FUSION OF INTACT HUMAN ERYTHROCYTES AND ERYTHROCYTE GHOSTS

HAVA PERETZ, ZIVIA TOISTER, YEHUDITH LASTER, and ABRAHAM LOYTER

From the Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

ABSTRACT

Sendai virus is able to induce the fusion of human erythrocytes. Bivalent cations or ATP are not essential for polyerythrocyte formation. High fusion indices were obtained when Sendai virus was added to cells incubated in the presence of both EDTA and iodoacetic acid. Human erythrocyte ghosts prepared by gradual hemolysis still retain the potential to undergo virus-induced fusion. Fusion of human red blood cells without the addition of viruses was obtained by incubation of erythrocytes at pH 10.5 in the presence of Ca^{++} (40 mM) or by addition of phospholipase C Clostridium perfringens preparations to cells previously agglutinated or polylysine.

INTRODUCTION

Virus-induced fusion of mammalian cells is a well-known phenomenon which has been studied in recent years by Okada (1) and Kohn (2) in particular, as well as in our laboratory (3). Okada showed that metabolic energy in the form of ATP, regenerated by oxidative phosphorylation, is essential to the fusion process (4). Using Sendai virus, Ehrlich ascites tumor cell systems, we have recently observed that fusion can also be driven by ATP generated via glycolysis (3). ATP is probably required in order to reduce virus-induced lysis and hence promote fusion. Furthermore, it was shown that high concentrations of bivalent cations such as Ca^{++} or Mn^{++} can partially substitute for internal ATP in the fusion systems, since they are also able to inhibit lysis caused by the viruses (3).

Poole et al. (5) succeeded in inducing fusion of chicken erythrocytes in the absence of virus by the addition of large amounts of lysolecithin to cells incubated at pH 5.4. Recently we also achieved the fusion of chicken erythrocytes, with or without the addition of virus (6–8). In the presence of bivalent cations such as Ca^{++} or Mn^{++}, virus-induced hemolysis was reduced and homopolykaryons were formed. The fusion of chicken erythrocytes, without any added virus, was obtained at pH 10.5 in the presence of Ca^{++} (7) or by the addition of phospholipase C Clostridium perfringens preparations to cells at pH 7.4 (8).

The human erythrocyte is virtually a sac which encloses soluble proteins. It is devoid of any membrane-bounded organelles such as nuclei or mitochondria, which are still retained in chicken erythrocytes (9). Its energy, in the form of ATP, is generated solely from glycolysis. The fusion of human erythrocytes is therefore of special import in the understanding of the fusion mechanism and in the clarification of the molecular changes which occur during the process. Lately, virus-induced fusion of human red blood cells has been observed in several laboratories (10–12) as well as in our own (13). Its characteristics, however, have not yet been studied. It seems to us that the fusion of human erythrocytes might well serve as a suitable

system in the study of several basic points related to the mechanism of membrane fusion.

The purpose of the present work was to study the correlation between ATP, bivalent metals, and the fusion reaction, using human erythrocytes as the fusion system. Furthermore, pure membranous vesicles, ghosts, could be prepared by osmotic shock of human erythrocytes. It was thus interesting to check whether these ghosts could still be fused by the virus, for the fusion of ghosts might help to resolve the question of whether or not intracellular components are required for the fusion reaction. In this work an attempt was made to fuse human erythrocytes without the addition of virus, either by high concentrations of Ca^{++} ions or by phospholipase C C. perfringens preparations. An abstract of the work has appeared elsewhere (13).

MATERIALS AND METHODS

Virus

A strain of Sendai virus, one of the parainfluenza group I, was used. The virus was propagated in the allantoic cavity of a 10-day old chicken embryo and was purified essentially as described previously (3). The washing medium, however, was buffered saline (see medium A), to which 2 mM EDTA was added in the second wash.

The hemagglutinin titer of the virus was determined by Salk's pattern method with 0.5 ml of 0.5% (vol/vol) chicken red blood cells in phosphate-buffered saline at pH 7.0.

Cells

Human blood, type O, was obtained from the Blood Bank of the Hadassah Hospital, Jerusalem. Blood aged for 4–8 wk was used in most of the experiments (stored blood). Fresh blood, type O, obtained from volunteers by venipuncture, was used in some experiments. The blood was washed three times with the buffered saline (medium A). The buffy layer containing the white cells was discarded, and the pellet was suspended in medium A to give a concentration of 5% (vol/vol). If not otherwise stated, stored red blood cells were used. Phospholipase C C. perfringens preparations were obtained from Worthington Biochemical Corp., Freehold, N. J. and polylysine (degree of polymerization = 24) from Miles-Yeda, Rehovoth, Israel.

Buffered Salt Solution

MEDIUM A: The medium used in the present work for the suspension of the virus and cells and for the fusion process contained: NaCl (160 mM) buffered with 20 mM Tricine-NaOH, pH 7.4 (buffered saline). Bivalent cations

were added to the medium for the fusion reactions and for the inhibition of hemolysis.

MEDIUM B: A high KCl medium for nonviral fusion at pH 10.5 contained KCl (135 mM), NaCl (5.4 mM), $MgSO_4$ (0.8 mM), and was buffered with 20 mM glycine-NaOH, pH 10.5.

