

COMPLEMENT AS AN ESSENTIAL FACTOR FOR INDIRECT IMMUNOFLUORESCENCE IN CASES OF PEMPHIGOID

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Abstract. Hitherto in the literature no proof has been published of a complement activation on the basement membrane in cases of pemphigoid. In this paper, for the first time, it is demonstrated, even if only in vitro, that complement, whose presence in the region of the basement membrane has long been known, plays an active role in a reaction. Through the action of the haemolytic system (amboceptor and sheep erythrocytes) and of anti C_3 on reaction specimens for indirect immunofluorescence in pemphigoid, it is shown that the indicator reaction of the fluorescein-labelled anti-IgG with the immune complex attached to the basement membrane is markedly dependent on complement. Free, dissolved IgG is precipitated by appropriate anti-serum, even without complement.

Key words: Bullous pemphigoid; Indirect immunofluorescence; Complement activation; Polymeric immune complexes

Investigations into the presence of complement factors on the basement membrane in pemphigoid patients started in 1968, when Chorzelski & Corman (3) discovered C_3 deposits on the basement membrane. By the indirect immunofluorescence method, Jordon et al. (7) subsequently demonstrated adherence to the immune complexes formed on the basement membrane. More recently, the presence of a series of complement factors has been demonstrated by means of corresponding antisera, but it has not hitherto proved possible to verify, by means of a complement-dependent reaction sequence, whether any specific significance can be attached to the complement adhering to the basement membrane. The objective of this present investigation is to provide proof, at least in vitro, of the occurrence of a reaction involving complement factors, i.e. to demonstrate that, in connection

with indirect immunofluorescence with fluorescein-labelled anti-IgG, the indicator reaction occurs only in the presence of active complement.

METHOD

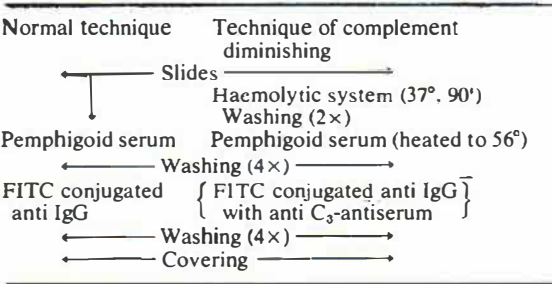
In order to prepare for the experiments using complement-free medium, slices of the antigenic substrate were incubated in a haemolytic system at 37 °C for 90 min. This haemolytic system consisted of 0.8 ml amboceptor, 0.8 ml sheep erythrocytes washed with physiological saline solution, and 2.4 ml Coons's complement buffer. Before use, the mixture was diluted with Coons's complement buffer in the ratio 1:4. Then the slices were rinsed with Coons's complement buffer for two periods, each of 5 min, and were then allowed to stand for 30 min under a layer of pemphigoid serum, which had previously been heated for 30 min to 56 °C. Then the actual immunofluorescence-microscopic investigations were undertaken by the indirect method, as described by Coons et al. (4) and by Beutner et al. (1) (Table I).

Relatively high-titre pemphigoid sera (titre $\geq 1:128$ against guinea pig tongue) were always used. Cryostat sections of guinea pig tongue were used as antigenic substrate. The dilution series for the pemphigoid serum can be seen in Table II. We used the fluorescein-labelled IgG diluted 1:10. When anti C_3 was to be added, one part of undiluted, labelled IgG solution was mixed with nine parts of anti C_3 (diluted 1:5 with buffer). If complement was to be added to the patient's serum in the control experiments, then each dilution stage received 25% complement solution (e.g. serum dilution 1:4:1 part serum, 2 parts Coons's complement buffer, one part complement).

Immuno-electrophoretic investigations were performed by the method of Grabar et al. (5). In this, we allow both an IgG solution and also a pemphigoid patient's serum to run against anti-IgG serum. All solutions had, beforehand, been brought to 56 °C for 30 min.

The reagents for the complement-binding reaction (guinea pig complement, amboceptor and sheep's blood) were supplied by the Behringwerke Marburg, and so were

Table I. Model of reactions



the fluorescein-labelled anti-IgG antiserum, the anti C₃ antiserum, and the IgG.

Characteristics of the anti-IgG: Protein concentration of the gamma-globulin fraction conjugated with FITC=about 10 ±3 mg/litre; Specific antibody fraction is about 10±5% of the gammaglobulin concentration; molar F-P ratio is about 2.5.

