THE PREVALENCE OF ANTIBODIES AGAINST DESMOGLEIN 1 IN ENDEMIC PEMPHIGUS FOLIACEUS IN BRAZIL

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ABSTRACT

Background Pemphigus foliaceus is an autoimmune skin disease mediated by autoantibodies against desmoglein 1. The endemic form is thought to have an environmental cause. The Terena reservation of Limão Verde in Mato Grosso do Sul, Brazil, is a recently identified focus of the disease, with a prevalence of 3.4 percent in the population. We tested the hypothesis that normal subjects living in an endemic area have antibodies against desmoglein 1.

Methods We used an enzyme-linked immunosorbent assay to detect antibodies against desmoglein 1 in serum samples from 60 patients with endemic pemphigus foliaceus (fogo selvagem) who lived in Limão Verde or elsewhere in Brazil, 372 normal subjects (without pemphigus foliaceus) from Limão Verde and surrounding locations, and 126 normal subjects from the United States and Japan.

Results Antibodies against desmoglein 1 were detected in 59 of the 60 patients with fogo selvagem (98 percent) but in only 3 of the 126 normal subjects from the United States and Japan (2 percent). Antibodies were also detected in 51 of the 93 normal subjects from Limão Verde (55 percent) and in 54 of the 279 normal subjects from surrounding areas (19 percent). Serum samples obtained one to four years before the onset of disease were available for five patients; all five had antibodies in the initial serum samples, and the onset of disease was associated with a marked increase in antibody values.

Conclusions The prevalence of antibodies against desmoglein 1 is high among normal subjects living in an area where fogo selvagem is endemic, and the onset of the disease is preceded by a sustained antibody response. These findings support the concept that the production of antibodies against desmoglein 1 is initiated by exposure to an unknown environmental agent. (N Engl J Med 2000;343:23-30.)

PEMPHIGUS foliaceus is an autoimmune disease characterized by superficial subcorneal blisters and autoantibodies that are specific for the extracellular region of the desmosomal glycoprotein, desmoglein 1 (Fig. 1).[1,2] The disease can be induced by the passive transfer of affinity-purified antibodies against desmoglein 1 in mice.[3,4] There are two forms of pemphigus foliaceus: a nonendemic form, first described in Paris in 1844, that occurs throughout the world,[5] and an endemic form, known as fogo selvagem, that was first reported in Brazil in 1903.[6] Other endemic foci have been reported in Colombia[7] and Tunisia,[8] as well as in Brazil.[9-11] The endemic and nonendemic forms of the disease are clinically, histologically, and immunologically similar,[1,12] but several features are unique to the endemic form, such as the geographic, temporal, and familial clustering of cases, the higher frequency of cases among children and young adults than among older persons, and an association with certain HLA-DR alleles.[13]

Several epidemiologic features of fogo selvagem strongly suggest that the production of pathogenic autoantibodies in patients with this disease is linked to exposure to one or more environmental antigens.[9-11,14] Endemic foci of fogo selvagem are almost exclusively located in rural areas of Brazil. People of many races and ethnic groups are affected,[9,14] including Brazilians of Portuguese, Spanish, German, African, and Japanese descent who live in the endemic areas. The incidence of fogo selvagem decreases dramatically as a region becomes urbanized.[9] There are no reported cases of person-to-person transmission,[9,14] and no microbes have been cultured or identified in skin lesions.

We recently identified an Amerindian settlement in Brazil, the Terena reservation of Limão Verde,[11] with a high prevalence of fogo selvagem (Fig. 2). Of the 916 people living on the reservation, 31 have fogo selvagem (prevalence, 3.4 percent). The first case was documented in 1979, and up to four new cases per year have been identified since then.[11] In contrast, the prevalence of the disease on nine other Terena reservations in the state of Mato Grosso do Sul is low. In recent field studies of the Terena reservations of Ipegue and Taunay, which have a combined population of approximately 3000 people, we found only three cases of fogo selvagem (prevalence, 0.1 percent).[11] These reservations are located 90 km west of Limão Verde and differ from it in terms of geography and ecology.[15] According to unconfirmed