Virus-Induced Fusion and Hemolysis of Human Erythrocytes

The fusion of human red blood cells was performed essentially as described previously in the case of chicken erythrocytes (6, 14). Aliquots of 0.5 ml of 5% (vol/vol) suspension of either intact human red blood cells or ghosts were added to 0.5 ml of medium which contained bivalent cations and virus. The resulting suspension was incubated in 20-ml glass flasks for 10 min at 4°C to allow agglutination, and was then incubated at 37°C in a rotating bath at 135 rpm. The suspension was then cooled at 4°C and 4 vol of cold medium were added. The data of the present investigation were obtained with an infective Sendai virus. However, a virus inactivated by ultraviolet light was almost as efficient as the infective one in fusing human red blood cells.[1]

The equation employed for the calculation of the fusion index was the one described by Okada (4).

Fusion index
$$= \frac{\text{Cell number in control tube without virus}}{\substack{\text{Cell number (mono- or polycells) in} \\ \text{experimental tube with virus}}} - 1.$$

During the present work some variations in the fusion were noted under identical conditions but in different experiments. This was probably due to differences in the fusion activity of different batches of virus. Nevertheless, the relative fusion indices under different conditions were reproducible from experiment to experiment. The data reported in this work represent one experiment in each case, although two to three experiments were performed under identical conditions. Fusion in the present work was very effective, and high fusion indices were obtained. Therefore, even large variations in the fusion index reflect only small changes in the number of the fused cells.

Hemolysis was determined essentially as previously described (6), by measuring the supernate's absorbance of the fusion reaction at 540 nm. Complete hemolysis was obtained by suspending 0.5 ml of 5% (vol/vol) human erythrocytes in 4.5 ml of distilled water to which 1 drop of concentrated ammonia was added.

Ca^{++}-Induced Fusion at pH 10.5

Fusion was performed essentially as described previously in the case of chicken erythrocytes (7). Stored red

---

[1] N. Zakai, A. Loyter, and R. G. Kulka, unpublished results.

blood cells were washed three times at room temperature in a high KCl medium (medium B) and buffered with 40 mM glycine-NaOH buffer at pH 10.5. The final pellet was suspended in the above medium to give a concentration of 5% (vol/vol). The cell suspension was incubated, unless otherwise stated, for 60 min at 37°C with gentle shaking. Afterwards the cells were cooled to 4°C and, after adding Ca^{++} or other bivalent cations, they were kept in the cold for 20 min to allow for agglutination. The cells were then incubated at 37°C with gentle shaking, and fusion was obtained within 10–40 min of incubation.

Fusion and Hemolysis Induced by Phospholipase C

C. perfringens Preparations

The conditions for fusion and hemolysis of human erythrocytes by phospholipase C were essentially the same as those previously described for chicken erythrocytes (8). Polylysine of degree of polymerization 24 (mol wt = 5,010) was used for the agglutination of cells, and 7.5 μg/ml of phospholipase C C. perfringens for the inducing of fusion. Hemolysis was determined on the supernate after the centrifugation of the fused cells.

Gradual Hemolysis (Preparation of Ghosts for the Fusion Reaction)

The method developed by Danon et al. (15) was modified as follows. Washed human erythrocytes, 2.5% (vol/vol), were dialyzed against 10 vol of 40 mM NaCl buffered with 10 mM Tricine-NaOH pH 7.4. After 2 h of dialysis at room temperature (20°–21°C), the cells were removed from the dialysis tubes, and 4 vol of medium A (buffered saline) were added to each volume of the cell suspension in order to reseal the ghosts. These were then collected by centrifugation at 12,000 g for 15 min in the cold. The extent of hemolysis was calculated from the difference in the amount of hemoglobin in the supernate and the pellet of the dialyzed cells.

Preparation of ATP-Depleted Cells

Washed fresh human erythrocytes (2.5% vol/vol) suspended in medium A were incubated in the presence of NaF 20 mM for 10 h at 37°C. At the end of the incubation period the cells were washed four times with the above medium, and the final pellet was suspended in the same medium to give either a 30% (vol/vol) concentration for ATP and protein determination, or a 5% (vol/vol) concentration for fusion experiments.

Restoration of ATP

ATP was restored essentially as described by Nakao (16). The packed pellet of washed, ATP-depleted cells was suspended to give 2.5% (vol/vol) in medium A, to which 5 mM glucose, 10 mM adenine, 2 mM inosine, and 5 mM sodium phosphate (pH 7.4) were added. The

suspension was incubated for 8 h at 37°C, and the cells were then washed four times in the buffered saline and resuspended for determination of ATP, protein, and fusion as described above.

Preparation of Sections for Electron Microscopy

Samples of fused cells were fixed with 4% glutaraldehyde and 2% OsO_4, embedded in Epon, and stained with uranyl acetate and lead citrate as described before (3). Thin sections were cut on an LKB Ultrotome III (LKB Instruments, Inc., Rockville, Md.) and viewed in a Philips 300 electron microscope.

Assays

Protein was determined in the presence of 0.1% sodium dodecyl sulfate according to Lowry et al. (17). ATP was determined by the luciferin luciferase method (18) as previously described (19), and hemoglobin according to Drabkin (20). Tricine was obtained from Calbiochem, San Diego, Calif. All other chemicals used were of the purest commercial grade available.

