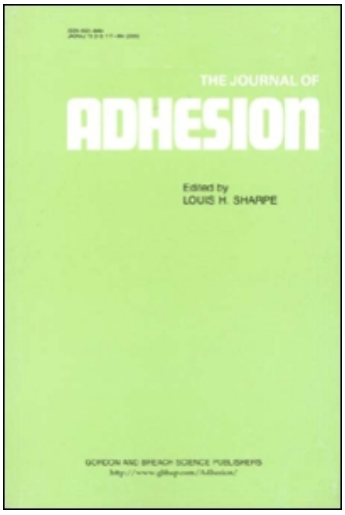

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Cell—Cell Adhesion of Erythrocytes

F. R. Attenborough[a]; K. Kendall[a]
[a] Birchall Centre, Keele University, Keele, UK

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Cell–Cell Adhesion of Erythrocytes

F. R. ATTENBOROUGH and K. KENDALL*

Birchall Centre, Keele University, Keele, Staffs UK ST5 5BG

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Three different species of red blood cells have been tested by a new image analysis method to determine cell–cell adhesion with a high level of precision. Various literature sources have suggested that horse and rat erythrocytes adhere to form aggregates more readily than human red cells but this paper provides the first accurate quantification of this phenomenon. Addition of surface active agents such as glutaraldehyde, papain and fibronectin was also tested in order to measure the effects on cell-to-cell adhesion. Glutaraldehyde reduced adhesion whereas both papain and fibronectin increased it.

Keywords: Erythrocyte; Adhesion measurement; Statistical mechanics; Aggregation

1. INTRODUCTION

Several measurement methods for determining cell adhesion have been reported over the years. Micro-pipette techniques [1, 2] gave the most direct observations, the cells being sucked onto glass probes and then pushed into contact to obtain deformation and adhesion results. The atomic force microscope has been employed more recently [3–5] to give a direct contact and measurement on the cell membrane. This has the distinct advantage that the probe can be located with nanometer precision in order to find patchy adhesion across the membrane surface. An innovation over the past three years has been the use of laser tweezers [6, 7]. In this test, a cell was held in a laser beam and brought into contact with another cell, also held by a laser. Very fine nano-manipulation of the ces was possible in this method, although

*Corresponding author. Tel: +44(01782) 548071, Fax: +44(01782) 715944, e-mail: k.kendall@chem.keele.ac.uk

the adhesion measurements were not simple to interpret. It was claimed that single macromolecular bonds could be detected, in principle, by this method. A similar claim was made by Bongrand [8, 9] for his cell flow device. He observed single cells moving over a surface in a controlled shear flow. The periodic stopping of the cell at the wall was said to be a measure of single molecule adhesion.

In 1998, a more interesting method of obtaining cell adhesion was reported, requiring no apparatus to produce the adhesion events [10]. The basis of this new idea was statistical mechanics. According to this theory, cells should move randomly with Brownian motion and should attach to neighbouring cells stochastically as random collisions occur. Doublets of cells are then produced, surviving for a certain time before Brownian collisions of sufficient force disrupt the adhesive bond between the cells. The number of cell doublets at equilibrium should, therefore, be a direct measure of cell adhesion. A high number of doublets indicates strong adhesion, whereas no doublets signify zero adhesion. This theory was verified by measuring human red cell dispersions by both optical and Coulter Counter methods [10–12]. An important feature emerging from these results was the dependence of the doublet numbers on the concentration. More doublets form at higher concentrations even though the adhesion remains constant. Therefore, it is essential in comparing cell adhesion to work at constant, known volume fraction of cells.

The purpose of this paper is to extend the studies above to compare the cell–cell adhesion of human erythrocytes with that of horse red cells, and that of other species. Various reports have suggested that horse red cells are more adherent to each other than human cells, but such observations have not been quantified [13, 14]. This paper provides the first precise measurements of horse red-cell-to-red-cell adhesion by the new Brownian method and also includes rat erythrocytes for comparison. The measurements have been interpreted using the theory outlined below.

2. THEORY

Consider a dilute dispersion of uniform spherical particles as shown in Figure 1.

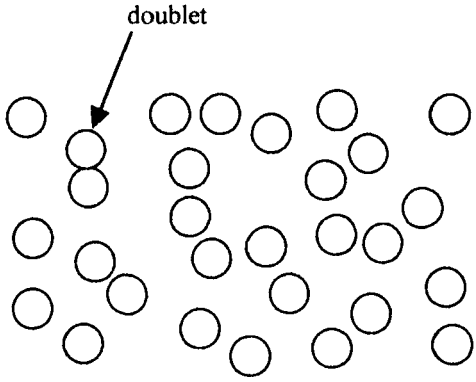

FIGURE 1 Dispersion of spherical particles.