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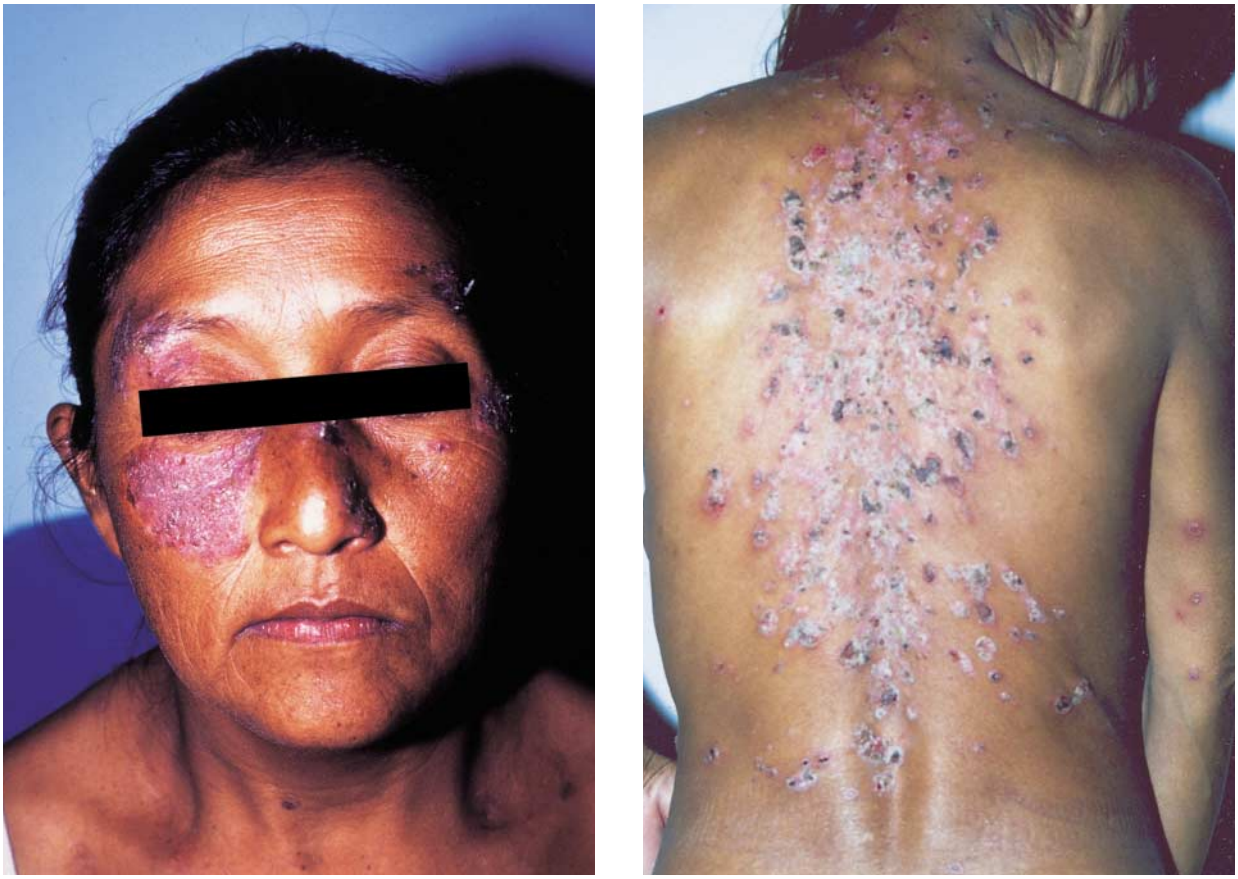

A B

Figure 1. Two Women from the Terena Reservation of Limão Verde, Brazil, Who Had Endemic Pemphigus Foliaceus (Fogo Selvagem). Panel A shows facial lesions on one woman. Subcorneal acantholysis, which is characteristic of the disorder, was present on biopsy of a lesion. Panel B shows lesions on the back of another woman.

information from the Hospital for Pemphigus (Hospital Adventista do Penfigo) in Campo Grande, which treats patients with fogo selvagem from all regions in the state of Mato Grosso do Sul, there have been very few or no cases of fogo selvagem on other Terena reservations. We conducted a study to determine the frequency of antibodies against desmoglein 1 in patients with fogo selvagem and subjects without the disease from the Limão Verde reservation and other locations in Brazil and in other countries.