RESULTS

Inhibition of Virus-Induced Hemolysis by Bivalent Cations

Mn^{++} (5–20 mM) and UO_2^{++} (0.1–0.5 mM) were found to have a potent protective effect against virus-induced hemolysis of human erythrocytes (Fig. 1). When these two metals were added separately to either fresh or stored erythrocytes under fusion conditions, large polyerythrocytes containing hemoglobin were formed (Figs. 2 and 3). Ca^{++}, which inhibits the hemolysis of chicken erythrocytes (14), had almost no effect on the virus-induced hemolysis of human erythrocytes (Fig. 1). In experiments where Ca^{++} was substituted for Mn^{++} or UO_2^{++} in the fusion medium, extensive lysis took place and only very few polyerythrocyte ghosts could be detected. Most of the cells lysed and formed single ghosts without any fusion.

The dependency of the fusion index on virus concentration in the presence of UO_2^{++}, in both fresh and stored cells, is demonstrated in Fig. 4. Fusion indices as high as 5–7 were obtained even with stored blood cells which contained practically no internal ATP (16).

Fig. 5 shows that the fusion reactions were found to be optimal at pH 6.0–8.0, whereas hemolysis increased slightly between pH 7.0–10.0. No clear
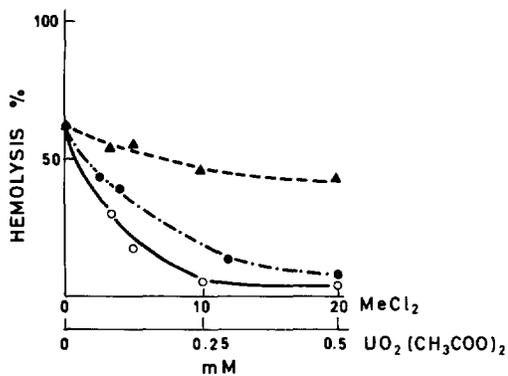
FIGURE 1 Effect of Ca^{++}, Mn^{++}, and UO_2^{++} on virus-induced hemolysis. The hemolytic system contained 1.8% (vol/vol) of washed erythrocytes, 200 hemagglutinating units of virus, and different cations all suspended in 3 ml of buffered saline. After 10 min in the cold to allow agglutination, the various systems were incubated with shaking at 37°C for 30 min. Hemolysis was determined as described in Materials and Methods. ▲, Ca^{++}; ●, Mn^{++}; O, UO_2^{++}.

correlation between the above two reactions can be seen.

Fusion in the Absence of Both ATP and Bivalent Cations

Okada has shown fusion to be dependent on both ATP and bivalent metals (1). The results presented in Table I demonstrate that the fusion of human erythrocytes can occur in the absence of internal ATP. The cells used for exp. 1 (Table I) were practically devoid of internal ATP, as they were stored in the cold for 7 wk. Nevertheless, in order to deplete them of any residual ATP they were further incubated for 3 h at 37°C in the presence of 2.5 mM iodoacetic acid. As can be seen in exp. 2, no significant differences were observed in the fusion index between fresh cells, ATP-depleted cells, and erythrocytes in which ATP was restored.

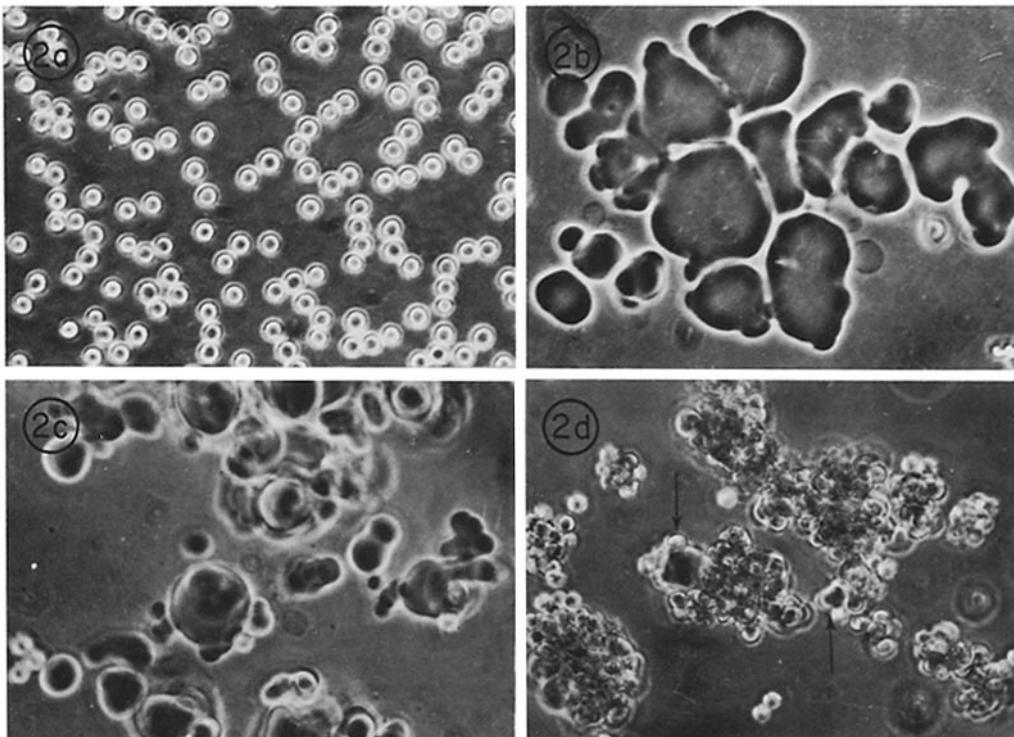Polyerythrocytes were also formed in the ab-

