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DEMONSTRATION OF ERYTHROCYTES MODIFIED BY LIPO-POLYSACCHARIDES, USING THE FLUORESCENT ANTIGENS TECHNIQUE

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The fluorescent antibody method according to Coons &c (1,2) has been widely used to demonstrate various antigens present in tissues or in isolated cells, including micro-organisms.

This method of investigation has recently been applied to certain immuno-hematological reactions in connection with erythrocytes. Alexander (3) has shown that there is a specific fluorescence in sheep erythrocytes agglutinated with homologous tracer anti-serum, but both the Waaler-Rose reaction and the Heller-Svartz tests, performed with serum from patients with rheumatoid arthritis mixed with fluorescein yielded negative results. The same Author was unable to find fluorescence in erythrocytes of sheep previously sensitized with rabbit hemolysin, and agglutinated with fluorescent antiglobulin rabbit antiserum, nor yet in those of men sensitized with incomplete anti-D antibodies and agglutinated with fluorescent antiglobulin human antiserum. In connection with this latter reaction, however, Cohen and his co-workers (4) found a very positive reaction. These Authors (Cohen, l.c.4; Whitaker &c, 5) also obtained positive reactions of a specific nature between antigens of the ABO group and fluorescein-tagged antibodies.

Furthermore, with the use of antibodies tagged with fetal anti-hemoglobin, the presence in vitro of this hemoglobin, in erythrocytes of the umbilical cord has been demonstrated (Meneghelli, 6).

From the foregoing, we can take it as proven that this method can also be applied to erythrocytes for investigating various cellular antigen constituents, as has been done for other types of cells, although the results dealing with their sensitizing with antibody globulin fractions are less clear.

A well-known phenomenon, different from these processes of erthrocyte sensitization, is that of coating them with polysaccharide antigens, which is the basis of the hemoagglutination and conditioned hemolysis reactions. Erythrocytes treated in this way take on a new antigenic pattern, as if the coating substances has become part of the antigen heritage proper to the cell. We therefore felt it might be fruitful to inquire whether the fluorescent antibody technique could be used to single out the new acquired serological traits of the erythrocytes after coating with lipo-polysaccharide materials.

Materials and methods. -- We used erythrocytes from sheep and from group O humans, preserved until the time of use in Alsever solution. As a polysaccharide antigen we used was a purified endotoxin of S. typhi, phenol- and water-soluble.[o] ([o] The endotoxin, 1138 C, was very kindly supplied by Prof. Gerlough, U.S.A.)

We obtained our antiserum by vaccinating rabbits of an average weight of 2 kilograms with intravenous injections of increasing quantities of endotoxin according to the following schedule: 100 µg for 2 consecutive days in the first week; 200 µg for 2 days in the second, and 500 µg for 2 days in the third. We used available serums with a high hemoagglutinating rating (at least 1:1024).

The fluorescent antibody technique was used according to the indirect method (Weller & Coons, 7; Gillin & coworkers, 8). The tagged antibody we used was the sheep globulin-rabbit anti-globulin fraction, linked with fluorescein from the Sylvana Company, of Orange, N.J. (USA). In all our serological procedures we used a saline solution buffered with m/15 phosphates at pH 7.2 (S.S.T.) The hemoagglutination reaction was produced with the usual techniques. The erythrocytes were treated with a 0.25% solution of endotoxin; the hemoagglutination reaction was produced in test batches with double dilutions of the serum, starting with 1:4 in a volume of 0.40 ml, then an equal volume of the erythrocyte suspension at 0.5%; the antiserum, inactivated at 56°C for 30 minutes was adsorbed with the erythrocytes used in the test, with a double treatment of 1 ml of serum with 0.20 ml of cellular sediment for 1 hour at 37°C.

On these agglutinated erythrocytes (1st or 2nd test) we produced a reaction with the fluorescent antibody on watch crystals or in test-tubes. In the first case, the total sediment, washed three times in S.S.T. went into suspension again up to a volume of 0.40 ml; we placed a drop of the suspension on a slide, dried the slide in air, and left it in an acetone bath for 10 minutes. We then added a drop of the 1:5 solution of fluorescent antibodies, and left the preparation for 30 minutes in a damp room at ambient temperature, shaking it from time to time. After two successive ten-minute washings in S.S.T., we allowed the slide to dry, and prepared it for observation by mounting it in buffered glycerol (9 parts of glycerol to 1 part of S.S.T). In the test-tube reaction, we added 3 drops of the 1:5 solution of fluorescent antibodies to the

thrice-washed erythrocyte sediment, let it stand 30 minutes at room temperature, washed it 3 times with S.S.T., and put a drop of the suspension on a slide. We then dried it, washed it in acetone for 10 minutes, and mounted it in buffered glycerol.

