FUSION OF HUMAN RED BLOOD CELL MEMBRANES

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The ability of the parainfluenza group of viruses to induce cell fusion with the production of polykaryocytes and heterokaryons of animal cells has been well documented (1, 2, 3, 4).

Much of the interest in cell fusion in recent years has centered on cell hybrids with nuclei of different species occupying the same cell. Such hybrid cells have proved useful for studies of nuclear-cytoplasmic relationships (5).

The use of fused cells for membrane studies has received less attention. Watkins and Grace (6) observed mixing of surface antigens on heterokaryons made between HeLa cells and Ehrlich ascites tumor cells, using bacteria and erythrocytes as markers. More recently, Frye and Eididin (7) used hybrids of mouse (C11D) and human (VA-2) tissue culture cells to demonstrate by fluorescence an apparent mobility of cell surface antigens.

The fusion of mammalian red cells has not been reported. Since a large amount of data on structure, function, antigenic determinants, etc. are available for the human red cell, the fusion of these cells would be an especially useful tool for membrane studies.

The purpose of this communication is to report the conditions under which fusion of human red cells takes place, with the formation of vesicles of various sizes, composed of as many as 2-50

Figure 1 Vesicles formed by human red-cell fusion, after 40 min of incubation at 37°C with attached Sendai virus. ~2 × 10⁹ red cells were mixed with ~2 × 10⁹ virions in 1 ml of isotonic phosphate-buffered saline and kept at 4°C for 1 hr. The number of single cells (S) has been reduced to 43% of the initial value. After hemolysis, single cells and vesicles are usually spherical. Phase-contrast microscopy. × 800.

Figure 2 After 20 min of incubation at 37°C, an apparent partial incorporation of two cells into a large vesicle is seen at top of figure (arrow). Fusion was arrested by rapid chilling. Phase-contrast microscopy. × 800.
individual red cells, and with volumes as much as several hundred times larger than the volume of a single erythrocyte.

**MATERIALS AND METHODS**

Fresh human erythrocytes (Hyland Blood Bank type O, Rh+ [Hyland Travenol Labs., Inc., Los Angeles, Calif.]) were washed three times with 10 times their own volume of phosphate-buffered saline (PBS, pH 7.4, 300 mosmols). Sendai virus was grown on chick allantoic membrane. The allantoic fluid, after harvesting, was clarified by low-speed centrifugation. The virus was then spun at 30,000 rpm for 1 hr and resuspended at 10 times the original concentration. The hemagglutination titer was measured by mixing 0.5 ml of serial twofold dilutions of the virus with 0.5 ml of 0.8% chicken erythrocytes. Agglutination was assayed visually. A typical batch of virus agglutinated after a 16,000-fold dilution. Assuming the end point for agglutination to be one virion per two red cells, 16,000 HA units corresponds roughly to $10^5$ virions/ml.

For cell fusion, 0.2 ml (~$2 \times 10^{11}$ virions) of virus was mixed with 0.2 ml (~$2 \times 10^9$) of packed red cells, the volume brought to 1 ml with PBS, and the mixture kept at 4°C for 1 hr. The agglutinated red cell suspension was then incubated at 37°-45°C in a water bath. Portions were taken at time intervals and slide-cover slip preparations made, which were examined and photographed through a Reichert phase-contrast microscope.

Time-lapse micrographs were made by allowing fusion to take place in a slide-cover slip preparation mounted on a warm stage. A drop of agglutinated cell suspension was mounted on a cold slide, and a cover slip was sealed in place. Micrographs of selected fields were made at time intervals during fusion.

Sections were prepared for the transmission electron microscope by fixing the incubation mixture with 2% glutaraldehyde in isotonic phosphate buffer. After 1 hr the cells were washed with PBS and fixation was continued with 1% buffered OsO4 for 2 hr. Centrifugation, dehydration, and embedding in Vestopal W (Madame Martin Jaeger, Geneva, Switzerland) was followed by thin sectioning on an LKB Ultratome.

Fused cells were prepared for scanning electron microscopy by glutaraldehyde fixation followed by washing with distilled water and air drying on an SEM specimen holder. The cells were uniformly coated with an evaporated film of gold-palladium to minimize surface charging in the microscope.

**RESULTS AND DISCUSSION**

The sequence of events during incubation of the agglutinated cells was hemolysis and then fusion. Hemolysis was initiated within a few minutes at 37°C, whereas fusion took as long as 45 min to complete at the same temperature. No fusion took place at temperatures less than 37°C; the rate of fusion increased rapidly above 37°C, going to completion within 1 or 2 min at 45°C.

Figs. 1 and 2 are typical micrographs of the vesicles after 40 min of incubation at 37°C. At this time.

### Table I

| $n$  | $(R/r)^2$ | $\text{sd}$ | $N$ |
|------|-----------|---------------|-----|
| 2    | 2.07      | 0.24          | 14  |
| 3    | 3.03      | 0.15          | 22  |
| 4    | 4.06      | 0.21          | 20  |
| 5    | 5.05      | 0.16          | 14  |
| 6    | 6.10      | 0.11          | 3   |
| 7    | 6.85      | —             | 1   |
| 8    | 8.02      | 0.15          | 5   |
| 9    | 9.00      | —             | 1   |
| 10   | 10.2      | —             | 1   |
| 11   | 11.0      | 0.06          | 4   |

$R$, vesicle radius; $r$, single cell radius; $n$, No. of cells fusing to form a vesicle; $N$, No. of vesicles counted; $\text{sd}$, standard deviation.