METHODS

Subjects

The Terena reservation of Limão Verde is divided into two subregions, the Limão Verde subregion and the Corrego Seco subregion (Fig. 2). All the patients lived in the Limão Verde subregion. We obtained serum samples from all 31 patients with fogo selvagem who lived in this subregion and from 29 patients with fogo selvagem who lived in Goiania, Brazil (26 males and 34 females; age range, 7 to 71 years). We also obtained serum samples from a total of 372 normal subjects (subjects without the disease) in Brazil (149 males and 223 females; age range, 5 to 90 years). These normal subjects were from São Paulo (24 subjects), Campo Grande (58), Aquidauana (108), the Terena reservation in Limão Verde (158), and the Terena reservation in Ipegue (24). Of the 158 normal subjects from Limão Verde, 65 were from the Corrego Seco subregion and 93 were from the Limão Verde subregion; 55 of the 93 subjects from the latter subregion were related to one or more of the patients with fogo selvagem. A dermatologist examined all patients and normal subjects at the time that blood samples were collected. The diagnosis of fogo selvagem was established according to the standard clinical, histologic, and immunologic criteria.[1,12]

We also obtained serum samples from 126 normal subjects who lived in other countries (101 from the United States [46 Choctaw Indians and 55 other U.S. residents] and 25 from Japan), 40 hospitalized patients with nondermatologic diseases, and 197 patients with dermatologic diseases other than fogo selvagem. Of these 197 patients, 37 had bullous pemphigoid (10 from Japan and 27 from the United States), 33 had herpes gestationis (all from the United Kingdom), 25 had systemic lupus erythematosus (all from the United States), 38 had the nonendemic form of pemphigus foliaceus (10 from Japan and 28 from the United States), and 64 had pemphigus vulgaris (10 from Japan and 54 from the United States). For 5 of the patients with fogo selvagem who were from the Limão Verde subregion, serum samples obtained before the

