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# TECHNIQUE OF LABELING ERYTHROCYTES WITH FLUORESCENT ANTIBODIES - APPLICATION TO FETO-MATERNAL TRANSFUSIONS

by F. Streiff, A. Peters, B. Genetet and D. Vincent

Laboratory of the Transfusion Center, Nancy
(Prof. F. Streiff)

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The method of fluorescent antibodies formulated by Coons in 1942 [1] has since been applied in many areas. By conjugating an antibody with a substance which makes the former fluorescent without making it lose its serological properties, this method makes it possible to combine the high degree of specificity of the serological reactions with the precision of microscopic investigations. It is surprising that this attractive technique has found only limited application in hematology. Thus, the transplacental passage of erythrocytes has been investigated mainly by histochemical methods. These methods make it possible to characterize, within the dominant population of maternal erythrocytes, a few red blood cells whose biochemical characteristics are peculiar to the child [2--6]. Unfortunately, the majority of these properties, particularly the existence of a fetal hemoglobin, are capable of appearing in the adult. Under these conditions, it seems that the best method would be that which applied itself to the antigenic differences which may exist between the mother and her child. The small extent of the transplacental passage does not permit the use of certain techniques such as differential agglutination. On the basis of other authors [9--13], too, we thought that the best method would be the use of fluorescent antibodies.

We shall describe here the technique which we have used and our results.

TECHNIQUE USED

A. Preparation of the Fluorescent Antisera

The anti-A, anti-B and anti-D sera were obtained from immunized subjects. The titer determined by the usual methods was 1/512 for anti-A and 1/256 for anti-B. Our anti-D contained complete antibodies active until a dilution of 1/128, but it also contained incomplete antibodies active until 1/512.

The conjugation technique was that used by Coons and Kaplan [13], as modified by Riggs [14].

After conjugation of the antiserum with fluorescein isothiocyanate, the excess dye was eliminated by passing it through a column of Sephadex 9 50 M, then through a column of Amberlite C 9,400.

This antiserum was frozen at -20°C and thawed only at the time of employment. It was then absorbed on organ powder. In reality, it seems that, since the nonspecific fluorescence of the erythrocytes is negligible, this manipulation is not necessary.

B. Demonstration of the Antigen

The erythrocytes originating from the mother are used as soon as possible after the sample is taken. So far we have used the bloods of O mothers whose child could be A or B.

A 2% suspension of erythrocytes washed once with buffered physiological saline was incubated with an equal volume of labeled antiserum. This incubation was carried out at the laboratory temperature for anti-A and anti-B and at 37° for anti-D with constant mechanical agitation.

The erythrocytes were collected with a Pasteur pipette and washed three times. They were then placed on a slide, covered with a slide cover sealed with paraffin and examined with a dark-field Leitz apparatus permitting a rapid passage from ordinary light to UV light.

RESULTS

1) Under these technical conditions, in the absence of an antigen corresponding to the antibody, we have never observed

any fluorescence of the erythrocytes in the numerous control reactions which we have carried out.

2) Artificial mixtures of O D- and AB or D+ bloods were prepared until a dilution of 1/10,000. At this extreme dilution, it is still possible to recognize, very easily, a few fluorescent erythrocytes. This seems to correspond to a transplacental passage of approx. 0.5 ml of fetal blood.

When the mixture is prepared at overly low dilutions, all the erythrocytes affected by the antiserum are agglutinated. The agglutinate appears with a sharp fluorescence giving an image resembling a bunch of grapes; the erythrocytes appear like well-drawn circles heaped on one another.

It is only at the highest dilutions that we note the appearance of isolated red fluorescent globules. It is important each time to make sure that the image is not an artifact, and in particular not to take into account elements which may have been hemolyzed during the manipulations. In these cases the fluorescence is no longer peripheral only, but colors the whole erythrocyte.

Erythrocytes $A_1$ and B are very sharply fluorescent, while erythrocytes $A_2$ are somewhat less so. With our technique, the use of the fluorescent anti-D gives a slight greenish-yellow coloration of erythrocyte D+. We believe that it would, perhaps, be useful to carry out, in addition, an overstaining by means of a fluorescent antiglobulin.

3) We have applied this technique to the study of transplacental passages by examining the blood of 28 women belonging to the O group during delivery:

- In 18 cases the study was negative, but the child itself was O;

- In ten cases where there was an incompatibility between mother and child, we noted, in five cases, the existence of a few fluorescent erythrocytes. In one case we had to do with a child belonging to group B.

