case the intercellular antibodies were found before the first histologically typical lesions developed.

It is evident that these observations do not constitute unequivocal proof of the etiologic role of the autoantibodies to the intercellular areas of stratified squamous epithelia in pemphigus. However, since gamma globulin and complement have been demonstrated to be bound *in vivo* on the surface of skin epithlial cells, the role of the autoantibody in the pathogenicity of the disease becomes highly suggestive. This body of evidence certainly pro-

vides bases for working hypotheses for the design and formulation of further studies and experiments.

A more general view of Brazilian pemphigus foliaceus affords the following generalizations:

(1) The epidemiology of the disease suggests that it is caused by an arthropod borne infectious agent.

(2) Clinically and histologically the disease resembles the nonendemic form of pemphigus foliaceus.

(3) Immunologically the disease also resembles nonendemic pemphigus. As in pemphigus vulgaris, there is a correlation between the titer of intercellular antibodies and the severity of the disease. However, the range of titers in the present series of cases was significantly higher (about 8- to 16-fold) than is generally observed in severe cases of pemphigus vulgaris.

As far as the origin of the autoantibody is concerned, several explanations may be offered. (a) The epithelial cells and the infectious agent may share antigenic determinants. Thus, infection with an agent could break immunological tolerance; (b) The infectious agent could liberate sequestered antigenic determinants of the epithelial cells and lead to autoantibody formation; and (c) A superimposed secondary infection, e.g., by beta-hemolytic streptococci could be the source of cross-reacting autoantibodies which would contribute to the self-perpetuation of the lesion. Obviously, these or other hypotheses remain in the realm of science fiction until some factual foundation becomes available.

The reproducibility of the titers observed in the double blind tests performed in the two participating laboratories is equal to that of any serologic test. It must be kept in mind that the factual foundation of all of the available information on autoantibodies in pemphigus rests upon the titration of the indirect IF staining with intercellular antibodies. Thus the reproducibility of the titers obtained is of obvious import.

Summary. Sera of patients with fogo selvagem or BPF (Brazilian pemphigus foliaceus) were found to contain antibodies reactive with the intercellular areas of stratified squamous epithelium of esophagus and skin as revealed by indirect IF (immunofluorescent) staining. These intercellular antibodies could not be distinguished histologically from those associated with nonendemic pemphigus (i.e., the forms of pemphigus which occur in rest of the world). The following observations were made on these antibodies: *(1)* Only sera of patients with pemphigus were found to contain these intercellular antibodies. None of 56 control sera yield such staining patterns. *(2)* Indirect IF staining titers of the intercellular areas could be determined reproducibly ± 1 doubling dilution in the two participating laboratories in a double blind study of 29 sera. (A 30th serum is discussed under point 5 below.) *(3)* The titers of intercellular antibodies appeared to be proportional to the severity of the disease process in BPF. *(4)* These titers were approximately 8-fold higher than those found in cases of nonendemic pemphigus of comparable severity. *(5)* One serum specimen from a patient with BPF yielded variable titers initially. Circumstantial evidence indicates that this may have been due to the presence of rheumatoid factor which acts as an inhibitor. Three subsequent serum specimens of this patient yielded titers in the range expected with the clinical condition of the patient.

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