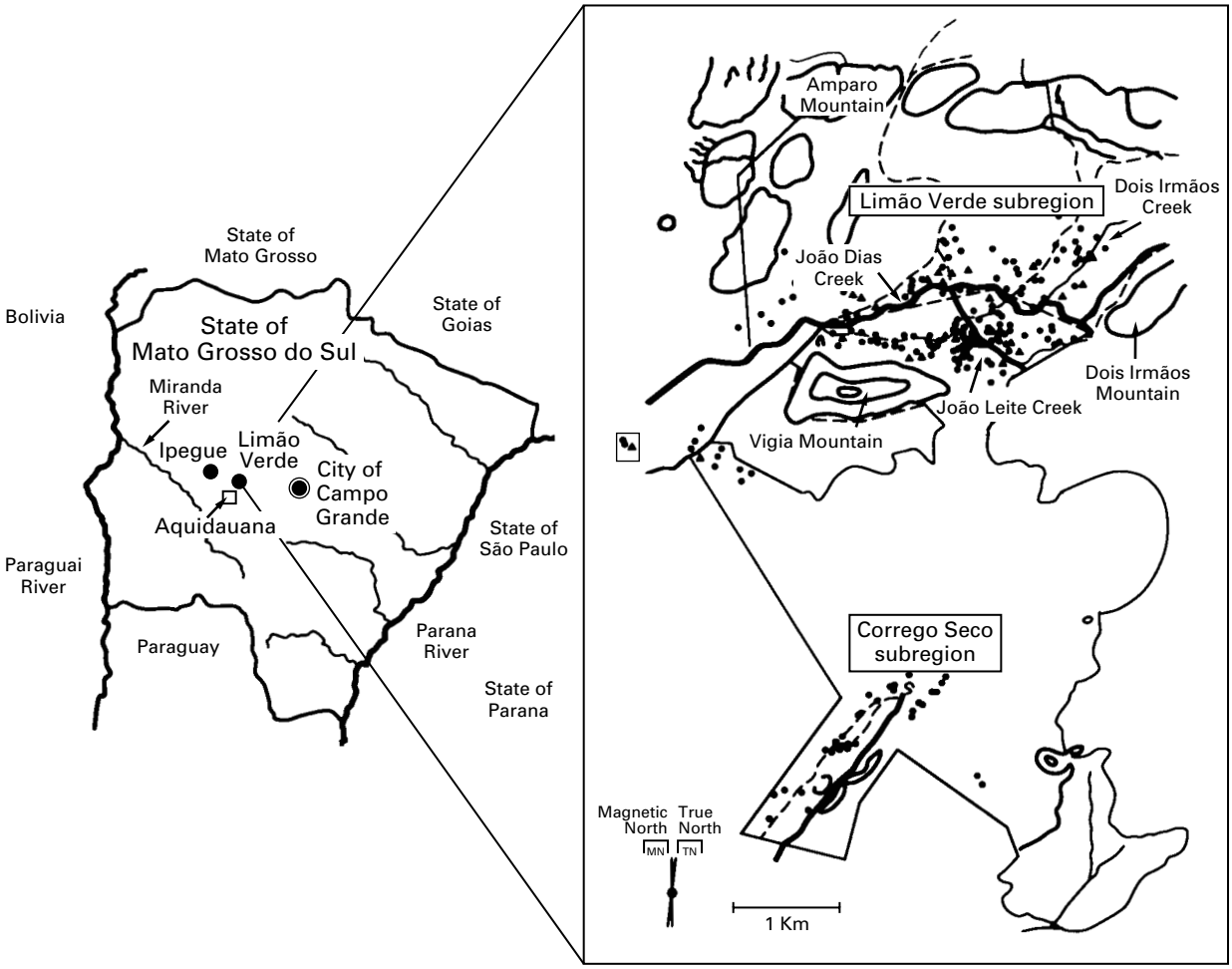

Figure 2. Maps of the Main Study Areas.

The map on the left shows the state of Mato Grosso do Sul in Brazil. The map on the right shows the Limão Verde reservation, the focus of fogo selvagem. The solid circles indicate the locations of houses, and the triangles houses where patients lived. Limão Verde is inhabited by the Terena tribe of Brazilian Indians and comprises two geographic subregions, Limão Verde and Corrego Seco. Broken lines indicate dirt roads. Modified from Hans-Filho et al.[11]

onset of the disease were available for analysis, and multiple serum samples obtained over a period of five years from 19 normal subjects living in the Limão Verde subregion were also available.

The study protocol was reviewed and approved by the institutional review committees at the Medical College of Wisconsin and the University of São Paulo, in Brazil. The studies of the patients and normal subjects from the Terena reservation of Limão Verde were approved by the government of the reservation as well as by Fundaçâo Nacional do Indio, which is recognized by the Brazilian government as the official organization representing Indian interests.

The blood samples were transported within 12 hours after collection to the Universidade Federal de Mato Grosso do Sul in Campo Grande, where the serum was separated and stored at −20°C. The frozen serum samples were then transported to the United States, where all assays were carried out.

Production and Purification of Recombinant Desmoglein 1

A recombinant form of desmoglein 1, consisting of the entire extracellular domain of this desmosomal protein and a C-terminal histidine tag, was generated in the baculovirus system and purified by nickel affinity chromatography according to the procedure of Ding et al.[16] Optimal conditions for this system were determined empirically as described by Liebmann et al.[17] The typical yield of protein was 20 μg per milliliter of culture supernatant.

Enzyme-Linked Immunosorbent Assay

The protocol for the enzyme-linked immunosorbent assay (ELISA) was based on that of Ishii et al.,[18] with the following modifications. Baculovirus-expressed desmoglein 1 was immobilized on 96-well polystyrene plates coated with nickel–nitrilotriacetic acid (Qiagen, Chatsworth, Calif.) by means of overnight incubation at 4°C, and the plates were then washed with TRIS-buffered saline (pH, 7.2) containing 3.7 mM calcium. Duplicate samples of a 1:200 dilution of serum were incubated for 35 minutes. The plates were washed and then incubated with a 1:3000 dilution of horseradish-peroxidase–labeled goat antihuman IgG (Bio-Rad, Hercules, Calif.) for 45 minutes. Additional dilutions beyond 1:200 were tested for any serum samples for which the results were above the readable range (i.e., >2). The assay was also carried out with the use of commercially available desmoglein 1–coated mi-

crotiter plates (Medical and Biological Laboratories, Chicago) according to the procedures described by Ishii et al.[18]