These spheres experience Brownian motion and, therefore, diffuse in all directions, causing collisions between the particles. If there is adhesion between the particles, then a collision has a chance of creating a doublet; that is, two particles adhering together at the point of contact. If the adhesive bond is weaker than kT, then thermal collisions can break this bond in a period of time. The spheres will then separate and move apart. Thus, there is a dynamic equilibrium between joining and separation, giving a certain number of doublets in the suspension at equilibrium, after a suitable time has elapsed for diffusion to take place. High adhesion should give a larger number of doublets and lower adhesion a smaller number. Hence, there is a definite connection between sphere adhesion and the equilibrium number of doublets observed in a dilute suspension.

Of course, there are several assumptions in this argument. The main premise is that the spheres are all identical. This is not true of red cells which are known to have distributions of various molecular species on their surfaces. However, it is possible, in principle, to filter out any rogue doublets formed by unusually tacky cells. Equilibrium can then be re-established. Repeating this filtering and equilibration procedure several times should lead to a point where the remaining cells are more nearly equal.

A second assumption is that the cells are spherical and equal in diameter. In fact, human red cells are dimpled and range in size between 6 and 8 μm. The errors in the argument caused by such

problems are not yet known but are being investigated by computer modeling [15].

The most interesting consequence of the above idea, that red cell adhesion may be measured by observing the number of doublets at equilibrium in a dilute suspension, is that an exact mathematical solution can be found under certain circumstances, depending on the interaction between spheres when they collide. The simplest situation is that shown in Figure 2, where a particle approaches its neighbour at constant speed until, at a certain separation, the particles are attracted to each other with an energy, ε. If this energy remains constant until the spheres touch rigidly at the point of contact, then the square well potential is revealed. The approaching sphere travels at constant speed, is accelerated into the potential well, reflects rigidly on contact, and then is decelerated as the particles move apart. This "hard sphere square well", which was first used by Alder and Wainwright [16] in 1961, can be solved exactly to predict the number of doublets in a suspension.

The mathematical result is that the ratio of doublets to singlets, N_2/N_1, is proportional to the volume fraction, ϕ, of the cells and depends on the range, λ, and the energy, ε, of the well according to the

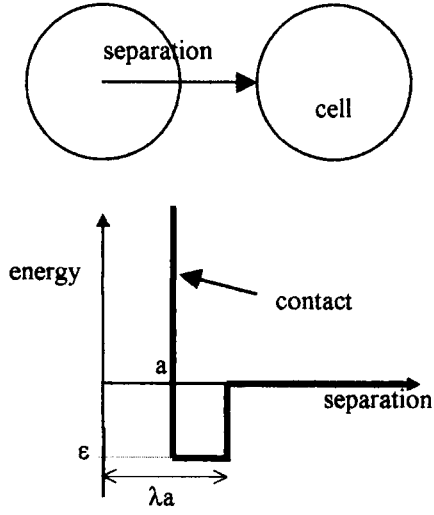

FIGURE 2 Interaction energy between approaching spheres.

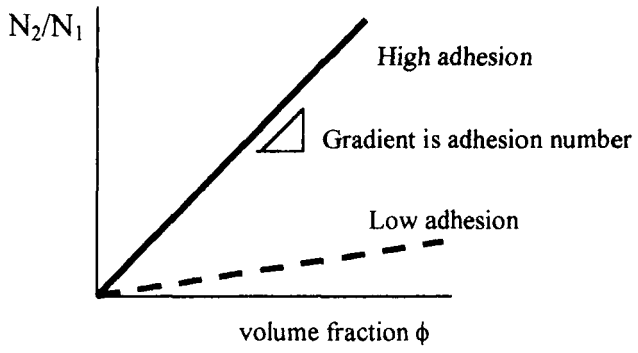

FIGURE 3 Defining the adhesion number for cell – cell interaction.

equation below.

$$NN_2/N_1^2 = 4\phi(\lambda^3 - 1)\exp(\varepsilon/\mathrm{kT}) \approx N_2/N_1 \qquad (1)$$

The conclusion of this argument is that a plot of doublet-to-singlet ratio *versus* particle volume fraction should yield a straight line passing through the origin. The gradient of the line is a measure of the adhesion which depends on range and energy of the interactions. Thus, a high gradient signifies high adhesion and a low gradient low adhesion as shown below in Figure 3. Thus, an adhesion number can be defined as the gradient of this plot, to give a measure of the bonding of the cells. The experimental objective of this paper is to define this non-dimensional adhesion number for three different species of red cells, horse, rat and human.