FIGURE 2 Phase micrographs of polyerythrocytes obtained from human red blood cells. (a) Control of human erythrocytes incubated in the presence of 0.25 mM uranyl acetate at 37°C during 25 min in the absence of virus. (b) Polyerythrocytes obtained after 25 min incubation of red blood cells in the presence of 0.25 mM uranyl acetate and 1,000 hemagglutinating units of Sendai virus. (c) Polyerythrocytes obtained with 1,000 hemagglutinating units of Sendai virus in the presence of 5 mM Mn^{++}. (d) Agglutination of human erythrocytes and polyerythrocytes obtained by incubation of cells for 30 min at pH 10.5 in the presence of 40 mM Ca^{++}. The arrows show areas in which fusion was initiated. × 560.
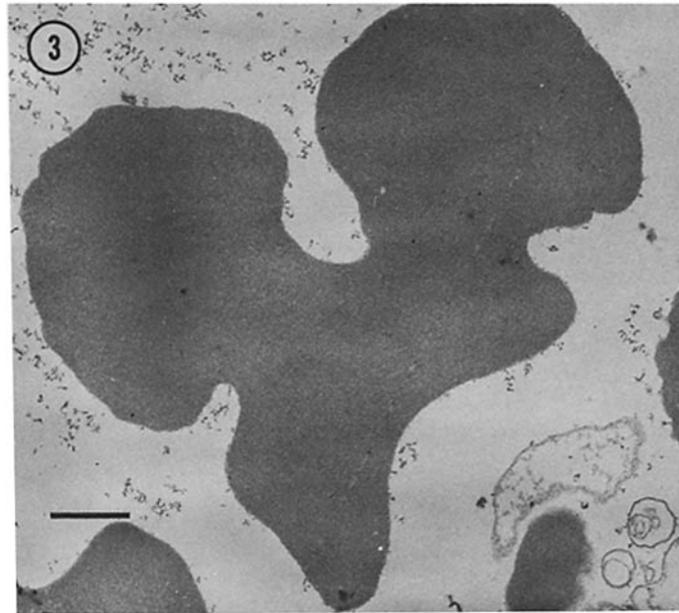
FIGURE 3   Electron micrograph of fused human red blood cells. Cells were fused with 1,000 hemagglutinating units of Sendai virus in the presence of 0.25 mM uranyl acetate. Conditions of fixation, embedding, and sectioning (for observations by electron microscopy) are described in Materials and Methods. Bar length is 1.6 μm. × 6,290.
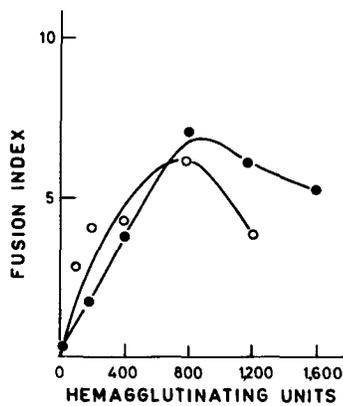

FIGURE 4   The dependency of the fusion index on virus concentration. Fusion was carried out in the presence of 0.25 mM UO_2^{++}. All other conditions were as described in Materials and Methods. O, fresh blood; ●, stored blood. Fusion was determined after 30 min at 37°C.

sence of bivalent metals or in the presence of EDTA, provided that low concentrations of virus were used. Fusion indices as high as 3-4 were obtained with cells which were washed and fused with saline containing 2 mM EDTA (Table II). High fusion indices were obtained in the presence
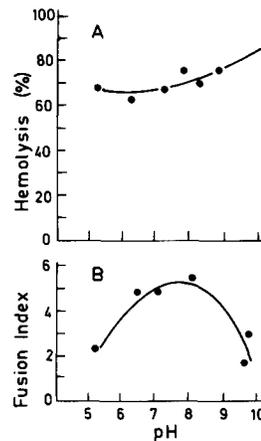

FIGURE 5   Virus-induced hemolysis and fusion as a function of the pH of the medium. (a) Virus-induced hemolysis. Washed cells were suspended in saline (0.9% NaCl) buffered with 10 mM piperazine-glycyl glycine which was titrated to the appropriate pH with either HCl or NaOH. All other conditions were as described in Materials and Methods. The degree of hemolysis without virus ranged at the various pH values between 0 and 9%. Concentration of virus: 200 hemagglutinating units. (b) Virus-induced fusion. Conditions as in Fig. 5 A, except 400 hemagglutinating units of the virus were used.

TABLE I

Fusion of ATP-Depleted Cells

System	Cells in the fusion reaction	Fusion index	μmol ATP/ mg protein
Exp. 1			
I	Stored cells	9.0	
II	Stored cells incubated with iodoacetic acid	8.7	
Exp. 2			
I	Fresh cells	4.7	0.21
II	ATP-depleted cells	6.0	0.013
III	Cells no. II in which ATP was restored	4.0	0.117

Fusion was carried out in the presence of 800 hemagglutinating units of the virus and 0.25 mM UO_2^{++}.

Exp. 1: (I) Blood suspended in acid-citrate dextrose was incubated at 37°C for 3 h and then washed and suspended in buffered saline to give 5% (vol/vol) concentration. (II) As in I, but the incubation at 37°C was carried out in the presence of 2.5 mM iodoacetic acid. After washing, the cells were suspended in buffered saline which contained 5 mM iodoacetic acid to give a concentration of 5% (vol/vol). Cells were fused in a final concentration of 2.5 mM iodoacetic acid.

