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FACTORS CONTRIBUTING TO OCCASIONAL FAILURES IN DEMONSTRATION OF PEMPHIGUS ANTIBODIES BY THE IMMUNOFLUORESCENCE TEST*

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ABSTRACT

The failure to demonstrate pemphigus antibodies in sera of some patients with active lesions may be due to their species or organ specificity, to prozones, to interference by other antibodies or to errors in technique. *In vivo* binding of IgG to the intercellular areas can, however, be demonstrated in sections of biopsies of lesions even when the indirect staining fails to reveal the presence of pemphigus antibodies. The available evidence indicates that all active cases of true pemphigus have pemphigus antibodies.

The detection and titration of pemphigus antibodies has proven to be a valuable tool in the diagnosis and control of corticosteroid therapy of patients with pemphigus (1, 2). Almost all sera of active cases have demonstrable immunofluorescent staining titers of pemphigus antibodies (3). However, some exceptions have been encountered (4). The studies set forth in this report show that such apparent discrepancies may be due to species and organ specificity of some pemphigus antibodies, to prozones of negative staining at low serum dilutions or to interference phenomena caused by the presence of other reactive tissue antibodies in the patients' sera.

METHODS

Immunofluorescent staining. Fresh frozen specimens of human, monkey, rabbit and guinea pig skin and/or lip, preserved for less than one week were sectioned in a cryostat by standard methods (5). Sera of selected cases were diluted immediately before use. Immunofluorescent staining for IgG type antibodies was performed with fluorescein labeled globulin fraction of an unabsorbed monospecific goat antiserum to human IgG. The molar fluorescein to protein ratio was 4:1 and the antibody to protein ratio was 0.4. The dilution used for staining contained 60 mcg antibody protein per ml or about ⅓ unit/ml. Further details of the methods are given elsewhere (6). Indirect staining for IgA type antibodies was performed with a conjugate having a molar F/P of 4.4 and 4 units/1%/ml at a dilution containing ½ unit/ml. For staining of IgM type antibodies, two conjugates were employed. One with molar F/P of 3.4 and 8 units/1% P/ml was used at a dilution of 1 unit/ml and another with a molar F/P of 1.6 and 16 units/ml was employed at a dilution containing 2 units/ml.

RESULTS

The serum of a patient (DYL) with clinically typical oral lesions of pemphigus vulgaris yielded consistently negative results for pemphigus antibodies when tested by standard indirect immunofluorescent methods on rabbit and guinea pig tissue. Similarly tests for IgA and IgM type of pemphigus antibodies were negative. However, histopathology revealed acantholysis typical of pemphigus. Furthermore, direct staining of biopsies of oral lesions for *in vivo* bound IgG showed intercellular staining typical of pemphigus. Interestingly, indirect immunofluorescent staining tests performed with the serum of this patient on monkey lip sections gave strongly positive reactions on oral mucosa and hair follicle epithelium (see Table I). On these epithelia, the serum had a titer of 160. No reactivity could be detected on human or monkey epidermis. Biopsies of interdental papillae were also examined. As has been observed before, such tissues do not afford satisfactory samples of oral mucosa because the intercellular antigens are lost in the areas in which pathologic changes appear. While some reactivity of pemphigus antibodies in the sera of DYL and MER could be detected, these oral biopsies were unsatisfactory for titration or other reliable studies. Serum of another patient with pemphigus vulgaris (MER) gave a similar species and organ specificity pattern of reactions to that of serum DYL (see Table I). Other tests performed on the same patient

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TABLE I

Summary of titers of pemphigus antibodies in sera of patients with active lesions