3. EXPERIMENTAL MEASUREMENT AND RESULTS

Blood cells were prepared from three species, human blood from North Staffordshire Hospital, fresh horse blood in EDTA and fresh rat blood from Central Animal Pathology Ltd. Each blood sample was washed six to seven times in phosphate-buffered saline to remove the non-red-cell components, before suspending in physiological saline solution, then examined by both optical and Coulter tests. Tests were carried out immediately after washing since the cells degraded with

time. Each species of cell was treated in three ways to judge the effect of surface adhesion molecules; by adding glutaraldehyde, fibronectin and papain.

The optical apparatus is shown in Figure 4. The cells were placed in an accurately defined 10 μm space within a glass chamber which was imaged using a video microscope at 40x magnification. Each cell could then be clearly seen moving around with Brownian movement, while not overheating as occurred at 100x magnification. Pictures of the cells were taken at random locations in the chamber and the numbers of doublets and singlets were counted by the image analysis software Sigmascan Pro [17]. By taking the ratio of doublets to singlets, the adhesion number was obtained. Cells were observed over a period of 30 minutes. They did not stick significantly to the walls of the glass cell. The assumption was made that the cells were moving randomly in three dimensions.

The collision and adhesion events could be observed in experiments as shown in Figure 5, which shows one field of view seen 30 seconds apart. Three doublets remained unchanged in this period, two broke up, while four new doublets and one new triplet were formed. The dynamic equilibrium could, thus, be evaluated.

The second set of experiments to measure the doublet numbers used the Coulter Counter, which was set up in standard mode to count the individual red cells, as shown by the results of Figure 6a. The strong peak showed a symmetrical distribution of single cells at a volume fraction near 10^{-5}.

At higher concentration, a shoulder appeared at a 13% higher diameter, and this was interpreted as a doublet peak. At still higher

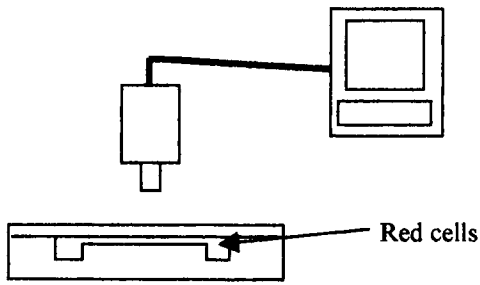

FIGURE 4 Video camera apparatus for observing red cells.