Immunofluorescence and Immunoprecipitation Techniques

Serum samples from all patients with fogo selvagem who were living in the Limão Verde subregion and from 142 normal subjects living in the Limão Verde subregion and elsewhere in Brazil were tested with the use of immunoprecipitation techniques and recombinant desmoglein 1, as described previously.[3,16] Serum samples from all patients and normal subjects who were living in the Limão Verde subregion were also tested by immunofluorescence techniques with the use of normal human skin as the substrate, according to procedures described previously.[19]

Statistical Analysis

Each ELISA plate contained three dilutions of a standard positive serum sample that were used to correct for plate-to-plate variability by linear regression analysis. Logarithmic transformation was used to analyze the assay results. Assay values were considered positive if they were higher than 0.92 optical-density unit at a wavelength of 492 nm. This cutoff point was based on a receiver-operating-characteristic analysis of values in serum samples from normal subjects in the United States (including Choctaw Indians) and in Japan.[20] The value of 0.92 optical-density unit was approximately equal to the mean plus 2.5 SD of the values in these subjects. Serum samples with values higher than 0.92 and lower than 1.25 optical-density units at a wavelength of 492 nm were classified as weakly positive, and those with values above 1.25 as strongly positive. Linear contrasts within a one-way analysis of variance were used to test for trends. For comparisons of groups, we used the Waller–Duncan adjustment for multiple comparisons.[21] All tests were two-sided.

RESULTS

Antibodies against desmoglein 1 were detected in 30 of the 31 patients with fogo selvagem from the Limão Verde subregion (97 percent) and in all 29 patients with fogo selvagem from Goiania, Brazil. The one patient in whom antibodies were not detected was in long-term remission and had a negative immunoprecipitation test. There was a significant correlation between the value on ELISA and the results of indirect immunofluorescence tests ($P<0.001$); however, all serum samples from patients with ELISA values below 2.95 optical-density units had negative indirect immunofluorescence tests.

Both the proportion of normal subjects from Brazil who had positive tests for antibodies against desmoglein 1 and the mean value of the positive tests increased progressively with the proximity of the subjects' residence to the Limão Verde subregion. Antibody tests were positive in 5 of the 24 subjects from São Paulo (21 percent), in 6 of the 58 subjects from Campo Grande (10 percent), in 12 of the 108 subjects from Aquidauana (11 percent), and in 18 of the 65 subjects from the Corrego Seco subregion (28 percent). In the Limão Verde subregion, 15 of the 38 normal subjects who were unrelated to the patients with fogo selvagem had positive tests (39 percent), as compared with 36 of the 55 subjects who were related to the patients (65 percent) (Table 1 and Fig. 3). A test for trend in the mean values of the positive tests for these groups was significant ($P<0.001$). A test for trend in the proportion of serum samples that were positive was also significant ($P<0.001$).

The Terena reservation of Ipegue, which is near the Limão Verde subregion, is also a focus of endemic fogo selvagem, but the prevalence of the disorder is lower in Ipegue and Taunay (3 cases in a population of 3000, or 0.1 percent) than in the Limão Verde subregion (31 cases in a population of 916, or 3.4

TABLE 1. POSITIVE TESTS FOR ANTIBODIES AGAINST DESMOGLEIN 1 IN PATIENTS WITH FOGO SELVAGEM AND NORMAL SUBJECTS, ACCORDING TO THE REGION IN BRAZIL.

GROUP AND REGION	DISTANCE FROM LIMÃO VERDE SUBREGION	TOTAL	POSITIVE ANTIBODY TEST	ELISA VALUE*	
				MEAN	RANGE
	km	no.	no. (%)	optical-density units	
Normal subjects					
São Paulo	1200	24	5 (21)	1.10	0.24–1.23
Campo Grande	160	58	6 (10)	1.15	0.18–1.33
Aquidauana	25	108	12 (11)	1.98	0.13–5.18
Corrego Seco subregion	6	65	18 (28)	2.03	0.23–14.0
Limão Verde subregion†	0				
Unrelated subjects		38	15 (39)	2.48	0.33–14.68
Related subjects		55	36 (65)	3.93	0.37–24.10
Patients with fogo selvagem					
Limão Verde subregion	0	31	30 (97)	16.95	0.59–163.70
Goiania	NA	29	29 (100)	28.06	0.24–240.40

*The mean values are for subjects with positive tests. ELISA denotes enzyme-linked immunosorbent assay. NA denotes not applicable.

