Segregation into separate rouleaux of erythrocytes from different species

Evidence against the agglomin hypothesis of rouleaux formation

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Erythrocytes from one species were labelled with fluorescein isothiocyanate and mixed with unlabelled erythrocytes from another species. Albumin polymers were added to generate rouleaux. The species of origin of erythrocytes in rouleaux was determined by fluorescence microscopy. Erythrocytes from different species segregated into independent rouleaux. However, fluorescent and non-fluorescent erythrocytes from one individual were mixed randomly in rouleaux. These results confirm, using a novel experimental approach, previous observations of Sewchand & Canham [(1976) *Can. J. Physiol. Pharmacol.* **54**, 437–442]. Since rouleaugenic agents are not species-specific, under the 'agglomin' hypothesis of rouleau formation they would be expected to form bridges between cells from different species. It follows that either the agglomin hypothesis is incorrect, or additional species-specific surface components are involved in the aggregation of agglomin-cross-bridged cells.

Erythrocytes aggregate into rapidly sedimenting 'piles of coins', or rouleaux, when suspended in media containing a rouleaugenic agent (Fahraeus, 1921). Rouleaugenic agents include a variety of higher polymeric forms of dextran (Thorsen & Hint, 1950), vinylpyrrolidone (Sewchand & Canham, 1976), hyaluronic acid (Bychkov & Kuzmina, 1977) and albumin (Forsdyke *et al.*, 1982). In certain diseases serum becomes highly rouleaugenic. This is associated with increases in the concentrations of various high-molecular-weight proteins, especially immunoglobulin M, α_2 -macroglobulin and fibrinogen (Scherer *et al.*, 1975). The phenomenon of rouleau formation provides the opportunity to examine the role of humoral factors in cell–cell interactions in a simple eukaryotic model system.

One hypothesis on the mechanism of rouleau formation is that rouleaugenic agents act as multivalent 'agglomerins', which react with the erythrocyte surface and cross-link cells (Ruhstroth-Bauer, 1961; Chien & Jan, 1973; Brooks *et al.*, 1980). Since rouleaugenic agents are not species-specific, the agglomin hypothesis predicts that they should form bridges between cells from different animal species. Addition of rouleaugenic agents to mixtures of erythrocytes from two different species should result in the formation of individual rouleaux containing erythrocytes from both species.

An alternative hypothesis has been derived from a consideration of the phase separation of high-molecular-weight polymers from mixtures of polymer solutions (Forsdyke & Ford, 1983). At high polymer concentrations similar polymer molecules can associate to form an independent phase. Considering the cell surface as resembling that of a large polymer, it is postulated that there should be a separation of a cell phase from the solution phase in the presence of high concentrations of a high-molecular-weight polymer. Under this scheme the process of cell aggregation has two components: (i) an extrusion of cells from the polymer phase (Bychkov & Kuzmina, 1977), so that cells associate more rapidly than predicted on the basis of Brownian motion (Rowlands *et al.*, 1982); and (ii) an attraction of similar cells on the basis of similarities in their polymer-like surfaces (Edwards, 1978). The phase-separation hypothesis predicts that there is no necessary reaction of rouleaugenic agents with the erythrocyte surface. Furthermore, addition of rouleaugenic agents to mixtures of erythrocytes from different sources should result in the segregation into independent rouleaux of erythrocytes with similar surfaces.

Evidence favouring the phase-separation hypothesis rather than the agglomin hypothesis was first provided by Sewchand & Canham (1976). They showed significantly more aggregation of like erythrocytes in mixtures of erythrocytes from

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different animal species. Careful size measurements were used to distinguish the cells of different species. In view of the importance of the result, we repeated the work using labelling with fluorescein isothiocyanate to distinguish erythrocytes.

Methods

Details of materials and methods are presented elsewhere (Forsdyke *et al.*, 1982). Since the studies of Sewchand & Canham (1976) were carried out with a variety of species combinations, we felt it would be adequate to use only one species combination (rabbit/human).

Labelling of erythrocytes with fluorescein isothiocyanate

Freshly collected washed erythrocytes (packed volume approx. 1 ml) were incubated at 37°C for 20 min in an equal volume of phosphate-buffered saline (5 mM-potassium phosphate/0.14 M-NaCl, pH 7.4) containing fluorescein isothiocyanate (final concn. 50 µg/ml; Butcher & Weissman, 1980). To wash the cells free of unattached dye, the mixture was diluted with 5 ml of phosphate-buffered saline at room temperature and centrifuged for 10 min at approx. 1200 g. The red cells were then resuspended in 20 ml of phosphate-buffered saline and three samples of 7 ml were each carefully layered over 1 ml of autologous serum. After centrifugation the red-cell pellets were pooled in 5 ml of phosphate-buffered saline, centrifuged again and finally suspended in a volume of phosphate-buffered saline equal to the packed cell volume. Control erythrocytes were subjected to the same preparative procedures, except that fluorescein isothiocyanate was omitted. In the case of human erythrocytes the blood group was usually group A, Rhesus-positive.

