Detection and Quantitation of Minor Erythrocyte Populations in an Admixture by Means of Fluorescent Antibody: Rhesus Monkey

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IN HETEROIMMUNIZATION STUDIES, to obtain markers for determining red cell repopulation after homotransplantation of erythropoietic tissues in irradiated rhesus monkeys, Owen and Anderson developed antisera reagents that recognized five red cell specificities. These were designated A, B, C, D, and E* and were tested by means of an antiglobulin technic in which goat antirabbit globulin serum provided the developing reagent. Although the reagents and the antiglobin technic proved amenable to the quantitative evaluation of chimerism in monkey blood samples following the transfusion of homologous bone marrow into irradiated recipients, there was a regrettable tendency for positive test cells to entrap negative ones in cell mixtures.

The studies forming the basis of this report were designed to duplicate the rabbit antirhesus erythrocyte serum reactivities of Owen and Anderson and to determine whether these antisera reagents would lend themselves to the detection and quantitation of minor cell populations in admixture with a major population when used in the indirect fluorescent antibody technic. Coincident with these studies, an effort was made to develop a simple and rapid method that would minimize cell counting and yet give accurate and reproducible estimations of minor to major cell population ratios well above 1:1000. The usefulness of the fluorescent antibody method in such studies needs no comment, having been demonstrated by Cohen, Whitaker, and Jankovic in the human erythrocyte system and by Möller with isoantigens in mice.

MATERIALS AND METHODS

Antisera were prepared by injecting the marginal ear vein of rabbits with 0.5 ml. of a 50 per cent suspension of washed, rhesus erythrocytes, 3 times weekly for a total of 21 injections, and the rabbits were bled 8 to 10 days later. Antisera prepared in this manner required from 9 to 16 absorptions with one-half volume of packed erythrocytes prepared from rhesus animals of predetermined phenotype for monoreactivity. They were, however, high-titered and could be diluted as much as 1:30 with buffered saline and still retain desired reactivity for immunofluorescence. Erythrocytes from venous blood collected in ACD† were

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*The rhesus specificities are not related to the human A, B blood grouping system.
†Acid citrate-dextrose solution (NIH Solution B) 14.7 Gm. dextrose, 13.2 Gm. sodium citrate, and 4.8 Gm. citric acid per liter of distilled water.

573
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washed 3 times and prepared as suspensions or packed by centrifugation for antiserum absorptions. Difco FA buffered saline (pH 7.2) was used for all cell washings, suspensions, and antiserum dilutions.

Glassware was standardized as follows: selected, precleaned microscope slides, 27 x 75 mm., and Corning cover-glasses, 22 x 30 mm. Reactions were carried out in 10 x 75 mm Kahn tubes, and a Gravex pipet of 0.1 ml. total volume with 1 lambda divisions was used to measure cell suspensions after fluorescent antibody staining.

Microscopy was done with a 10 x wide-field ocular into which was fitted a Howard mold count disc, 21.15 mm. DIA (American Optical Company), and a high-dry 45 x objective. The ultraviolet light source was an AO Fluorolume illuminator with HBO-200 watt mercury arc lamp used in conjunction with a 2 mm. thick exciter filter (Corning #5113) and a barrier filter (Schott GG-1).

The various steps of the procedure for fluorescent antibody staining and counting are numbered and presented in the recommended sequence.

I. \textit{Fluorescent Antibody Staining of the Minor Cell Population}

Wash the admixture of erythrocytes 4 times with buffered saline and then prepare a 10 per cent suspension.

Mix 0.1 ml. of the erythrocyte suspension with 0.1 ml. of the desired antiserum reagent in a Kahn tube. Allow to mix on a rotator at 160 to 180 r.p.m. for 30 minutes at room temperature.

Wash cells twice to remove all unreacted rabbit antiserum reagent. After the final centrifugation on the saline should be carefully and \textit{completely} decanted.

To the packed cells add 0.1 ml. of the fluorescein isothiocyanate conjugated sheep antirabbit globulin globulin that had been previously absorbed 2 times with equal volumes of packed rhesus erythrocytes.

Resuspend the cells by shaking and allow to mix on rotator at 160 to 180 r.p.m. for 30 minutes at room temperature.

Remove excess, unreacted fluorescein-labeled sheep globulin by washing the cells twice. After the final centrifugation the packed cells are resuspended in 0.125 ml. of the buffered saline.

