Indirect cutaneous immunofluorescence¹

II Clinical significance

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SYNOPSIS Sera of 532 patients with bullous diseases, connective tissue diseases and malignancies were tested for pemphigus epidermal intercellular fluorescence (ICF) and for the bullous pemphigoid 'tubular' band by the indirect fluorescent antibody technique. Human normal skin cryostat sections were used.

The band and ICF were seen primarily only in bullous pemphigoid and pemphigus respectively. Some indirect band and ICF-negative patients demonstrated positive direct results in involved skin, suggesting that direct tests should be performed in indirect negative patients clinically thought to have pemphigus or bullous pemphigoid.

No close correlation was found between disease activity and positive or negative indirect tests in bullous pemphigoid and pemphigus. Steroids did not interfere with positive results of this diagnostically extremely valuable test.

Direct and indirect immunofluorescence has become a useful diagnostic tool in confirming the clinical diagnoses of bullous pemphigoid and pemphigus. Beutner and Jordon (1964) first demonstrated antibodies in the sera of patients with pemphigus vulgaris to an intercellular substance of stratified squamous epithelium by indirect immunofluorescence. Epidermal intercellular fluorescence (ICF) consisting of smooth polygonal lines of fluorescence around the epidermal cells was noted. Intercellular fluorescence was also demonstrated by the direct technique in involved and uninvolved skin of pemphigus patients (Beutner, Lever, Witebsky, Jordon, and Chertock, 1965). These investigators also found that sera of patients with bullous pemphigoid reacted with the basal zone of stratified squamous epithelium producing a 'band' of fluorescence at this site with the indirect technique (Beutner *et al*, 1965). Jordon first demonstrated a similar band in involved and uninvolved bullous pemphigoid skin by the direct technique (Jordon, Beutner, Witebsky, Blumenthal, Hale, and Lever, 1967). This band, although occurring at the same site, the dermal-epidermal junction, as

that first reported by us in LE skin lesions (Burnham, Neblett, and Fine, 1963) differed morphologically from it in being 'tubular' due to a dark centre in places producing a hollow tubular appearance both by the direct (Burnham, Fine, and Neblett, 1970) and indirect techniques (Burnham and Fine, 1972). Chorzelski, von Weiss, and Lever (1966) and Beutner, Jordon, and Chorzelski (1968a) reported that indirect ICF titres were related to disease activity in pemphigus with higher titres in patients with extensive disease. Fluctuations in serial titres were also found in individual patients related to activity of the disease (Chorzelski *et al*, 1966) with ICF titres falling in remission and rising with relapse of the disease in a particular patient. Conversely, in bullous pemphigoid no such relationship between indirect band titres and disease activity was found by Chorzelski, Jabłońska, Błaszczak, and Jarzabek (1968) although Jordon and his coworkers (1969) found a closer relationship between the extent of disease and the complement-fixing bullous pemphigoid antibody titre. However, Katz, Halprin, and Inderbitzin (1969) reported that the titres of both ICF and the band-inducing antibodies were related to activity of the disease in both pemphigus and bullous pemphigoid, respectively, while Beutner *et al* (1968a) found that positive indirect band tests were more common in bullous pemphigoid patients with active lesions.

The aims of this report are (1) to confirm that

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indirect cutaneous immunofluorescence is a valuable diagnostic confirmatory test for the clinical diagnoses of bullous pemphigoid and pemphigus; (2) to emphasize that direct tests should be performed in patients strongly suspected of having bullous pemphigoid or pemphigus if the indirect test is negative; and (3) to evaluate the relationship between the duration and activity of the disease and the effects of drugs with the results of the indirect technique.

Materials and Methods

CLINICAL MATERIAL

Sera of 532 patients with bullous diseases, malignancies, and connective tissue diseases were tested (table I).

STAINING TECHNIQUE

Indirect technique

The sera, stored at -20°C , were tested within two weeks by the indirect fluorescent antibody technique. Four micron-thick human normal skin cryostat sections were employed as described previously (Burnham and Fine, 1972).

Direct technique

The cryostat cut skin sections were prewashed in veronal-buffered saline (VBS) pH 7.2-7.3 and distilled water to remove any unbound globulins. They were then dried in air and incubated with the various conjugates diluted as for the indirect technique (Burnham and Fine, 1972). They were washed again as above, air-dried, and stored at 4°C until examined.

EXAMINATIONS

The slides were examined without coverslips. The equipment consisted of a Leitz Ortholux microscope with an Osram HBO 200 watt mercury lamp, a BG12 excitation filter, paired Schott OG4 and GG4 barrier

filters, and a $95\times$ fluorite oil immersion objective (Burnham and Fine, 1971, 1972).

ANTINUCLEAR FACTOR (ANF) TESTS

Antinuclear factor determinations were performed with the immunofluorescent tumour imprint technique (Burnham, Neblett, and Fine, 1966; Burnham, Neblett, Fine, and Bank, 1969) modified by mainly employing touch imprints of human normal and lymphoma spleen imprints as nuclear substrate for indirect immunofluorescence.