Tissues	Titers and intensities of indirect immunofluorescent staining of pemphigus antibodies									
	DYL.		MER.		DEV.		MAR.		JOR.	
	Titer	1:10*	Titer	1:10*	Titer	1:10	Titer	1:10	Titer	1:10
Human epidermis	Neg.	−	Neg.	−	5120	+++	Neg.	−	80	++
Monkey lip epidermis	Neg.	−	Neg.	−	5120	++++	Neg.	−	10–20	+
Hair follicles	160	++	160	+	2560	++++	40	+	20	+
Mucosa	160	+++	160	+	2560	++++	80	++	80	+
Guinea pig lip epidermis	Neg.	−	Neg.	−	10,240†	++++	Neg.	−	40	+
Hair follicles	Neg.	−	Neg.	−	10,240	++++	80	+	80	+
Mucosa	Neg.	−	Neg.	−	10,240	++++	80	++	40	±
Rabbit lip epidermis‡	Neg.	−	Neg.	−	2560‡	++++	Neg	±	40	+ Neg.
Hair follicles	Neg.	−	Neg.	−	2560	+++	10‡	+	40	+ Neg.
Esophagus	Neg.	−	Neg.	±	1280	++++	80	++	40	+++ Neg.

* 1:10 Intensity of intercellular staining.

† DEV serum 10,240 highest dilution tested

‡ Sections of epidermis from some rabbits yielded significantly lower titers. (See text.)

at earlier times did, however, give some limited staining on nonprimate epithelia. This was never the case with patient DYL.

The fact that serum from a patient with Brazilian pemphigus foliaceus (DEV) reacts with all antigens listed in Table I demonstrates that all of the epithelia tested did contain antigenic determinates capable of combining with at least some of the pemphigus antibodies. Many strongly reactive sera from patients with pemphigus areas react in this way. Serum from a patient with pemphigus vulgaris (JOR) is an example of a weakly reactive serum which yielded low to moderate titers on all epithelium tested.

Another serum from a patient with Brazilian pemphigus foliaceus (MAR) illustrates the organ specificity and a lack of species specificity of a fairly typical weak serum. Reactivity of this serum could be detected on monkey, guinea pig and rabbit lip mucosa and hair follicle epithelium, but not on the epidermis of these species. Tissues of different rabbits yielded variable reactions as indicated at the bottom of Table I. For example, serum DEV gave a titer of 320 and sera JOR and MAR were negative when tested on sections of some rabbit tissues.

Nuclear antibodies may interfere with the demonstration of pemphigus antibodies at some serum dilutions. Table II summarizes the results of an experiment with serum (SVE) which contained such a high titer of nuclear antibodies that pemphigus antibodies were not demonstrable. Table II also illustrates the effect of partial absorption of the antinuclear factor with guinea pig liver sediment.

As indicated in Table II, absorption of the serum (SVE) with nuclear antigen reduced the titer of the antinuclear antibodies from 320 to 40. Concurrently the absorption allowed detection of the pemphigus antibodies. The observed titer of the latter was 80, although a prozone of negative reactions still obtained at a lower dilution. This prozone was attributed to interference by the residual nuclear antibodies.

Essentially all fresh sera from patients with active Brazilian pemphigus foliaceus give prozones of negative staining in lower serum dilutions in titrations. This is illustrated in a series

TABLE II

Demonstration of interference phenomenon with serum (SVE) containing muscle, nuclear and pemphigus antibodies, from a pemphigus erythematosus patient

IF tests on monkey esophagus sections

Serum dilutions	Indirect IF staining reactions					
	Unabsorbed serum			Absorbed serum†		
	I.C.*	Nuclei	Stria	I.C.*	Nuclei	Stria
10	−	+++	+++	−	+	+++
20	−	+++	+++	−	+	+++
40	−	++	++	−	+	++
80	−	+	+	+	±	++
160	−	+	±	±	−	+
320	−	+	−	−?	−	−
Titers	Neg.	320+	80	80	40	160

* I.C. = Intercellular areas (pemphigus antigen).

† Absorption 1:1 of serum (1/5) and washed sediment of guinea pig liver.

TABLE III

Preliminary titrations of pemphigus antibodies in fresh frozen sera from active cases of Brazilian pemphigus foliaceous (BPF)

Dilutions tested	BPF Sera			
	Pel	Per	DgG	SIL
10	−	−	±	−
40	−	±	+	−
160	++	++	+/++	+/++
640	+/++	+++	+	++
2,560	+/++	++	+	+/++
10,220	−	−	−	−

of preliminary titrations of four sera shown in Table III.