II. \textit{Slide Preparation}

Transfer 15 lambda of the cell suspension to the center of a glass slide and carefully spread the drop by covering with a coverslip. Completely seal edges with clear fingernail polish.

III. \textit{Counting the Fluorescent Minor Cell Populations}

The fluorescent cells are counted \textit{first} because of the lability of the fluorescence under exposure to light.

Place slide preparation under high-dry power of the microscope, with a 10 x wide-field ocular fitted with a Howard mold count disc. Counting is done under ultraviolet light with the substage dark-field condensor in place and a drop of oil between condensor and slide.

Scan the slide preparation as illustrated in Figure 1, counting only the fluorescent cells and the number of fields studied. Slides should be scanned methodically from top to bottom, moving from left to right. The number of fields to be scanned in each vertical movement, as well as the number of ascending and descending vertical movements necessary to scan the entire slide preparation from left to right, depends upon the size of the cell population the microscopist wishes to study.

Slides prepared in the manner described have an average cell density of 280 cells per field. Therefore, if one scans 100 fields, for example, a cell population of approximately 28,000 cells have been studied for fluorescence.
Fig. 1.—An illustration of the method for scanning a slide preparation for fluorescent cells.

Fig. 2.—A microscopic field as seen through a Howard mold count disc mounted in the ocular.

IV. Estimating the Average Cell Density per Field

Visualization of the entire cell population is accomplished simply by replacing the exciter filter with an opal glass filter using the same slide preparation and optics. With the Howard mold count disc mounted in the ocular, the microscopic field is divided into 16 squares, as shown in Figure 2.

Count the cells within the 4 squares running diagonally from upper left to lower right. Squares to be studied are marked with "X's" in Figure 2. All cells within 4 lines of a square are counted, including those cells which overlap the boundary lines on the top and left side of the square (as in hemacytometer counting). Three separate fields are thus counted. Fields are chosen as shown in Figure 3 so that samplings of the slide preparation are made of the left upper portion, the center, and the lower right portion.

The average cell density per field of a particular slide preparation is a simple calculation which considers N as the sum of the cells counted in 4 squares of a field as outlined in the Howard mold count disc (Fig. 2).
Fig. 3.—A slide preparation indicating the 3 general areas from which a microscopic field is chosen for sample counting the major cell population.

\[
\frac{N_1 + N_2 + N_3}{3} \times 4 = \text{average number of cells per field}
\]

N1 = field in upper left
N2 = field in center
N3 = field in lower right

The average number of cells per field, multiplied by the number of fields scanned for fluorescent cells under ultraviolet light, gives the total cell population studied.

The ratio of minor to major cell population, then, is simply number of fluorescent cells counted:total cell population studied.

**RESULTS**

The reactivities developed in this laboratory confirmed the A, B, C, D, and E specificities of Owen and Anderson. An additional antiserum reactivity, designated "F," was developed. A summary of the results of screening several small rhesus populations for reactivity with the various reagents is presented in Table 1. Although hemagglutinin F appears to be as common as B, one can be present or absent independently of the other.

Experiments using the various reagents and the corresponding homogeneous cell populations demonstrated consistently strong and specific fluorescence with the A and B systems but revealed variability in both production and intensity of fluorescence with C, D, E, and F. The method was then applied to the detection and quantitation of minor cell populations in artificial mixtures of erythrocytes, differing primarily in respect to the A and B antigens.

Figures 4A and 4B illustrate the same microscopic field of a minor population of A cells in B cells as seen under high power (oil immersion) with tungsten and ultraviolet light, respectively. It is evident that the fluorescence of the minor populations is "all-or-none," and the distinction is clear-cut. In working with the erythrocyte antigens studied and the A and B reagent specificities, one did not face the problem of having to discriminate between weak and strong fluorescence as a means of defining specific reactions.

Figure 4C is an example of a microscopic field as seen when scanning for fluorescent cells under high-dry power of the microscope. A fluorescent cell is clearly outlined against the black background. Figure 4D is the same field and magnification as viewed under tungsten light, and illustrates the cell density of a slide preparation with approximately 200 cells per field.
Table 1.—Population Frequencies of Reactions with Antirhesus Reagents

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Frequency (%)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>34.7</td>
</tr>
<tr>
<td>B</td>
<td>91.2</td>
</tr>
<tr>
<td>C</td>
<td>33.3</td>
</tr>
<tr>
<td>D</td>
<td>30.6</td>
</tr>
<tr>
<td>E</td>
<td>25.2</td>
</tr>
<tr>
<td>F</td>
<td>91.2</td>
</tr>
</tbody>
</table>

*Total of 143 animals tested.

