Fluorescent stains in protein detection on immunoblots

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Transfer of proteins to nitrocellulose paper and subsequent staining with an immunoprint technique, is an efficient method for identification of specific proteins as well as for detection of genetic variations. Proteins may be transferred to nitrocellulose paper from different kinds of gels used for separation, and in our experience proteins are readily transferred by passive blotting both from agarose gels and from polyacrylamide gels. The immunoprint technique implies a series of antigen-antibody reactions forming immunocomplexes on the nitrocellulose surface. The first antibody is directed against the protein in question, and protein staining is usually achieved by an enzyme-linked second antibody combined with a suitable substrate. Horseradish peroxidase and alkaline phosphatase are the two most widely used enzymes in commercially available enzyme-linked antibodies. The reaction between enzyme and substrate usually involves the formation of insoluble, coloured products that indicate protein band position on the blot. This staining method is suitable when only one protein on each blot is examined, or when stained proteins are so well separated that their band patterns do not interfere.

Most techniques for electrophoretic separation and staining of serum proteins allow only one or two genetic marker systems to be studied. Several proteins may, however, be detected on one blot if an immunoblot staining method were available in which the stain could (i.e. after photographic documentation) be washed off leaving the blot "clean" for another protein to be detected. Such a method would require a selection of suitable combinations of primary and enzyme-linked secondary antibodies.

The test system we selected in order to establish a method for successive staining of proteins on one blot was neuraminidase treated serum proteins separated on agarose gels, and transferred to nitrocellulose paper by passive blotting. The electrophoretic procedure employed was a slight modification of the method described by O'Neill (1) for separation of complement factor C4. With these experimental conditions, satisfactory separation of coagulation factor 13B phenotypes as well as separation of complement factor C3 phenotypes were also achieved. In figur 1 is seen the electrophoretic band pattern of these three serum proteins detected on nitrocellulose blots with specific antibodies and peroxidase/4-chloro-1-naphtol/H_2O_2 staining.

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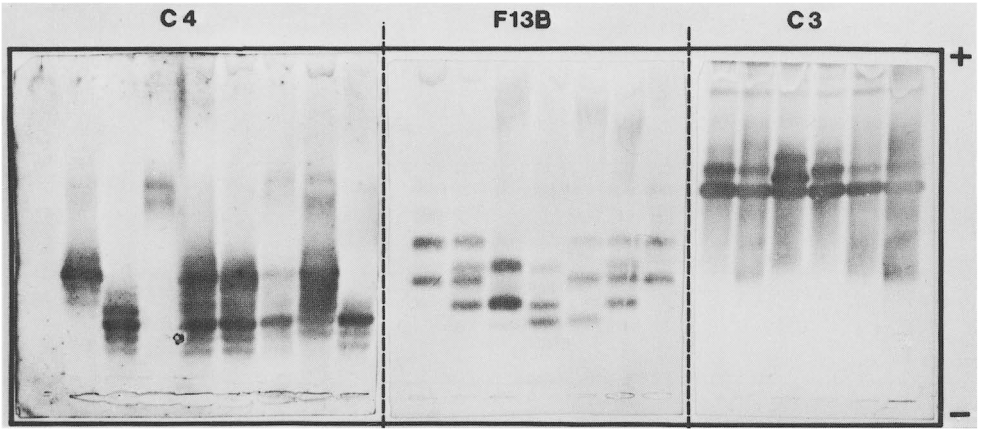

Figure 1. Complement factor C4, coagulation factor 13B and complement factor C3. Neuraminidase treated serum samples are separated on agarose gel and transferred to nitrocellulose paper. One third of the blot is subjected to anti human C4, one third to anti human F13B, and one third to anti human C3. Protein detection: Peroxidase conjugated secondary antibody/4-chloro-1-naphtol/H_2O_2.

Coagulation factor 13B and complement factor C3 are well separated and may be stained on the same blot without interference. Complement factor C4 and factor 13B have similar band positions and cannot be typed simultaneously.

As a test system for protein detection with soluble and "washable" staining was chosen swine anti rabbit antibody linked to alkaline phosphatase combined with 4-methylumbelliferyl dihydrogen phosphate as substrate. The reaction between phosphatases and 4-methylumbelliferyl phosphate produces the highly fluorescent substance 4-methylumbelliferone. This reaction is well known in the detection of acid phosphatase (2) and is also used in ELISA techniques (3).

Our access to suitable combinations of antibodies was limited, and the experiments were designed to avoid crossreactions. The following detection procedure was used on the blots obtained after agarose separation of neuraminidase treated serum samples:

Immunoprint procedure

Step	Antibody directed against	Antibody raised in	Conjugated enzyme	Enzyme substrate	Detection
1.	Human 13B and C3	Rabbit			
2.	Rabbit	Swine	Alkaline-phosphatase		
3.				4-methylumbelliferyl dihydrogen phosphate	Fluorescent bands of factor 13B and C3
4.	Human C4	Goat			
5.	Goat	Rabbit	Peroxidase		
6.				4-chloro-1-naphtol/H_2O_2	Blue bands of C4

The following antibody dilutions were used: anti C3 1:2000, anti factor 13B 1:500, anti C4 1:1000, swine anti rabbit antibody linked to alkaline phosphatase and rabbit anti goat antibody linked to peroxidase 1:1000. The washing procedure was as described for Hp-subtyping (4). Fluorescent bands were obtained by immersing the blot for approximately 1 minute in 50 ml diemal buffer, pH 8.6, containing 20 mg 4-methylumbelliferyl dihydrogen phosphate. The blot was then dried between two sheets of filterpaper and photographed in UV-light.