Exp. 2: ATP was depleted, restored, and determined as described in Materials and Methods.

TABLE II

Fusion of Human Erythrocytes in the Presence of Iodoacetic Acid and EDTA

Additions	Fusion index	
	Exp. I	Exp. II
Uranyl acetate	3.5	5.5
Iodoacetic acid	3.2	4.1
EDTA	3.2	3.7
Iodoacetic acid + EDTA	2.5	4.5

In exp. I and in exp. II, 400 and 800 hemagglutinating units of the virus were used, respectively. Before the addition of the virus, the cells were incubated at 37°C in the presence of iodoacetic acid, EDTA, or both for 140 min in exp. I and for 60 min in exp. II. Fusion was terminated after 7–10 min of incubation with the virus. Concentration: uranyl acetate, 0.25 mM; iodoacetic acid, 2.5 mM; and EDTA, 2 mM.

of EDTA with cells previously incubated with iodoacetic acid. When high concentrations of virus, 2,000–5,000 hemagglutinating units, were used in the absence of bivalent cations or in the presence of Ca^{++} ions, lysis with very little fusion took place.

Virus-Induced Fusion of Human Erythrocyte Ghosts

Cells suspended in 70 mM NaCl, a hypotonic solution in which 30% hemolysis occurred, could still be fused to form polyerythrocytes, whereas cells completely hemolyzed in 10 mM NaCl, rapid hemolysis, did not fuse. Ghosts prepared either by rapid hemolysis or by gradual hemolysis in the absence of albumin were agglutinated by the virus but their membranes remained unfused (Fig. 6 *a*). However, ghosts prepared by gradual hemolysis in the presence of albumin alone or albumin + Mg^{++} could be fused by Sendai virus and did form "polyghosts" (Table III, Fig. 6 *b*). An electron micrograph of the fused ghosts is shown in Fig. 7.
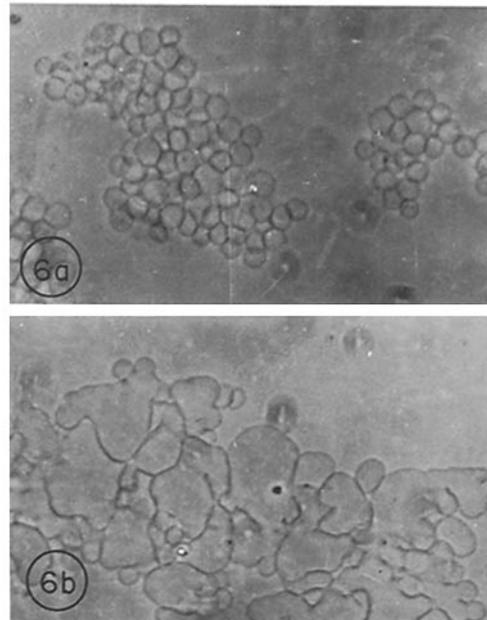

FIGURE 6 Phase micrographs of polyerythrocyte ghosts. Cells were suspended in buffered saline which contained 3% bovine serum albumin and then dialyzed against 40 mM NaCl buffered with 10 mM Tricine-NaOH, pH 7.4. All other steps of preparing of ghosts are as described in Materials and Methods. Fusion was performed in the presence of 800 hemagglutinating units and in the absence of bivalent metals. (*a*) Ghosts prepared by gradual hemolysis but in the absence of bovine serum albumin. No polycells were formed and only agglutination of ghosts can be observed after the addition of the virus. (*b*) Ghosts prepared by gradual hemolysis in the presence of 3% bovine serum albumin (conditions as in Table III). After incubation with the virus large polyghosts were formed. × 560.

As can be seen in Table III, only slight differences in the degree of hemolysis could be detected between cells hemolyzed with or without albumin, but only cells hemolyzed with albumin could be fused. Dextran (average mol wt 40,000) at a concentration of 32 mg/ml failed to act as a substitute for albumin, even though it reduced the extent of hemolysis to 75%.[2] It is thus inferred that the effect of albumin is more than merely to increase the external colloid-osmotic pressure and hence to reduce hemolysis.

Nonviral Fusion of Human Erythrocytes

CA^{++}-INDUCED FUSION: Ca^{++} at high pH induces agglutination and hemolysis of human erythrocytes (see Fig. 8). The extent of hemolysis is highly dependent on the Ca^{++} concentration, pH of the incubation medium, and on the preincubation of the cells without Ca^{++} at the high pH. Cells incubated for 30 and 60 min, before the Ca^{++} addition, were hemolyzed to a greater extent than cells which were not preincubated. The Ca^{++}-induced hemolysis was highly specific, and Mg^{++} ions failed to induce hemolysis when added to cells incubated between pH 10–11.5. Other bivalent cations such as Mn^{++} (1–10 mM) or UO_2^{++} (0.05–0.5 mM) also failed to cause either agglutination or hemolysis of human erythrocytes. Very little hemolysis (0–5%) was obtained at high pH's without the addition of any bivalent metals.

Large polyerythrocytes were formed during the Ca^{++}-induced agglutination and hemolysis at high pH values (Fig. 2 d). The optimal Ca^{++} concentration for inducing fusion was 10–40 mM in the pH range of 10.0–11.0. No dissociation of the fused cells occurred at pH 10.5, and the polyerythrocytes remained in big clumps. The experiments reported here on fusion at pH 10.5 were carried out in a high KCl medium (medium B), but similar results were obtained with cells incubated in a high NaCl medium.