Table 2.—Hemagglutinogen Profile of Rhesus Erythrocyte Mixtures in Which Minor Cell Populations Were Successfully Quantitated

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Anti-A</th>
<th>Anti-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major population</td>
<td>BDE</td>
<td>AEF</td>
</tr>
<tr>
<td></td>
<td>BF</td>
<td>ADEF</td>
</tr>
<tr>
<td></td>
<td>ABCF</td>
<td>BF</td>
</tr>
<tr>
<td></td>
<td>ADEF</td>
<td>ABF</td>
</tr>
<tr>
<td>Minor population</td>
<td>ABCF</td>
<td>ADEF</td>
</tr>
<tr>
<td></td>
<td>AE5</td>
<td>ABDEF</td>
</tr>
<tr>
<td></td>
<td>AEF</td>
<td>BDE</td>
</tr>
</tbody>
</table>

Table 2 summarizes the hemagglutinogen profile of rhesus erythrocyte mixtures in which minor cell populations were successfully quantitated. It is apparent that the phenotype of erythrocyte donor or recipient, as reflected in these blood group specificities, does not influence the use of anti-A or anti-B reagents in the indirect fluorescent antibody technic. The problem of specificity is readily controllable by the inclusion in all experiments of properly selected erythrocyte recipients lacking the antigen to be demonstrated and the use of antiserum reagents specific for donor erythrocytes.

No matter how exacting the slide preparation, artifacts such as air bubbles or uneven spreading of the cell suspension under the cover glass may occur. A grossly poor slide is, however, readily visible macroscopically and should be discarded. A too light or too heavy suspension of cells is not readily detectable until sampling counts of the total cell population are made. When a slide is properly prepared, the total number of cells counted in the 4 squares running diagonally in a single field (Fig. 3) will generally average about 70 cells, but may vary between 54 and 84 cells. A total of 50 cells or less is a thin preparation, whereas a total of 90 cells or above is too thick a preparation for accurate counting.

A statistically significant number of slide preparations and samplings, as herein described, were made. That the average of 3 samplings represents the average cell density of any one field in a slide preparation was indicated by the correlation between sample averages of 3 fields and 25 fields \((r = 0.89)\). The significance of this correlation had a p value < 0.01.

To scan 100 fields, counting the fluorescent minor cell population, and to estimate the total cell population studied from the sampling counts—i.e., to study one slide preparation adequately—requires from 10 to 12 minutes.

Table 3 summarizes the results of 3 separate studies, comparing the expected and actual counts of fluorescent cells made from 7 different in vitro mixtures of
Fig. 4A.—A high power field (oil immersion), showing an admixture of A cells in B cells, as seen with tungsten light.

Fig. 4B.—The same field as seen with ultraviolet light. Only the A cell fluoresces (minor cell population), illustrating the “all-or-none” reaction observed with both A and B reagents and rhesus erythrocytes of corresponding phenotype.

Fig. 4C.—An example of a fluorescent cell as seen with ultraviolet microscopy while scanning under high-dry magnification.

Fig. 4D.—The same field as seen with tungsten light under high-dry magnification. This is an illustration of the cell density of a slide preparation averaging 212 cells per field. The entire microscopic field is not shown here.

minor and major cell populations. Erythrocyte suspensions used in this procedure were 10 per cent, in contrast to the 2 per cent suspensions found to be optimal when estimating minor to major cell population ratios less than 1:1000. It is evident from the data presented in Table 3 that there was a tendency of the minor cell population to form clumps with artificially prepared ratios under 1:1000, creating a marked discrepancy between expected and actual values. With 2 per cent suspensions, clumping became a major source of errors in ratios of 1:250 or less. One could, however, verify specific agglutination due to fluorescence of the clump. Another factor influencing the disparity between expected and actual counts, which would not be a consideration in studies on
DETECTION OF MINOR ERYTHROCYTE POPULATIONS

Table 3.—Comparison of the Expected and Actual Counts of Fluorescent Cells Made From in Vitro Preparations of Minor and Major Cell Populations