All four of the sera shown in Table III yielded negative reactions at a dilution of 1:10; three of the four sera were even negative at a dilution of 1:40. However, all the sera were strongly positive at a dilution of 1:160. Such prozones usually disappear when sera are stored at refrigeration temperatures. Similar prozones have also been observed from time to time with sera of patients suffering from non-endemic pemphigus.

DISCUSSION

The results of these investigations on some selected sera from patients with pemphigus demonstrate clearly the existence of a broad spectrum of specificities of pemphigus antibodies. That is, different antisera contain antibodies reactive with different antigenic determinants in the intercellular substance of different stratified squamous epithelia. Some differences also occur between tissues of individual rabbits. Some undocumented observations also point to individual variations in the reactivity of monkey epithelia and possibly that of humans. This problem remains to be investigated.

Direct immunofluorescent staining with labelled pemphigus antibodies and the blocking thereof, also point to variations in the specificity of pemphigus antibodies (7). This latter study demonstrated that some, but not all, sera could block the direct staining of the intercellular area with given labelled pemphigus antibodies.

Several reports (1, 2, 3) now demonstrate that almost all cases of active pemphigus have in their circulation detectable levels of pemphigus antibodies. Some unexplained exceptions have, however, been encountered. It is evident from our present studies that one reason for such apparent exceptions is the species specificity of some pemphigus antibodies. For example, patient DYL had been investigated for many months. All tests for pemphigus antibodies on rabbit and guinea pig tissues were completely negative. Yet *in vivo* bound IgG could be demonstrated in the intercellular areas in biopsies of the patients' lesions. The clear demonstration of pemphigus antibodies on primate tissue served to resolve this apparent discrepancy.

Interestingly, case #3, reported by Peck *et al.* (4) as a patient with active pemphigus and no demonstrable pemphigus antibodies, also had specific pemphigus antibodies. In our studies this serum had a titer of 40 on monkey esophagus and it gave a prozone on monkey lip. Previous studies on guinea pig tissue had yielded consistently negative reports. An even more extreme case encountered after the completion of these studies had a titer of 2560 on monkey esophagus but was negative on rabbit tissue.

It is tempting to speculate on the clinical

relevance of the observed differences in organ specificity of different patient sera. For example, patient DYL had only oral lesions for many years but developed a few skin lesions after the sera were taken, although she was not treated with corticosteroids prior to blood sampling. Interestingly, her antibodies failed to react with epidermis even of primates. On the other hand, patient MAR had only skin lesions but her serum reacted with hair follicles and mucosa but not epidermis. Thus, it is hazardous to draw conclusions from the organ specificity of pemphigus antibodies relating to the pathogenic potential of pemphigus antibodies.

Prozones of the type set forth in this report constitute an additional phenomenon which may cause false negative immunofluorescent staining reactions. The mechanisms of such prozones remain to be elucidated. Interestingly, such prozones can be produced by mixing fresh normal human sera with positive sera containing high titers of pemphigus antibodies. Various mechanisms might be considered here: (a) The prozones might be due to the presence of intercellular antigens in sera. (These, of course, would lead to the formation of antigen-antibody complexes in the serum.) (b) They might be due to blocking antibodies. (c) They could possibly be due to rheumatoid factor which is present in most pemphigus sera. (d) They could be due to enzymatic degradation of labile intercellular antigens either by enzymes in fresh sera or by the serum activation of enzymes present in the cryostat cut sections which serve as substrate.

Interference phenomena, due to the presence of multiple tissue antibodies, have been observed in several fluorescent antibody staining systems (8, 9, 10). This interference phenomen appears to be due to the consumption of the labelled antiglobulin by one of the reactive tissue antibodies to the exclusion of the others. Efforts to reveal the other antibodies by repeated incubation of sections with fresh conjugate produced only strong nonspecific staining which masks specific immunofluorescent patterns.

Errors in technique may also lead to false negative results. These may include inadequately reactive conjugate, improper handling of antigen or sera. These problems are discussed in other reports (6, 11). It should be stressed that *in vivo* binding of IgG to the intercellular substance can be demonstrated even when problems arise in the detection of the circulating antibodies, which appear to be present in all active cases of pemphigus.

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