PHOSPHOLIPASE C-INDUCED FUSION: Fig. 9 illustrates that UO_2^{++} and Mn^{++} somewhat reduced the hemolysis of human erythrocytes caused by phospholipase C $C.$ $perfringens$ while Ca^{++} increased it. The stimulation of hemolysis by Ca^{++} ions was probably due to their effect on the hydrolytic activity of the enzyme (22). Table IV shows that these phospholipase C $C.$ $perfringens$ preparations were able to induce fusion of human

---

[2] H. Peretz and A. Loyter, unpublished results.

TABLE III

Virus-Induced Fusion of Human Erythrocyte Ghosts

Additions to the dialysis tubes	Hemolysis	Fusion*
	%	
None	89	–
1.5% bovine serum albumin	92	±
1.5% bovine serum albumin + 2 mM Mg^{++}	90	+
3% bovine serum albumin	87	++

Cells were hemolyzed by dialysis against 40 mM NaCl buffered with 10 mM Tricine-NaOH pH 7.4 by the gradual hemolysis method (15). Virus concentration in the fusion medium was 800 hemagglutinating units. No bivalent metals were present. Incubation time 25 min.
* Since quantitative estimation of the fusion ghosts in the phase microscope was impossible, fusion indices were not calculated.
– No fusion.
± Only a few fused ghosts can be seen in the field.
+ Every field contains a fair amount of fused cells, consisting of three to five cells.
++ Good fusion, polyghosts of five to ten cells.

erythrocytes which had been agglutinated by polylysine (23). Polyerythrocytes were formed only in the presence of Ca^{++} or Mn^{++}, whereas in their absence hemolysis of the agglutinated cells occurred without fusion. Since hydrolysis of phospholipids by phospholipase C takes place only in the presence of bivalent metals (22), it seems that fusion of agglutinated red blood cells by this enzyme occurs only when the membrane phospholipids are hydrolyzed. A picture of polyerythrocytes formed by the fusion of human erythrocytes with phospholipase C and polylysine is not presented since the fused cells are identical to those formed by the virus (Figs. 2 and 3).

DISCUSSION

The three major findings of the present work are: (a) Sendai virus induces fusion of both human erythrocytes and ghosts prepared from them. (b) Fusion of human erythrocytes by Sendai virus requires neither energy nor bivalent cations. (c) Human erythrocytes can be fused with either Ca^{++} at pH 10.5 or with phospholipase C at pH 7.4 without any addition of virus. Polyerythrocytes which still contain most of their hemoglobin were formed when Sendai virus was incubated at 37°C with human red blood cells, in the presence or absence of bivalent metals.

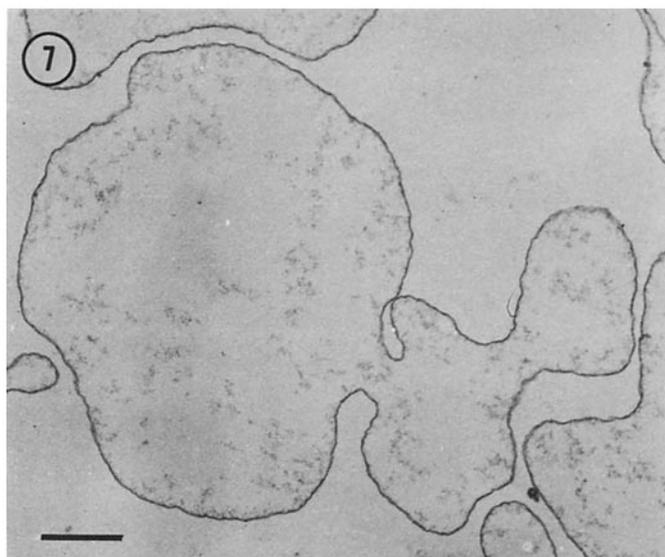Bivalent cations are known to be potent inhibi-

FIGURE 7 Electron micrograph of polyghosts formed by Sendai virus. Preparations of ghosts and fusion conditions are as described in Table III. Bar length 1.6 μm. × 6,290.
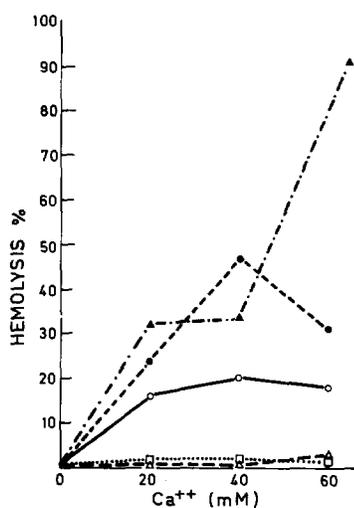