### Table II

| $t$ | $S$ | $2$ | $3$ | $4$ | $5$ | $6$ | $N$ |
|-----|-----|-----|-----|-----|-----|-----|-----|
| 2   | 100 | 0   | 0   | 0   | 0   | 0   |     |
| 5   | —   |     |     |     |     |     |     |
| 10  | 82.1| 7.2 | 6.8 | 3.9 | —   | —   | 222 |
| 20  | 60.0| 16.0| 10.3| 4.6 | 5.7 | 3.4 | 175 |
| 40  | 43.1| 19.8| 8.6 | 10.4| 7.3 | 5.7 | 228 |

$N$, No. of cells counted; $n$, No. of cells fusing to form a vesicle.
Conversion of clumps of intact cells into clusters of vesicles and single cells (ghosts) by time-lapse photography. Equal volumes of red cell and virus suspensions (virus/cell ratio 100) were mixed on a microscope slide, sealed with cover slip, and left at 4°C for 15 min. Slide heated on microscope stage with infrared lamp. Temperature measured with thermistor. Elapsed time between a and b was 7 min at 42°C. Phase-contrast microscopy. X 500.

The fusion of two cells into doublets is seen at top and bottom of the figure. The intact cells of Fig. 4 a are seen partially hemolyzed in Fig. 4 b after 3 min at 42°C. The two lower cells have started to fuse. Spherical vesicles (n = 2) are seen in Fig. 4 c after 9 min at 42°C. Diameter ratio of doublet to single cell is √2, confirming a two-cell fusion. Phase-contrast microscopy. X 900.
stage the vesicles were usually spherical in shape, deformable, and stable in isotonic buffer.

If it is assumed that membrane areas are additive during fusion, then the number of red cells that fuse to form a vesicle may be determined by measuring the ratio of the vesicle diameter to the single-cell diameter: \( n = \left(\frac{R}{r}\right)^2 \), where \( n \) is the number of cells fusing, \( R \) is the radius of the vesicle, and \( r \) is the radius of an unfused single cell. The single cells were almost always spherical.

A test of the above assumption is summarized in Table I. The good fit of the measured \( n \)'s, using the above relation between \( n, R, \) and \( r \), to the set of integers between 2 and 11, is evidence that membrane area is not lost during fusion. Furthermore, Table I is evidence that the vesicles are not being formed by some process other than cell fusion, since in this case there would be no reason to expect a whole-number ratio for \( (R/r)^2 \).

Table II summarizes the distribution of vesicle sizes as a function of time in a typical experiment. Nearly 60\% of the cells had fused after 40 min at 37°C. It seems reasonable to believe that vesicle-size distribution would be a function of, among other variables, the virus/cell ratio and the diffusion kinetics prevailing in the agglutination stage. The time-lapse micrographs (Figs. 3, 4) are evidence that clusters of intact red cells are directly converted to clusters of vesicles of various sizes. Time and equipment limitations have thus far precluded a more complete analysis by cinematography of the fusion process, on the light microscope level.

Fig. 5 illustrates a typical intercellular bridge at an intermediate stage of development. Close apposition between adjacent membranes apparently precedes the formation of a bridge. Unfolding of the two apposed membranes is the next step in the formation of a spherical vesicle. At lower magnification (Fig. 6) membrane unfolding is nearly complete in the fusion of the two cells shown, and the last step in fusion is a change in shape to spherical. A three-dimensional view (Fig. 7) of the vesicles was obtained by scanning electron microscopy. The role of Sendai virus in bridge formation remains unclear.

To summarize, there are four reasons for believing that a true fusion process is being observed. These are: the reduction with time in the number of remaining single cells; the whole number relationship involving \( (R/r)^2 \); the time
lapse results; and the low-magnification micrographs of thin sections.

According to Okada et al. (8), cell fusion depends on virus attachment to the cell surfaces, with membrane disconnection and reconnection mediated in some unknown manner by the virus envelope. Electron microscope evidence for membrane disconnection and fusion of Sendai viral envelope with the membrane of the human erythrocyte has been reported by Howe and Morgan (9). Hemolysis occurred at the disconnection stage. Fusion was, however, not observed by these workers. Lucy (10) has pointed out that leakage of intracellular material does not normally occur in virus-induced cell fusion, and this fact is hard to reconcile with a membrane disconnection or hole-making mechanism. It is of course possible that the mechanics of cell fusion may vary from one cell type to another.

Although the mature mammalian erythrocyte lacks the intracellular metabolic machinery of those animal cells in which fusion has been demonstrated, it is clear that under certain conditions, membrane repair does take place without the synthesis of new membrane components. The rescaling of the red cell ghost after osmotic hemolysis (11) and the fragmentation and repair known to occur in vivo (12) are examples of this interesting and important property of the mammalian red cell membrane.

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