RESULTS AND DISCUSSION

Our results show that, when pemphigoid patient's serum pre-heated to 56°C is used, after addition of haemolytic system, the fluorescence observed with fluorescein-labelled anti-IgG on the basement membrane was diminished in comparison with the "normal series". When this system was treated not only with the labelled anti-serum but also with anti C₃, the basement membrane could no longer be seen, as was found in six experiments performed independently of each other. The fact that a complete quenching can be produced only by the addition of an anti-serum directed against a complement component can probably be explained by the circumstance that our labelled anti-IgG contained impurities of complement. A summarized account of our experimental results is shown in Table II.

The site of action of the complement system can be clearly defined. Not only do the literature reports show that the complement factors cannot be fixed until anti-basement-membrane antibodies have been added, but our own experiments also show that the fluorescence of the basement membrane cannot be eliminated by the combined action of haemolytic system and anti C₃, whereby the incubation of the antigenic substrate with the antiserum together with the anti-basement-membrane antibodies is ensured. If, however, the experimental procedure is altered slightly, in such a way that the anti C₃ is added to the test-sample not with the pemphigoid antibodies, but with the

fluorescein-labelled anti IgG instead, then the fluorescence can no longer be detected. Consequently, the indicator reaction must be dependent on complement. In contrast thereto, the dissolved, free IgG could be precipitated with anti-IgG even in the absence of complement, as was in fact shown by a simple, immuno-electrophoretic investigation.

These findings are in agreement with the literature reports, namely that free immuno-globulins are not able to fix complement, whereas polymeric immune complexes, and also non-specific aggregates of immuno-globulins, certainly are capable of so doing (6, 8, 9). In the direct (and also in the indirect) immunofluorescence with anti-basement membrane anti-bodies, it has been demonstrated that these antibodies attach to antigenic structures of the basement membrane, and this can definitely involve

Table II. Survey of our experiments on complement inactivation

Explanation of symbols: 56° serum=pre-incubation of the pemphigoid serum at 56°C; 37° substrate=pre-incubation of the guinea pig tongue at 37°, etc. Whereas a pre-incubation with haemolytic system produces only a partial quenching of the basement membrane fluorescence, the addition of anti C₃ to the fluorescein-labelled anti-IgG can intensify this effect and lead to a complete quenching of the fluorescence. After the haemolytic system has exerted its effect, a fluorescence comparable to that of the normal series can be produced by simultaneous incubation with pemphigoid serum and complement. Similarly, after the haemolytic system has exerted its effect, a simultaneous incubation with pemphigoid serum and anti C₃ produces a fluorescence that corresponds to the reaction without anti C₃

Serum dilution	Normal series	56° serum, pre-incubated with haemolytic system	56° serum, pre-incubated with haemolytic system + anti C ₃ with antiserum
1:4	+++	++	0
1:8	+++	+	0
1:16	++	(+)	0
1:32	+	(+)	0
1:64	+	0	0
Controls: Deviations from the normal series			Result
56° serum			As normal series
56° serum+complement			As normal series
56° serum+37° substrate			As normal series
56° serum+56° substrate			Quenching
56° serum+56° substrate-complement			Quenching
Haemolytic system+56° serum+complement			As normal series
Haemolytic system+(56° serum-anti C ₃)			Anti C ₃ has no effect

polymeric immune complexes. Therefore it is by no means surprising that these immune complexes, in contrast to the free antibodies, can combine with complement, but it is surprising that the antiglobulin reaction on the basement membrane is dependent on complement, whereas the precipitation of the dissolved IgG by corresponding antiserum is not complement-dependent.

In this connection, it seems noteworthy that, by involving complement in the course of the indicator reaction in the indirect immuno-fluorescence in cases of pemphigoid, even if only in vitro, proof has been provided that the complement factors, which in the last few years have repeatedly been demonstrated immuno-optically in the basement membrane, are not non-specifically adsorbed there, but are in fact specifically involved in a reaction sequence of antibody fixation. In the recent report of Provost & Tomasi (10), it is particularly emphasised that no proof has hitherto been provided of complement activation on the basement membrane. At present, there exists nothing but hypotheses concerning the involvement of complement cascade in the pathomechanistic processes that eventually lead to subepidermal vesication, though our own experimental results may provide a certain amount of support for these hypotheses. Whilst we have recently (2) discussed the question of complement activation of pemphigoid, no definite statement can at present be made in this connection.

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