FIGURE 8 Ca++-induced hemolysis of human red blood cells. Cells were preincubated at 37°C for 60 min, then agglutinated by Ca++ in the cold, and incubated again at 37°C for another 40 min as described in reference 7 and Materials and Methods. At the end of the second incubation time, the cells were centrifuged and the extent of hemolysis was determined by measuring the absorbancy of the supernate at 540 nm (the buffer used at pH 7.5 was Tricine-NaOH and at pH 9.0–11.0, glycine-NaOH). □, tricine-NaOH buffer, pH 7.5; glycine-NaOH buffer: △, pH 9.0; O, pH 10; ●, pH 10.6; ▲, 11.0.
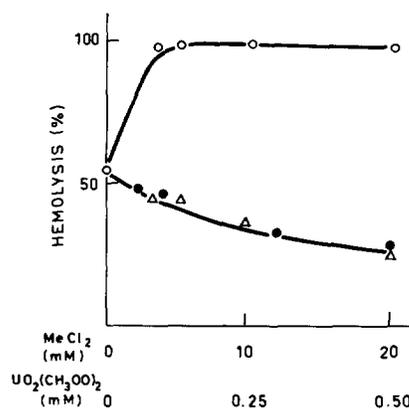

FIGURE 9 The effect of bivalent cations on hemolysis of human erythrocytes caused by phospholipase C C. perfringens. The concentration of the enzyme was 2.5 μg/ml. Degree of hemolysis as described in Materials and Methods. O, Ca++; ●, Mn++; △, UO₂++.

tors of lysis caused by several lytic agents (24). At least part of their effect in the fusion system may be attributed to the inhibition of the virus-induced lysis. Indeed, in previous investigations (6, 14) and in the present one, we have shown that Mn++ and UO₂++ greatly reduce virus-induced hemolysis of both chicken and human red blood cells. UO₂++ is

the most effective cation which at very low concentrations completely inhibits the hemolysis of human erythrocytes caused by Sendai virus. It should be added here that in the presence of UO_2^{++}, human erythrocytes were fused with Ehrlich ascites cells with almost complete prevention of hemolysis (25).

It is apparent from the present investigation that fusion of human erythrocytes can proceed in the absence of bivalent cations, provided that low concentrations of virus are used. Furthermore, fusion was induced in the absence of bivalent cations (or in the presence of EDTA) even in

ATP-depleted cells. The necessity of bivalent metals and ATP for fusion of mammalian cells was principally revealed by Okada (1) who showed that in the presence of respiratory inhibitors or EDTA the virus induced lysis of cells rather than their fusion. Based on these and other results, it has been suggested by Poste and Allison (26) that membrane fusion, in general, depends on the activity of Ca^{++}-dependent ATPase. However, from the present work it appears that neither ATP nor bivalent metals are required in the presence of low concentrations of Sendai virus for the fusion of human red blood cells. It seems unlikely that the mechanism by which the virus induces fusion in human erythrocytes differs significantly from that by which it induces fusion in other mammalian cells. ATP and bivalent metals might affect the fusion reaction by controlling the degree of virus-induced lysis, as has been shown in the case of Ehrlich ascites tumor cells (3) or chicken erythrocytes (14).

Sendai virus is about 800–1,000 Å in diameter (21) and occupies an area of about 0.8–1.2 μm^2. Therefore, each square micrometer of the plasma membrane can tolerate only one virus particle at any one time. From the data presented in Table V, it seems that many more virus particles are present in each of the fusion systems than could be in contact with the plasma membrane. As can be seen from Table V, while as many as 64 virus particles must be present for each square micrometer of the

TABLE IV

Fusion of Human Erythrocytes by Polylysine and Phospholipase C C. perfringens

System	Hemolysis	Fusion*
	%	
Polylysine	0	–
Polylysine + phospholipase C	93	–
Polylysine + phospholipase C + Ca^{++}	92	+
Polylysine + phospholipase C + Mn^{++}	90	+
Polylysine + phospholipase C + mM^{++} + Ca^{++}	83	+ +

* Since the agglutination was irreversible, determination of fusion indices was impossible. Reading of signs as in Table III. Concentrations: Ca^{++}, 5 mM; Mn^{++}, 0.6 mM.

TABLE V

The Correlation Between Virus Concentration and the Fusion Reaction in Different Systems

Type of cells	Concentration of cells in the fusion system, cells/ml*	Hemagglutinating units/ml	Numers of particles/ml‡	Numbers of virus particles/cells	Area of cell	Number of virus particles/μm^2 area of cell
					μm^2	
Ehrlich ascites tumor cells	2×10^6 (ref. 3)	2,000	5×10^{10}	25.000	314§	64
Chicken erythrocytes	1.5×10^7 (ref. 6)	4,000	10×10^{10}	3.000	225 (ref. 31)	13
Human erythrocytes	3×10^7	800	2×10^{10}	700	163 (ref. 31)	4

* Number of cells was determined by Clay Adams Cytometer (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.).
‡ Number of virus particles was calculated as described in reference 30. One hemagglutinating unit $= 2.4 \times 10^7$ virus particles.
§ Calculation of area in square micrometers was made by assuming that the average diameter of a cell is 10 μm, and considering as a complete sphere.

PERETZ ET AL. *Fusion of Intact Human Erythrocytes and Erythrocyte Ghosts* 9

plasma membrane in order to induce fusion in Ehrlich ascites tumor cells, only four are necessary for optimal fusion in human erythrocytes. This might reflect the differences in the virus receptors of these two cells and might also explain the high sensitivity of human erythrocytes to lysis by the Sendai virus. Thus, eight to ten times less virus particles are required to initiate agglutination and fusion in human erythrocytes than for agglutination and fusion of Ehrlich ascites tumor cells.