Rouleau formation

Defatted bovine serum albumin (40 mg/ml in phosphate-buffered saline, pH 7.4) was preheated at 68°C for 20 min to generate polymers (Forsdyke *et al.*, 1982; Takeda *et al.*, 1982). This is a different rouleaugenic agent from that employed by Sewchand & Canham (1976). To initiate rouleau formation, 0.3 ml of albumin polymers was added to a mixture of 0.1 ml of human erythrocytes (50% haematocrit) and 0.1 ml of rabbit erythrocytes (50% haematocrit). The erythrocytes of one of these species had been fluorescein-labelled. After 30 min at room temperature, 20 µl samples were placed under 22 mm-square cover slips for examination by fluorescence microscopy.

Results

Fig. 1 shows the result of mixing equal quantities of labelled and unlabelled erythrocytes from one

donor in the presence of a rouleaugenic agent, polymeric albumin. Fluorescent and non-fluorescent cells appeared to aggregate randomly. The fluorescein label did not appear to affect rouleau formation. Counts of the percentage of fluorescent cells in rouleau lengths of about ten erythrocytes showed a unimodal distribution, with the average rouleau containing approx. 50% fluorescent cells and 50% non-fluorescent cells (Figs. 2a and 2b). However, when fluorescent cells from one species (human) were mixed with non-fluorescent cells from another species (rabbit), the distribution became bimodal (Fig. 2c). This indicated that the fluorescent cells were preferentially associating with each other and that the non-fluorescent cells were preferentially associating with each other. Similar results were obtained when fluorescent rabbit cells were mixed with non-fluorescent human cells (Fig. 2d). The experiment shown in Fig. 2 was performed on two occasions with different preparations of rabbit and human cells. We also carried out one experiment with cells from two human donors, one of blood-group A, Rhesus-negative and one of blood-group B, Rhesus-positive. In this case a unimodal distribution was observed.

Discussion

Our results confirm the observation of Sewchand & Canham (1976) that, in the presence of rouleaugenic agents, cells from different species are less likely to aggregate with each other than with cells from the same species. Since the rouleaugenic agents are not species-specific, under the agglomerin hypothesis they would be expected to form bridges between cells from different species. It follows that either the agglomerin hypothesis is incorrect or additional species-specific surface components are involved in the aggregation of agglomerin cross-bridged cells.

The data are consistent with the phase-separation hypothesis which predicts that, in the environment created by the rouleaugenic agent, cells with similar surface components will form more stable aggregates than cells with dissimilar surface components (Forsdyke & Ford, 1983). Studies of the aggregation of non-erythroid cells by polymers are also consistent with the phase-separation hypothesis. Morris (1979) reported that such aggregation is directly proportional to polymer size and is not accompanied by appreciable binding of polymer to cells.

In the present work we have considered the separation, within one polymer phase, of like erythrocytes from mixtures of the erythrocytes of two animal species. In the presence of two polymer phases, conditions can be arranged such that the erythrocytes of one species will partition in one phase and the erythrocytes of the other species will

Fig. 1. *Random aggregation into rouleaux of fluorescent and non-fluorescent erythrocytes from a single donor*
Freshly collected, washed human erythrocytes were labelled with fluorescein isothiocyanate and then mixed with an equal quantity of unlabelled erythrocytes. To initiate rouleau formation, defatted bovine serum albumin, which had been preheated at 68°C for 20 min to generate polymers, was added (25 mg/ml). Samples were examined (a) under normal light and (b) at an exciting wavelength for fluorescence microscopy.

Fig. 2. *Non-random distribution among rouleaux of fluorescent erythrocytes from one species when mixed with an equal quantity of non-fluorescent erythrocytes from another species*

'+F' indicates erythrocytes labelled with fluorescein isothiocyanate. Thus, in (a), 'human/human+F' indicates that human cells from one donor were mixed with an equal quantity of fluorescein-labelled erythrocytes from the same human donor. A rouleau length of approx. ten cells was first identified under normal light and then the cells were examined at an exciting wavelength to determine the proportion which were fluorescein-labelled. Rouleaux were grouped into sets corresponding to 0–10% fluorescent cells, 11–20% fluorescent cells, 21–30% fluorescent cells, etc. A total of ten rouleaux were examined in each case.

partition in the other phase (Fisher, 1981). It is possible that this partitioning is facilitated by an initial formation of independent rouleaux.

Our method of detecting the segregation of erythrocytes with similar surfaces should be applicable to a variety of studies on cell and molecular interactions. In particular, it would be of interest to examine the effect of chemical or enzymic changes of the erythrocyte surface on the ability of erythrocyte populations to segregate independently.

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