Results

MORPHOLOGY

The bullous pemphigoid 'tubular' band (fig. 1) (Burnham and Fine, 1972) and epidermal intercellular fluorescence (ICF) (fig. 2) were seen in the bullous pemphigoid and the pemphigus group respectively.

INCIDENCE OF THE BULLOUS PEMPHIGOID TUBULAR BAND AND ICF

The band was seen essentially only in bullous pemphigoid. Only two patients, clinically not bullous pemphigoid, demonstrated a band by the indirect technique out of the 532 patients studied (table I). Bullous erythema multiforme remained the preferred clinical diagnosis in both these indirect band-positive patients. These two patients possibly represented 'drug-induced bullous pemphigoid' as described by Bean, Good, and Windhorst (1970).

Only three patients not in the pemphigus group demonstrated ICF out of the 532 tested (table I). One patient with bronchogenic carcinoma demonstrated ICF with the initial test only and was negative on repeated retesting. One patient with erythema multiforme had coexisting myasthenia gravis and rheumatoid arthritis. This patient also demonstrated ICF in involved and uninvolved skin by the direct technique.

Diagnosis	Number Tested	Number Positive for Band	Number Positive for Epidermal Intercellular Fluorescence
Pemphigus vulgaris	7	0	2
Pemphigus erythematosus	2	0	2
Pemphigus foliaceus	1	0	0
Bullous pemphigoid	20	12	0
Bullous erythema multiforme	14	2	0
Erythema multiforme	15	0	1
Erythema annulare centrifugum	1	0	1
Tumours	108	0	1

Table I Results from 532 patients tested by the indirect technique¹

Systemic lupus erythematosus (74), discoid lupus erythematosus (14), scleroderma (38), suspected connective tissue disease (53), polymorphous light eruption (34), Jessner's lymphocytic infiltrate (8), rheumatoid arthritis (33), miscellaneous medical diseases and dermatoses (88), cicatricial pemphigoid (2), Hailey-Hailey (4), dermatitis herpetiformis (9), herpes zoster (2), toxic epidermal necrolysis (2), pressure bullae (1), subcorneal pustular dermatosis (1), dysplastic epidermolysis bullosa (1).
Numbers in parentheses represent number of patients tested and negative by indirect technique.

Fig. 1

Fig. 1 Bullous pemphigoid tubular band

Normal skin incubated with bullous pemphigoid serum diluted 1:10 followed by incubation with fluorescein conjugated goat antihuman IgG. A convoluted sharply demarcated band with a dark centre in places producing a hollow tubular appearance is present at the dermal-epidermal junction (arrow). Epidermis in upper part of photograph.

Fig. 2

Fig. 2 Pemphigus epidermal intercellular fluorescence (ICF)

Normal skin incubated with pemphigus erythematosis serum diluted 1:10 followed by incubation with fluorescein conjugated goat antihuman IgG. Smooth polygonal ICF is seen in the intercellular space. Note the diminished fluorescence intensity of ICF in the basal cell layer (lower part of photograph) with complete absence of fluorescence at the basal cell dermal border.

The photographs were taken with a 95 × fluorite oil immersion objective, 10 × ocular, Leica MD body, magnified × 3.

RELATIONSHIP OF INDIRECT VERSUS DIRECT RESULTS IN BULLOUS PEMPHIGOID AND IN THE PEMPHIGUS GROUP

The bullous pemphigoid tubular band was seen in only 12 out of 20 bullous pemphigoid patients by the indirect technique (table II). However, the direct test was positive in the involved skin in nearly all the patients tested (one inconclusive) with at least one of the conjugates. The tendency for positive direct results in involved skin, despite negative indirect tests, was also seen with ICF in the pemphigus group (table III).

	Indirect Technique	Direct Technique	
		Lesions	Normal Skin
No. positive of no. tested	12/20	10/11	7/10
Maximum titres with anti-IgG and/or anti-Igs	1280 (active) 640 (remission)		
Anti-IgA	0/12	0/11	0/10
Anti-IgM	0/12	2/11	0/10
Anti-B ₂ C, B ₁ A	3/12	9/11	5/10
Anti-IgG and/or anti-Igs	12/20	8/11	7/10

Table II Comparison of results of tests with indirect and direct techniques for tubular band.

	Indirect Technique with Anti-IgG and/or Anti-Igs			Direct Technique with Anti-IgG and/or Anti-Igs			No. Clinically Normal Skins Positive
	No. Tested	No. Positive	Maximum Titres	No. Lesions Tested	No. Lesions Positive	No. Clinically Normal Skins Tested	
Pemphigus vulgaris	7	2	20 320	3	3	4	0
Pemphigus erythematosus	2	2	640 2560	2	2	2	2
Pemphigus foliaceus	1	0	0	0	0	0	0

Table III Indirect versus direct ICF results¹¹All had active lesions at time of test.

RELATIONSHIP OF THE BAND AND ICF TO DISEASE DURATION AND ACTIVITY AND DRUGS

Duration of disease

There was no relationship between duration of disease and indirect band and ICF results.