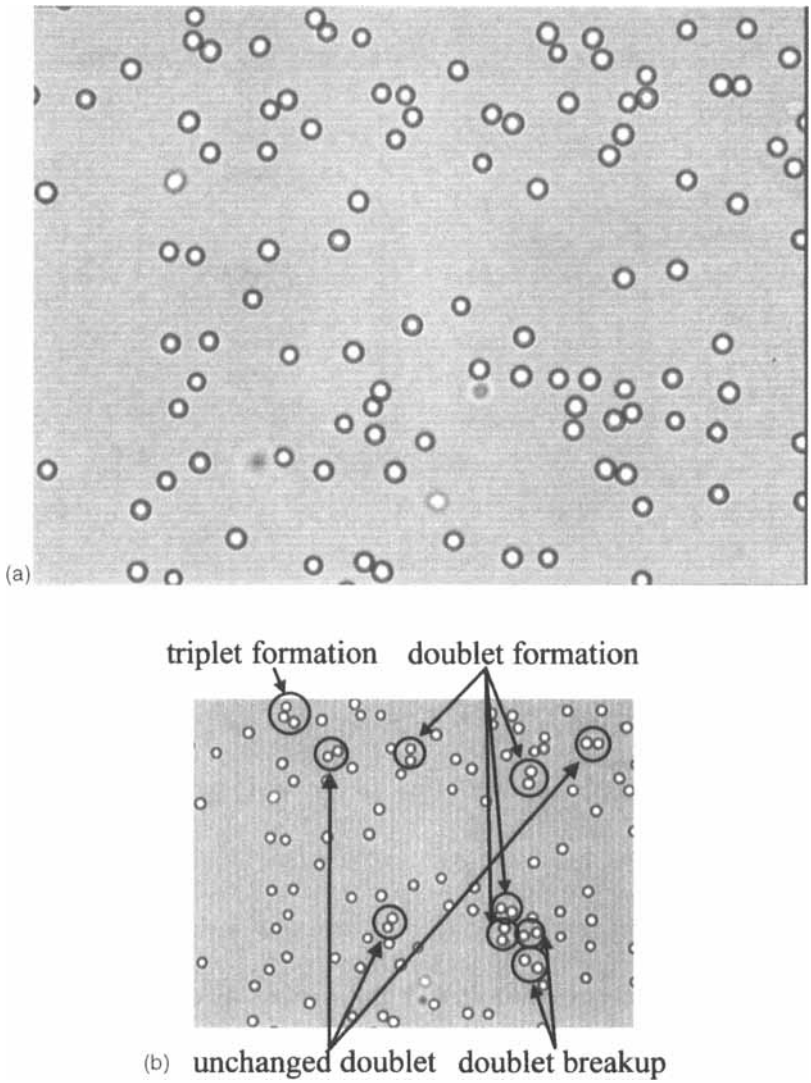

FIGURE 5 Images of horse red cells in Isoton solution; (a) time zero; (b) 30 seconds later.

concentration of the red cells, the shoulder increased in size, indicating that more doublets formed as the blood cells became more numerous. The number of doublets was measured and divided by the singlet peak

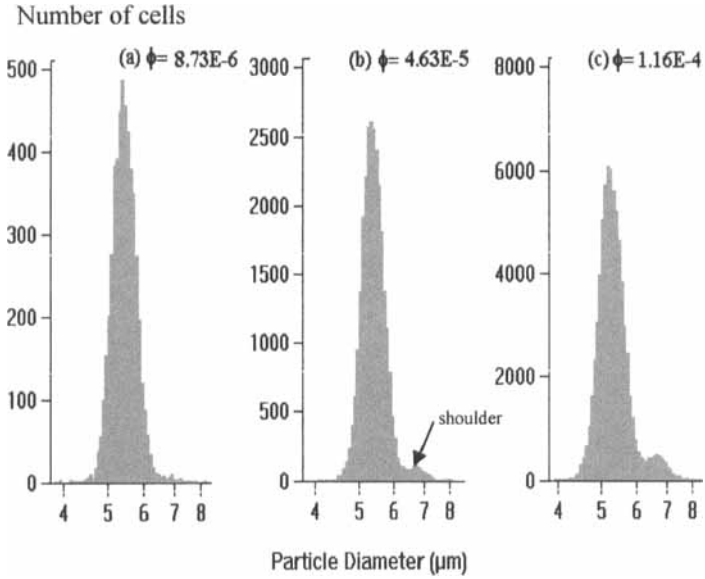

FIGURE 6 (a) Coulter counter results for human red cells; (b) Result at higher concentration showing shoulder; (c) Larger shoulder at higher concentration.

to obtain the ratio N_2/N_1. This was then plotted as a function of cell volume fraction to give the curve shown in Figure 7. The results showed the doublets increasing in proportion to concentration and allowing the adhesion number to be found by determining the gradient. For human cells this was 420.

Horse and rat erythrocytes were then tested in the same way and shown to give significantly higher adhesion. Baskurt *et al.* [13] have shown that the aggregation of such cells is increased over human cells, but volume fraction effects were not taken into account. Popel *et al.* [14] recognised that horse cells stick better and this was attributed to the athletic nature of the animal. Table I quantifies the difference of adhesion in terms of the adhesion number $N_2/N_1\phi$.

These results show conclusively that rat cells are almost twice as sticky as human red cells, while horse erythrocytes are almost twice as adhesive as rat cells. Whether this can be explained in terms of the higher energy of the bonds, as defined by Eq. (1), or in larger range of bonds remains to be determined.