<table>
<thead>
<tr>
<th>Numbers of Fluorescent Cells*</th>
<th>Study #1</th>
<th>Study #2</th>
<th>Study #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical Ratio Minor:Major Cell Population</td>
<td>Expected</td>
<td>Actual</td>
<td>Expected</td>
</tr>
<tr>
<td>1:250</td>
<td>141</td>
<td>421</td>
<td>115</td>
</tr>
<tr>
<td>1:500</td>
<td>100</td>
<td>551</td>
<td>63</td>
</tr>
<tr>
<td>1:1000</td>
<td>37</td>
<td>43</td>
<td>28</td>
</tr>
<tr>
<td>1:5000</td>
<td>6</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>1:10000</td>
<td>4</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>1:20000</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1:40000</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*Expected: number of fluorescent cells based upon sampling of cell population for each slide preparation. Actual: fluorescent cells actually seen in 100 high-dry fields scanned.
†Artificially prepared admixtures.
‡Clumping of minor cell population.

blood from chimeric animals, is the technical error innate to the preparation of artificial mixtures of cell populations. As would be expected, this factor becomes increasingly evident in the lower cell population ratios (Table 3). A good correspondence between actual and expected fluorescent cell populations in ratios 1:1000 or above is evident. Furthermore, if one compares only the expected values, a good reproducibility of cell density from slide preparation to slide preparation is also evident.

DISCUSSION

The use of fluorescent antibody as a method for detecting a minor cell population in an admixture is considerably more sensitive than hemagglutination, since it does not require the presence of enough cells for agglomeration as a means of identification. The results of these studies have demonstrated that the indirect fluorescent antibody technic can be used with rabbit antirhesus reagents not only to detect but also to quantitate minor erythrocyte populations in the rhesus monkey.

The extent to which blood group factors other than A and B can be demonstrated by this method is now under study. Apparently the number and perhaps the position of antigenic sites are factors determining the ease with which fluorescence can be achieved. The selection of a potent antiserum also seems to be an essential factor. Our observations have confirmed those of others, that far more antibody must be attached to the cell to produce fluorescence than is required for agglutination. An antiserum reagent optimally effective for fluorescence at dilutions of 1:30 had a hemagglutination titer of 1:256.

The sensitivity of the fluorescent antibody technic for detecting and quantitating minor cell populations appears to be limited only by the time and effort an investigator wishes to devote to counting cells. Time and eye fatigue become major problems, however, when multiple samples are to be studied concurrently. In the method presented, counting of the total cell population is unnecessary. A sampling of only 3 microscopic fields permits an estimation of
the cell density of any field in a particular slide preparation. The use of heavier erythrocyte suspensions and the high-dry objective makes possible the scanning of larger microscopic fields, and consequently larger populations of cells are sampled per field.

From the technical point of view it is important to stress that specific fluorescence was achieved with wet, unfixed preparations, and was "all-or-none."

**Summary**

The five rabbit antirhesus monkey erythrocyte specificities of Owen and Anderson\(^1\) have been confirmed. An additional specificity, designated "F," has been found in 91.2 per cent of rhesus animals tested. Antiserum reagents with the A and B reactivities have been used in the indirect fluorescent antibody technic to detect and quantitate minor rhesus erythrocyte populations in admixture with a major population. Specific fluorescence was achieved with wet, unfixed preparations and was "all-or-none." A rapid method for the estimation of minor to major cell population ratios well above 1:1000 is presented.

**Summario in Interlingua**

Le cinque specificitates de conilio anti erythrocytos de rheso describite per Owen e Anderson esseva confirmate. Un specificitate additional, designate como "F", esseva trovate in 91,2 pro cento del rhesos testate. Reagentes a antiserco con le reactivitates A e B esseva usate in le indirecte technica a anticorpore fluorescente pro deteger e quantificar minor populationes erythrocytic de rheso immixte con un population major. Fluorescentia specific esseva obtenite con humide, non-fixate preparatos e esseva o complete o absente. Un rapide metodo pro le estimation de proportiones de minor a major populationes cellular de ben in ultra de 1:1000 es presentate.

**Acknowledgments**

The authors wish to thank Doctors Donald R. Anderson and Ray D. Owen for providing reagents to pretype erythrocyte donors and to verify reagent specificities.

**References**

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