Activity of disease

There was no close correlation between activity of disease and positive and negative indirect results in the bullous pemphigoid or the pemphigus group (table II). This lack of relationship with clinical activity also extended to the results with the anti-B₁C, B₁A conjugate in bullous pemphigoid. Not enough data were obtained in the bullous pemphigoid and pemphigus groups to determine whether variations in the titre with sequential testing of individual patients were similarly unrelated to changes in disease activity.

Drugs

Strongly positive indirect results with titres as high as 640 were seen both in bullous pemphigoid and pemphigus patients on high doses of corticosteroid.

Immunoglobulin components of tubular band and ICF tubular band

Only three patients were positive by the indirect technique with anti-B₁C, B₁A as well as with anti-IgG and all were negative with anti-IgA and anti-IgM (table II). The lesions of two bullous pemphigoid patients demonstrated a tubular band by the direct technique with the anti-B₁C, B₁A conjugate only, and were negative with all the other conjugates. Conversely, the lesion of one bullous pemphigoid patient was positive only with the anti-IgG and anti-Igs conjugates by the direct technique.

ICF

All the patients in the pemphigus group were negative by the indirect and direct techniques with anti-IgA and anti-IgM. The only ICF-positive result with anti-B₁C, B₁A in this group was seen with the direct technique in the uninvolved skin of one of the two

patients with pemphigus erythematosus. This patient was also ICF positive with anti-IgG in the clinically normal skin. The results are set out in table III.

Discussion

DIAGNOSTIC VALUE

A positive indirect test is of great value in confirming a clinical diagnosis of bullous pemphigoid or pemphigus. However, negative indirect tests do not rule out these diagnoses. All the patients negative by the indirect technique who had the direct technique performed were positive by the direct technique in involved skin (one case of bullous pemphigoid was inconclusive). Direct tests should therefore be performed in patients strongly suspected of having bullous pemphigoid or pemphigus to confirm these diagnoses if the indirect tests are negative. Steroids did not interfere with the positive indirect band or ICF results. Indirect tests are therefore of diagnostic value even in patients on steroids. The two indirect band positive bullous erythema multiforme patients raise several questions. Do these patients represent 'drug-induced bullous pemphigoid', an entity suggested by Bean *et al* (1970)? Should clinical or immunological criteria be paramount in the final analysis? Although it is tempting to classify these patients as bullous pemphigoid because of the positive indirect band tests, this would not be justified in view of the clinical picture. However sophisticated our laboratory tests become, they should merely be employed to confirm a clinical diagnosis and not be used as a reason to change to a clinically untenable diagnosis. To quote an editorial by Fiumara (1971), 'In this scientific age, physicians need to be reminded that a laboratory test is not a diagnosis. The latter is a judgment based on the patient's history and the results of the physical and epidemiologic examinations. Laboratory tests merely confirm the judgment.'

The patient with erythema multiforme, rheumatoid arthritis and myasthenia gravis who was ICF positive is reminiscent of Beutner's patient with myasthenia gravis and pemphigus erythematosus

(Beutner, Chorzelski, Hale, and Hausmanowa-Petnusewitz, 1968b). However, a diagnosis of pemphigus in our patient could not be confirmed clinically or histopathologically.

LACK OF RELATIONSHIP BETWEEN POSITIVE INDIRECT RESULTS AND DISEASE ACTIVITY

The lack of close correlation between activity of disease and indirect results in bullous pemphigoid has been reported by some authors (Chorzelski *et al*, 1968; Sams, 1970), but other investigators found a correlation (Beutner *et al*, 1968a; Katz *et al*, 1969). Absence of correlation extended to the findings with the anti-B₁C, B₁A conjugate in our series. This casts some doubt on the possibility of using titres of the complement-binding antibody as a therapeutic guide in bullous pemphigoid as suggested by the findings of Jordon *et al* (1969). However, it is still possible that those patients who are positive with anti-B₁C by the indirect technique may be found to have a correlation between disease activity and titre of the complement-binding antibody with sequential testing.

There was also no close correlation between disease activity and positive and negative indirect results in the pemphigus group which has not been the finding of other authors (Chorzelski *et al*, 1966; Beutner *et al*, 1968a).

We did not obtain sufficient data to determine whether there is a relationship between serial fluctuations of the ICF titre and changes in disease activity in following an individual patient as none of the patients were in remission at the time of any of their indirect tests.

PATHOGENESIS

The question whether these band and ICF-inducing antibodies are pathogenetic is still unresolved. Katz *et al* (1969) and Schroeter, Sams, and Jordon (1969) noted that the drop in ICF titre lagged by several weeks behind clinical remission in pemphigus. This favours the idea that these antibodies may be a result rather than the cause of the lesions. However, against this theory, Katz *et al* (1969) noted that a rise in ICF titre preceded exacerbation of the skin disease in one pemphigus patient. Schroeter *et al* (1969) even suggested that relapses might be predicted by a rise in ICF titre in pemphigus.

Despite our ignorance regarding the pathogenetic role, if any, of these antibodies, their demonstration by both the direct and indirect techniques provides an extremely valuable diagnostic aid in confirming the clinical diagnoses of bullous pemphigoid and pemphigus.

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