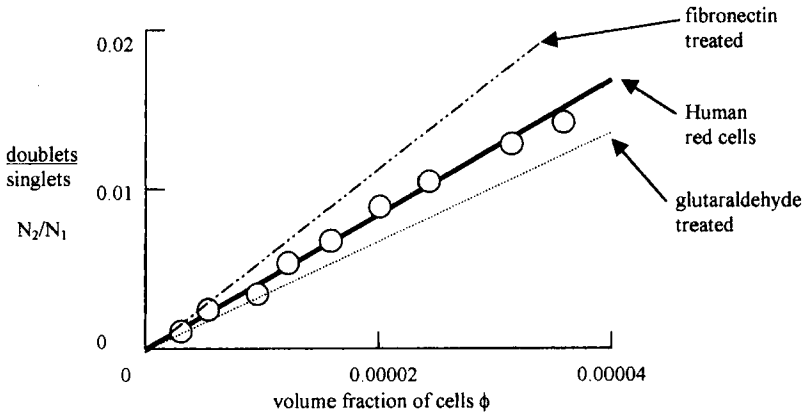

FIGURE 7 Increase in doublets with higher concentration of human red cells.

TABLE I Comparison between adhesion of various red cells

Animal	*Adhesion number* $N_2/N_1\phi$
Horse	1488 ± 200
Rat	750 ± 4
Human	420 ± 5

TABLE II Effect of surfactants on horse red cell adhesion

Horse cell treatment	*Adhesion number* $N_2/N_1\phi$
Isoton	1279 ± 203
Isoton+glutaraldehyde	1020 ± 162
Isoton+fibronectin	1399 ± 184
Isoton+papain	1513 ± 295

Addition of surface active molecules to the cell suspension was also studied. The results for human cells are illustrated in Figure 7 which shows that fibronectin increased the adhesion whereas glutaraldehyde reduced it. The effect of surfactants on horse erythrocytes is shown in Table II.

The control sample of horse cells in Isoton (Coulter Electronics, Luton, UK) showed somewhat weaker adhesion than the sample shown in Table I. Such variation was found to be common in different samples of horse blood. Differences between animals in type, age, *etc.*, and also in bod cell conditioning, had a distinct influence which will be

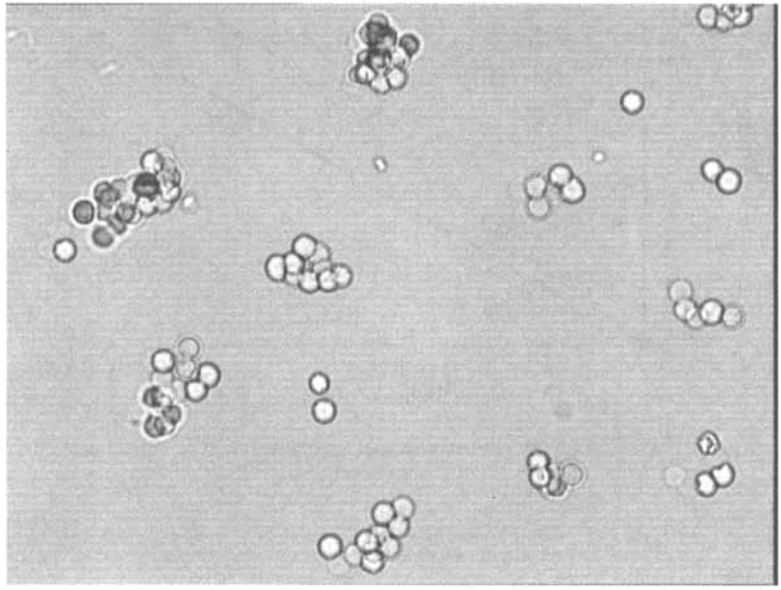

FIGURE 8 Large aggregates of horse red cells after soaking in papain.

described in separate papers. It is evident from the results that glutaraldehyde reduced the adhesion by about 25%, whereas fibronectin increased the adhesion by 10% and papain by 20%, changes which were comparable with the effects seen on human red cells but disappointingly small compared with the effects anticipated.

When the red cells were left in papain at high concentration, much larger aggregates of cells were observed, as shown in Figure 8. It is clear that adhesion has been changed substantially in this experiment. The large aggregates were also not dispersible on dilution with saline solution in this case, suggesting that the reversibility of the adhesion process had been compromised. Under such irreversible conditions, the equilibrium theory of Eq. (1) is no longer valid.

4. CONCLUSIONS

The adhesion of red blood cells has been tested by both optical analysis and by Coulter techniques. In particular, the differences between

horse, rat and human cell – cell adhesion have been quantified with improved precision. Horse red cells were almost twice as adhesive as rat erythrocytes and almost four times as adhesive as human cells, under the conditions used in the experiments. Addition of surface active molecules has also been tested. Glutaraldehyde treatment decreased the adhesion significantly, whereas fibronectin and papain increased adhesion substantially, causing strong aggregation and irreversibility at high concentration.

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