†Normal subjects from the Limão Verde subregion were divided into two groups according to whether they were related to one or more of the patients with fogo selvagem.

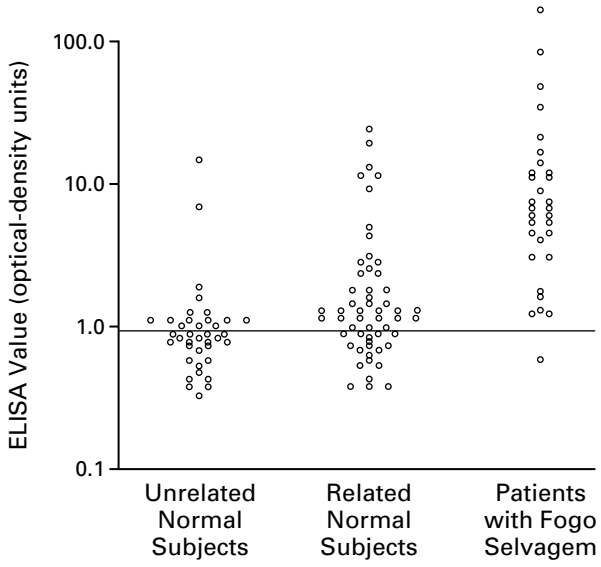

Figure 3. Results of Enzyme-Linked Immunosorbent Assay (ELISA) for Antibodies against Desmoglein 1 in Normal Subjects and in All 31 Patients with Fogo Selvagem in the Limão Verde Subregion.

Normal subjects are divided into those who were unrelated to the patients and those who were related to one or more of the patients. The ELISA values are shown on a logarithmic scale. The horizontal line shows the cutoff point for a positive result (0.92 optical-density unit).

percent). Of the 24 normal subjects from Ipegue, 13 (54 percent) had positive tests for antibodies against desmoglein 1.

Tests for antibodies against desmoglein 1 were negative in 123 of the 126 normal subjects from the United States (including the Choctaw Indians) and Japan, in 39 of the 40 patients with nondermatologic diseases, and in 91 of the 95 patients with bullous pemphigoid, herpes gestationis, or systemic lupus erythematosus (Table 2). The serum samples from three Choctaw Indians, one patient with a nondermatologic disease, two patients with bullous pemphigoid, and two with systemic lupus erythematosus were weakly positive; only one of these serum samples, from a patient with bullous pemphigoid, was positive on immunoprecipitation testing.

Antibodies against desmoglein 1 were detected by ELISA in 36 of the 38 patients with nonendemic pemphigus foliaceus (95 percent). The overall sensitivity of the assay for both endemic and nonendemic forms of pemphigus foliaceus was 97 percent. Of the 64 patients with pemphigus vulgaris, 25 had positive assay results (39 percent), a finding that is consistent with previous reports of the frequency of antibodies against desmoglein 1 in such patients.[3,16,18]

Among the 372 normal subjects from Brazil, serum samples from 84 subjects with positive ELISA results and 58 subjects with negative ELISA results were tested by immunoprecipitation techniques. Thirty-nine of the 84 samples with positive ELISA values (46 percent) also contained antibodies that precipitated desmoglein 1, as compared with 2 of the 58 samples with negative ELISA results (3 percent). Serum samples from eight normal subjects with positive ELISA results and three with negative ELISA results, all of whom were from the Limão Verde subregion, were tested with a commercially available ELISA kit for antibodies against desmoglein 1, and yielded results that were similar to those for our own assay.

We tested patients and normal subjects for reactivity to other, unrelated epidermal antigens. Serum samples from 10 patients with fogo selvagem and 20 normal subjects from the Limão Verde subregion (10 of whom had antibodies against desmoglein 1) did not react with BP180 (a hemidesmosomal antigen targeted in patients with bullous pemphigoid), as determined with the use of an ELISA.[22] In addition, serum samples from all subjects from the Limão Verde subregion (patients with fogo selvagem and normal subjects) showed no immunoreactivity against other nuclear, cytoplasmic, or basement-membrane antigens on indirect immunofluorescence testing.