DISCUSSION

Although the principle and the procedures of the method just discussed appear relatively simple, we should not forget that the labeling of erythrocytes with fluorescent antisera encounters considerable difficulties, since until 1959 all the

attempts to apply this reaction to the erythrocytes were negative (Cohen et al. [9]).

To explain this failure, certain authors had formulated the hypothesis that an extinction phenomenon took place on the erythrocyte level due to structural and metabolic factors. One author demonstrated, in effect, that the fluorescent antibody fixed on the erythrocytes gave a positive Coombs test. It was then possible to elute the antibody fixed on the erythrocytes and to obtain a fluorescence of the eluate. It seems indeed that neither the bond between the antigen nor that between the protein of the antibody and the dye are responsible for this phenomenon, and that we have to do with a veritable temporary extinction phenomenon. In 1958 Alexander [14], incubating erythrocytes A in a labeled anti-A rabbit serum, also did not observe any fluorescence. This phenomenon seemed to him all the more curious since one year before [15] Glynn, Holborow and Johnson had successfully used this method for demonstrating the presence of antigens A on certain human tissues. According to him the difficulties encountered may in part be attributed to the presence of hemoglobin which, absorbing the UV radiations, would in this way reduce the total quantity of radiations capable of exciting the conjugated antibody. Without taking any position as to the exact role played by the hemoglobin in the disappearance of fluorescence [16] Whitaker nevertheless obtained in 1958 a slight fluorescence of erythrocytes A previously incubated for 30 minutes with different chelating agents such as versene or BAL, before being placed into contact with the conjugated antiserum. Finally, in 1960, Cohen, Zuelzer and Evans -- continuing Whitaker's work -- obtained, by means of an antiserum of very high titer and labeled with isocyanate, a fluorescence of the corresponding erythrocytes, without the use of chronic artifices such as BAL or versene.

Studying the presence of group antigens on blood cells by means of fluorescent antibodies, we have come up against the same obstacles as those which stopped Alexander and Whitaker at the beginning of their studies. Our conjugation procedure did not seem to be responsible for this failure, and our conjugated serum had preserved its specific agglutination power, while on the other hand, in our study of the group antigens on the leukocytes we were disturbed, when using the same sera, by a nonspecific fluorescence which gave us false images, since the erythrocytic agglutinates were not fluorescent.

Abandoning the method of incubation in a tube on 2% erythrocyte dilutions, we made the conjugate act on erythrocytes fixed on a glass plate. Different fixation procedures were tested: Methanol, ethanol, formaldehyde and acetone were

successively employed. We have also varied the fixation time and the incubation time in the antiserum, but without any result.

We then recalled Alexander's hypothesis according to which the difficulty in bringing the fluorescence to light could be attributed to the absorption of UV by hemoglobin. For this reason we eliminated it by various artifices. When a first attempt at a hemolysis of the erythrocytes in a 2% suspension by successive freezing and thawing was found to be insufficient, we hemolyzed the erythrocytes directly by the addition of distilled water to the erythrocyte residue. The washed stromas were incubated for 30 minutes with the corresponding antiserum. We have also obtained masses which were very fluorescent in UV light, but in which it was very difficult to identify any erythrocytes; moreover, this fluoresence did not seem to be specific because we had obtained similar images with the control erythrocytes.

Resuming our work with the 2% erythrocyte dilutions we finally obtained positive results by mechanically agitating the erythrocytes diluted with the conjugate during the entire incubation period.

It seems that the fluorescence of the erythrocytes requires a considerable and firm fixation of the antibody, with the most complete occupation of the antigenic sites. It then exceeds a certain threshold beyond which the fluorescence becomes visible. This implies the use of very avid and powerful antibodies, and the execution of the reactions under optimum conditions of temperature and with constant mechanical agitation.

As far as the study of the feto-maternal transfusions are concerned, so far we have used conditions in which the transplacental passage of erythrocytes has had the greatest probability of taking place, i.e., the moment of delivery.

It remains to be proved by a more systematic study that this passage is frequent during pregnancy, and that these erythrocytes belonging to an incompatible group persist in the mother's blood. We propose to undertake this study at a later date.

However, already at this time, we can state that Coons' technique may be used for the erythrocytes. The sensitivity of this technique is at least equal to that of other methods such as the method of Kleihauer's acid elution method; its specificity is certainly higher than that of these methods.

SUMMARY

The authors describe a technique for labeling erythrocytes with fluorescent anti-A, anti-B and anti-D sera. The first results of this method applied to the transplacental passage of erythrocytes are presented.

This study shows that this technique presents undeniable advantages compared with the procedures which had been available up to now for the study of these phenomena.

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