For our fluorescence observations, we used the Zeiss apparatus, with the HBP mercury vapor lamp as our light source. Dark-field vision was obtained with 2 BG-12 excitation filters and OG5 and GG4 stop filters.

We arranged various tests for each trial. For this purpose, we performed the fluorescence reaction on the following: a) erythrocytes treated with the endotoxin and suspended in S.S.T.; b) erythrocytes left untreated and brought into contact with the specific antiserum; c) treated erythrocytes, brought into contact with normal rabbit serum, or with a non-specific rabbit antiserum (anticandida, for example).

Furthermore, in order to see if it were possible to use the fluorescence reaction on a suspension of normal erythrocytes to identify a minute quantity of cells treated with endotoxin, we mixed treated erythrocytes with normal ones at ratios of 1 to 10 and 1 to 20, and then applied the procedures described above.

Results and Conclusions. -- The fluorescent antibody technique, applied with the indirect method, according to the above procedures, invariably showed a brilliant green fluorescence in erythrocytes treated with endotoxin and agglutinated with the specific antiserum. (Figures 1 and 2) The fluorescence was diffuse throughout the entire cell body, and particularly marked on the periphery, so as to produce a pattern of thin rings, almost always continuous and regular. Fluorescence was noted not only in erythrocytes crowded into large clots, but also in the isolated cells. We observed the same appearance in erythrocytes treated and dispersed in among a majority of normal cells, but which could nonetheless be readily identified in both the 1 to 10 and the 1 to 20 dilutions.

All controls gave negative results. In agreement with Alexander's findings via the direct method, we found no difficulty in distinguishing between true fluorescence and the weak and non-brilliant fluorescence that was sometimes found in erythrocytes treated with normal serums or with non-specific antiserums.

Human and sheep erythrocytes behaved identically. We found no difference in behavior under the two methods of procedure, test-tube or glass, in the fluorescence reaction.

These results enable us to draw the following conclusions:

1) The fluorescent antibody technique is a suitable one to indicate the presence of polysaccharide antigens bonded to the surface of erythrocytes, in the same way as are the antigen compounds proper to the cell (3-6). This is of particular interest if we consider that it provides a feasible manner for studying the phenomenon from the morphological aspect, which was hitherto impossible except by the indirect serological method. If we admit that there are, on the surface of the erythrocytes, receptors for the polysaccharide antigens, just as there are for certain viruses, we can assume that the regular and uniform fluorescence we found on the entire surface of the erythrocyte indicates either a homogeneous distribution of these receptors all over the cell surface, or a chemico-physical adsorption mechanism on the cell surfaces, by means of which the antibody globules become a thinly spread film after antigen fixation.

2) In view of the consistent negative reaction in the control experiments, we must consider the reaction as strictly specific. Furthermore, the fact that isolated fluorescent elements are present, in addition to the clot formations, is enough to rule out any trapping artefacts. (4)

3) This method makes it possible to identify, at least in vitro, in a large quantity of normal erythrocytes, a minority of elements whose antigen character has been altered by the attachment of polysaccharide antigens. Cohen and his co-workers (4) have already used the study of fluorescent erythrocytes in vivo to identify Rh+ erythrocytes injected into Rh- subjects, and Rh+ fetal erythrocytes in Rh- pregnant women. In our case, we could use the antigen modifications of erythrocytes and the possibility of distinguishing them readily from other non-treated cells as a special type of tracer, very quickly identifiable. Furthermore, the recognition in vivo of erythrocytes modified by antigens of bacterial or other origin might be an approach to the study of problems still unsolved, such as the pathogenesis of certain types of hemolytic anemia from auto-antibodies.

References

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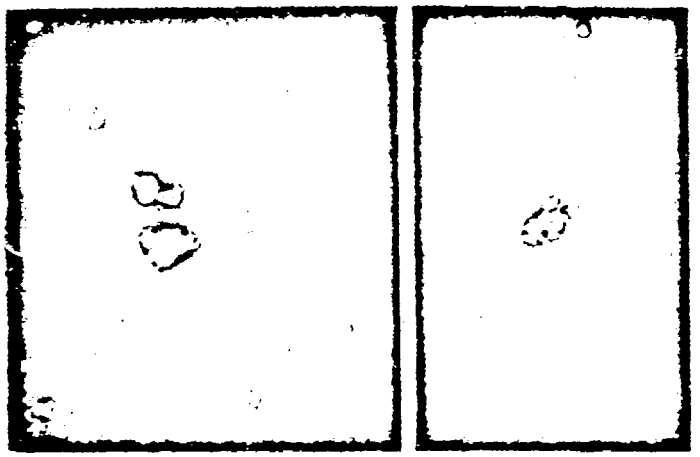

Figure 1. Sheep erythrocytes treated with endotoxin and agglutinated with the specific antiserum. Reaction on a slide.

Figure 2. Human Group O erythrocytes treated with endotoxin and agglutinated with the specific antiserum. Reaction on a slide.