Serial Studies in Residents of the Limão Verde Subregion

We tested serum samples obtained one to four years before the onset of the disease in five patients with fogo selvagem. All these samples had positive ELISA values (Fig. 4). In four of the five patients, there was a marked increase in antibody levels when histologic and clinical manifestations of the disease developed. The mean antibody level in these five patients increased by a factor of 10.8. We also tested serial serum samples obtained over a period of up to four years from 19 normal subjects in the Limão Verde subregion (8 with negative or weakly positive ELISA results and 11 with strongly positive results). An average of 2.8 samples per subject were available for analysis. Of the eight normal subjects with initially negative or weakly positive ELISA results, seven had no change or a slight decrease in the antibody level. Of the 11 normal subjects with strongly positive results initially, 6 had no change or a decrease in the antibody level and 5 had an increase. For this group, the mean antibody level increased by a factor of 1.5.

DISCUSSION

Our findings provide support for the hypothesis that the production of antibodies against desmoglein 1 is an early step in the pathogenesis of fogo selvagem. This conclusion is based on our finding that 51 of 93 normal subjects (55 percent) living in the Limão Verde subregion had a humoral immune response to desmoglein 1. Moreover, five patients

TABLE 2. POSITIVE TESTS FOR ANTIBODIES AGAINST DESMOGLEIN 1 IN NON-BRAZILIAN NORMAL SUBJECTS, PATIENTS WITH NONDERMATOLOGIC DISEASES, AND PATIENTS WITH VARIOUS AUTOIMMUNE SKIN DISEASES.

GROUP	TOTAL	POSITIVE ANTIBODY TEST	ELISA VALUE*	
			MEAN	RANGE
	no.	no. (%)	optical-density units	
Normal subjects				
United States				
Choctaw Indians	46	3 (7)	0.50	0.15–1.20
Other	55	0	0.36	0.13–0.84
Japan	25	0	0.42	0.19–0.88
Patients with nondermatologic diseases	40	1 (2)	0.35	0.13–1.07
Patients with various autoimmune skin diseases				
Bullous pemphigoid	37	2 (5)	0.50	0.21–1.35
Herpes gestationis	33	0	0.47	0.18–0.84
Systemic lupus erythematosus	25	2 (8)	0.51	0.23–1.11
Nonendemic pemphigus foliaceus	38	36 (95)	29.16	0.63–206.10
Pemphigus vulgaris	64	25 (39)	1.55	0.30–13.79

*ELISA denotes enzyme-linked immunosorbent assay.

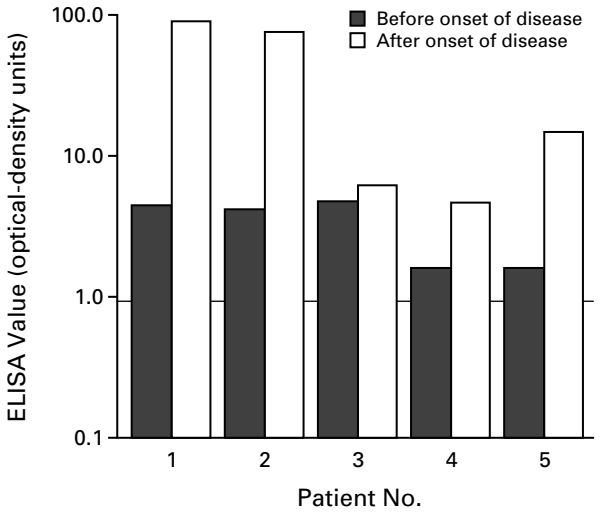

Figure 4. Results of Enzyme-Linked Immunosorbent Assay (ELISA) for Antibodies against Desmoglein 1 in Serum Samples Obtained One to Four Years before the Onset of Disease and after the Onset of Disease in Five Patients with Fogo Selvagem from the Limão Verde Subregion.