In figure 2 is seen the fluorescent band patterns of factor 13B and complement factor C3 photographed in UV-light. In figure 3 is seen the same blot after peroxidase/ 4-chloro-1-naphtol/H_2O_2 staining of complement factor C4.

The fluorescent detection technique was further tested on a nitrocellulose blot of an isofocused polyacrylamide gel intended for Hp-subtyping (4,5). Before Hp-detection, the blot was subjected to

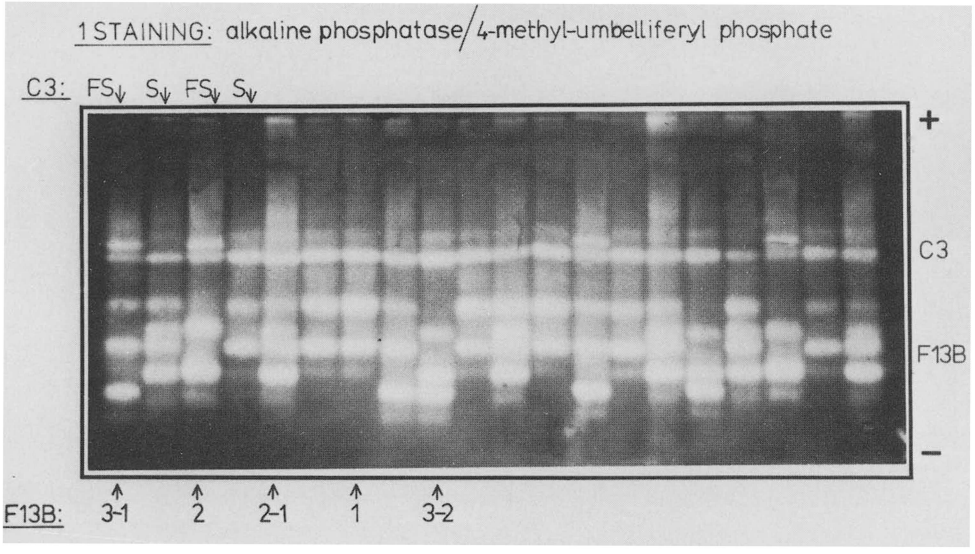

Figure 2. The electrophoretic band patterns of coagulation factor 13B and complement factor C3. The method is described in the text.

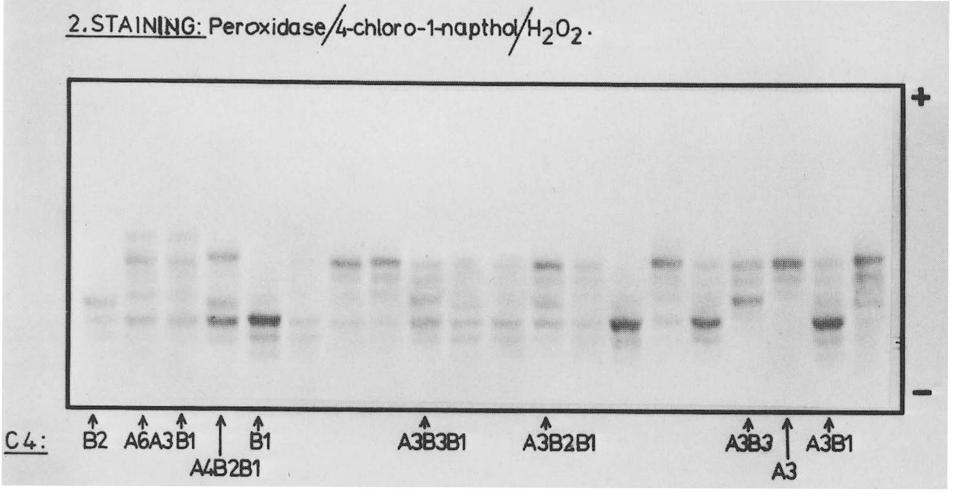

Figure 3. The electrophoretic band patterns of complement factor C4. The staining is performed on the same blot as seen in fig. 2. The method is described in the text.

rabbit anti human orosomucoid followed by swine anti rabbit antibody linked to alkaline phosphatase. The fluorescent band pattern of isofocused, neuraminidase treated orosomucoid is seen in figure 4. Normal Hp-subtyping could subsequently be performed on the same blot.

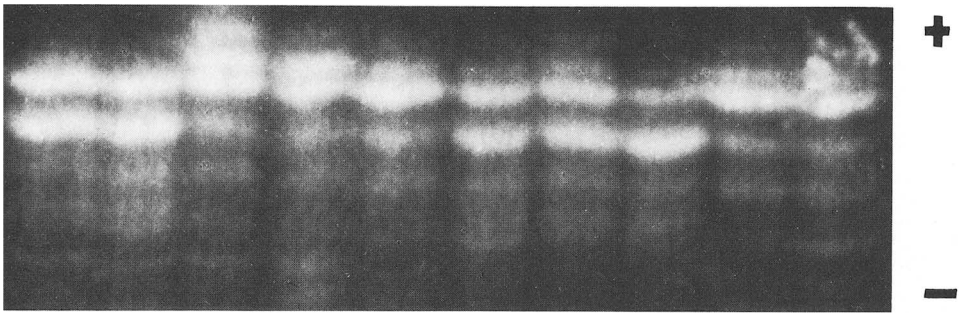

Figure 4. Neuraminidase treated orosomucoid, isofocused and immunoblotted. Staining: Alkaline phosphatase/4-methylumbelliferyl phosphate.

Conclusion

Detection of specific proteins on nitrocellulose blots is easily performed using secondary antibodies linked to alkaline phosphatase and 4-methylumbelliferyl phosphate as substrate. The method is sensitive. The fluorescent band pattern thus obtained does not interefere with subsequent colour detection of other proteins on the same blot.

References

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