The addition of high concentrations of virus to human erythrocytes in the absence of bivalent metals resulted in complete hemolysis with no fusion, as occurs under similar conditions with chicken erythrocytes or Ehrlich ascites tumor cells (6, 1). However, if the fusion process has already commenced and precedes lysis, which is the case in the presence of low concentrations of virus, either polycells containing hemoglobin or polyerythrocyte ghosts will form, depending on the fusion conditions (time of incubation and bivalent cations).

The failure of cells to fuse after virus-induced lysis could be explained by the following hypotheses. During hemolysis either a membrane constituent obligatory to fusion may leak out of the membrane or the membrane may undergo structural changes. Indeed, the virus failed to induce fusion of ghosts prepared by the regular drastic osmotic shock even though agglutination was not disturbed. Fusion was permitted only in cells hemolyzed in the presence of albumin by the gradual hemolysis procedure. It has been well established (27) that during drastic osmotic shock various membrane components, especially some of the proteins, are solubilized and escape from the surface of the erythrocyte membrane. The solubilization might be accompanied by structural changes of the phospholipids as well as of the proteins which are still retained in the membrane. Bivalent cations might prevent this leakage as has been demonstrated by Burger et al. in the case of the membranous acetylcholinesterase (28). Albumin and other proteins, when present during hemolysis, might also prevent the leakage of some of the membrane components and hence help to retain the correct structure for fusion of the erythrocyte membrane.

The present results imply that intracellular components are not involved in the virus-induced fusion, which appears to be an intermembranous process. Evidently, fusion of ghosts takes place in the absence of ATP or bivalent cations.

Fusion by Ca^{++} at pH 10.5 or by phospholipase C might mean that the major groups undergoing changes during fusion are the phospholipids. Substantial changes in the phospholipids of the chicken erythrocyte membrane have been revealed after incubation with Ca^{++} at high pH or with phospholipase C C. perfringens preparations (14, 29). Experiments to examine the changes in the phospholipids resulting from such treatment in human erythrocytes are under way.

REFERENCES

1. OKADA, Y. 1969. Curr. Top. Microbiol. Immunol. 28:102.
2. KOHN, A. 1965. Virology. 26: 119.
3. YANOVSKY, A., and A. LOYTER. 1972. J. Biol. Chem. 247:4021.
4. OKADA, Y. 1962. Exp. Cell Res. 26:119.
5. POOLE, A. R., J. I. HOWELL, and J. A. LUCY. 1970. Nature (Lond.). 227:810.
6. TOISTER, Z., and A. LOYTER. 1970. Biochem. Biophys. Res. Commun. 41:1523.
7. TOISTER, Z., and A. LOYTER. 1971. Biochim. Biophys. Acta. 88:620.
8. DE BOER, E., and A. LOYTER. 1971. FEBS (Fed. Eur. Biochem. Soc.) Lett. 15:325.
9. HARRIS, J. R., and J. N. BROWN. 1971. J. Ultrastruct. Res. 36:8.
10. BAKER, R. F. 1972. J. Cell Biol. 53:244.
11. BACHI, T., and C. HOWE. 1972. Proc. Soc. Exp. Biol. Med. 141:141.
12. APOSTOLOV, K., and G. POSTE. 1972. Microbios. 6:247.
13. PERETZ, H., and A. LOYTER. 1971. 41st Annual Meeting of the Israel Chemical Society. 78. (Abstr.).
14. TOISTER, Z., and A. LOYTER. 1973. J. Biol. Chem. 248:422.
15. DANON, D., A. NEVO, and Y. MARIKOVSKY. 1956. Bull. Res. Counc. Isr. Sect. E Exp. Med. 6:36.
16. NAKAO, O. 1960. J. Biochem. (Tokyo). 47:661.
17. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. J. Biol. Chem. 193:265.
18. STANLEY, P. E., and S. G. WILLIAMS. 1969. Anal. Biochem. 29:381.
19. FRISH, A., I. GAZITH, and A. LOYTER. 1973. Biochim. Biophys. Acta. 291:690.
20. DRABKIN, D. L. 1958. In Standard Methods of Clinical Chemistry. D. Seligson, editor. Academic Press, Inc., New York. 52.

21. FENNER, F. 1968. *In* The Biology of Animal Viruses, Vol. I. Academic Press, Inc. New York. 65.
22. BANGHAM, A. D., and R. M. C. SAMSON. 1962. *Biochim. Biophys. Acta.* 59:103.
23. MARIKOVSKY, Y., and D. DANON. 1966. *Biochim. Biophys. Acta.* 125:154.
24. BURT, D. H., and J. W. GREEN. 1971. *Biochim. Biophys. Acta.* 225:46.
25. ZAKAI, N., A. LOYTER, and R. G. KULKA. 1974. *J. Cell Biol.* 61:241.
26. POSTE, G. H., and A. C. ALLISON. 1971. *J. Theor. Biol.* 32:165.
27. MARCHESI, S. L., E. STEERS, V. T. MARCHESI, and T. W. TILLACK. 1970. *Biochemistry.* 9:50.
28. BURGER, S. P., T. FUJII, and D. J. HANAHAN. 1968. *Biochemistry.* 7:3682.
29. SABBAN, E., Y. LASTER, and A. LOYTER. 1972. *Eur. J. Biochem.* 28:373.
30. OKADA, Y., S. NISHIDA, and J. TADOKORO. 1961. *Biken J.* 4:209.
31. EYLAR, E. H., M. A. MADOFY, O. V. BRODY, and J. L. ONCLEY. 1962. *J. Biol. Chem.* 237:1992.