The ELISA values are shown on a logarithmic scale. The horizontal line shows the cutoff point for a positive result (0.92 optical-density unit).

with fogo selvagem had antibodies against desmoglein 1 in blood samples obtained one to four years before the onset of the disease, with the mean antibody level increasing by a factor of 10.8 at the time of onset. In contrast, among the normal subjects from the Limão Verde subregion who had initially negative or weakly positive tests and in whom the disease did not develop, there was no such increase in seven of the eight subjects tested.

We confirmed the results of our ELISA for antibodies against desmoglein 1 by testing a subgroup of serum samples with an immunoprecipitation assay and a commercially available ELISA kit for desmoglein 1. There was complete agreement between the results of the two ELISA protocols; furthermore, for all serum samples with strongly positive ELISA results (value greater than 4.2), the immunoprecipitation assay was also positive. In serum samples that had weakly positive or negative ELISA results, no antibodies were detected by immunoprecipitation or immunofluorescence techniques. These findings indicate that our assay is more sensitive for the detection of antibodies against desmoglein 1 than is either immunoprecipitation or indirect immunofluorescence.

Our findings further suggest that the environmental trigger for the initial production of antibodies is clustered in this area of endemic disease. This conclusion is based on our finding that the proportion of normal subjects with positive tests increased with the increasing proximity of their place of residence to the endemic area. The highest frequency of positive tests and the highest antibody values were found in the group of normal subjects from the highly endemic Limão Verde subregion. The results in the group of normal subjects from São Paulo deviated slightly from this trend, with a somewhat higher proportion of subjects with positive tests than in the groups closer to the Limão Verde subregion (Campo Grande and Aquidauana). However, the trend was still statistically significant because of the relatively

small number of normal subjects in São Paulo who were tested. In addition, the average ELISA value was lower in São Paulo than in the areas closer to the Limão Verde subregion. The disease was previously endemic in the state of São Paulo, but with increasing urbanization since the 1950s, the incidence of disease has fallen substantially.[9] This change may account for the somewhat higher proportion of subjects with positive tests in São Paulo than in Campo Grande or Aquidauana.

Although our results suggest that cases of fogo selvagem are triggered by an environmental factor that has a restricted geographic distribution, genetic factors such as HLA alleles associated with susceptibility to the disorder may also be involved.[13] Genetic factors are unlikely to provide a complete explanation for the geographic clustering of cases of fogo selvagem, however, because the disease can develop in persons of any race or ethnic group who live in areas where the disease is endemic.[9,14] Of the 31 patients with fogo selvagem in the Limão Verde subregion, 2 were non-Terena Indians, as were 7 of the 69 normal subjects from the Limão Verde and the Corrego Seco subregions who had positive tests for antibodies against desmoglein 1. Furthermore, among genetically related Terena Indians living in other areas of the state of Mato Grosso do Sul, either there have been no cases of fogo selvagem or the prevalence of the disorder has been much lower than in the Limão Verde subregion.

The mechanisms involved in the development of fogo selvagem are unknown. However, our results suggest that the disease has a subclinical phase of one to four years, characterized by the presence of antibodies against desmoglein 1 without other signs or symptoms, and that the onset of the clinical phase is marked by a substantial increase in the antibody level. The transition from the subclinical to the clinical phase may involve spreading of epitopes,[23] with the recognition of pathogenic epitopes. Preliminary studies suggest that the transition from the subclinical to the clinical phase may also involve HLA susceptibility alleles, since the known HLA susceptibility alleles (HLA-DRB1* 1042, 1406, and 0404) are not found more frequently in normal subjects with positive tests for antibodies against desmoglein 1 than in normal subjects with negative tests (unpublished data). The clinical phase of the disease appears to develop only in antibody-positive persons with the HLA susceptibility alleles, suggesting that the HLA susceptibility alleles may be relevant in the phenotypic expression of the disease.

In conclusion, in a Brazilian region with a high prevalence of fogo selvagem, we found that many subjects without the disease had antibodies against desmoglein 1, suggesting that an initiating factor is present in the region where the disease is endemic. Our findings also point to a two-step mechanism in the pathogenesis of fogo selvagem: an initial period of sensitization (possibly mediated by antigenic mimicry between the environmental antigen and desmoglein 1)[24] and the subsequent progression to the clinical phase of the disease, with a marked increase in serum levels of antibodies against desmoglein 1 in a small subgroup of sensitized persons.

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