ENHANCEMENT OF SENDAI VIRUS-MEDIATED CELL FUSION BY CUPRIC IONS

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ABSTRACT

The effect of divalent cations on cell fusion by concentrated Sendai virus, inactivated by beta-propiolactone, was investigated using Vero and mouse L-929 cells in monolayers. With both cell lines, which are normally resistant to exogenous viral fusion, Cu^{2+} in sublethal concentrations was found to promote polykaryon formation to a marked degree. The simultaneous presence of Cu^{2+} and virus was required for this effect, which was thought to be related to the cytotoxic action of Cu^{2+} on the cell membrane. Accordingly, under standard conditions and in the absence of virus, leakage of isotopically labeled intracellular protein was shown to bear a quantitative relationship to Cu^{2+} concentration. Concomitant changes in the membrane were seen electron microscopically to consist of loss of microvilli and the appearance of numerous vesicles on, or adjacent to, the membrane. The relationship of enhanced fusibility to these toxic changes was not further elucidated. The fusion-promoting effect of Cu^{2+} far exceeded that of Ca^{2+}; and other cations tested had no effect.

INTRODUCTION

Since the discovery by Okada (10) that Sendai virus possess the ability to fuse cells into syncytia, this phenomenon has been extensively studied. Okada (11) and Murayama and Okada (7), working with Ehrlich ascites tumor cells, showed that fusion occurs optimally at about pH 7.6 and 37°C, in the presence of calcium ions with energy available in the form of nucleoside triphosphates.

The fusion of many types of cells, including HeLa, FL amnion, KB, HEp-2, Madin-Darby canine and bovine kidney, Vero and mouse L-929 cells, has been reported by various investigators (2, 13, 15, 17). The strain of Sendai virus maintained in our laboratory by propagation in *ovo* for over 45 passages can efficiently fuse each of these cell types, except Vero and L-929. To obtain insight into the mechanism of the syncytiogenic process, various experimental conditions were investigated which might facilitate the formation of polykaryons in naturally resistant cell populations. This report describes the promotion of Sendai-induced polykaryocytosis in Vero and L cells by cupric ions (Cu^{2+}).

MATERIALS AND METHODS

Beta-propiolactone (BPL)-inactivated Sendai virus concentrates were prepared as previously described (18). Briefly, seed Sendai virus, diluted 10^{-3}, was inoculated into the chorioallantoic cavity of 11-day old chicken embryos. Infective chorioallantoic fluid (CAF) was harvested after 48 h at 36°C and clarified by low speed centrifugation and filtration through gauze. Virus was pelleted by centrifugation for 2 h at 32,000 g and the pellet resuspended in 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4. A 40-fold concentration of hemagglutinating units (HAU) was thereby achieved.

0.01% BPL was used to abolish the infectivity of Sendai virus concentrates according to the method of Neff and Enders (9). Preparations were tested

for residual infectivity by inoculation onto replicate tube monolayers of primary chicken embryo fibroblasts, and examining for hemadsorption after 3 days at 37°C. BPL-inactivated Sendai virus was devoid of any infectivity detectable by these means.

Human amnion (FL) cells, Vero and mouse L-929 cells were grown in Eagle's minimal essential medium in Hanks' balanced salt solution (MEM), supplemented with 10% newborn calf serum. When indicated, the number of viable cells in a preparation was determined by the trypan blue exclusion technique. After a 10-min incubation period in 0.1% trypan blue in PBS, cell sheets were washed three times in Earle's balanced salt solution (BSS) before detachment of the monolayers with 0.1% trypsin in 10^{-3} M disodium ethylenediaminetetraacetic acid (EDTA) in PBS. A single cell suspension was achieved by gentle pipetting, and unstained cells were counted.

Monolayers grown to confluence on cover slips in Leighton tubes (Bellco Glass, Inc., Vineland, N. J.) were challenged with 0.2 ml of BPL-inactivated Sendai virus diluted in either BSS or isotonic 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.6. Virus was left in contact with cells for 2 h at 37°C; cells were fixed in methanol, and stained with Lillie's azure-eosin. For quantitative assays, cover slips were placed in 5×10^{-4} M EDTA in PBS for 2 min before fixation and staining to cause the retraction of mononucleated cells from syncytia and thereby facilitate cell and nuclear counts (3). Fusion indices were expressed as the number of nuclei counted per number of cells in which they were found. At least 500 nuclei and four different fields were examined on each stained cover slip. Unfused monolayers served as controls.

In order to assess the effects of various cations, including copper, on the susceptibility of cells to exogenous viral fusion, monolayers were exposed to the chloride salts of each cation incorporated into maintenance medium for a 2-h period before or simultaneously with a viral challenge. The exact experimental conditions used for examining the effects of Cu^{2+} are detailed among the results subsequently presented. Because BSS contains phosphate ions which complex with divalent cations, isotonic 0.02 M phosphate-free Tris buffer was employed for comparing the effects of different ions on cell fusion. However, cultured cells maintained in BSS appeared healthier than those maintained in Tris buffer. The former medium was therefore used when small discrepancies in actual cation concentrations were relatively unimportant.

Leakage of protein from cells into the medium was measured by the amount of ^{14}C in acid-insoluble material (0.1 mCi/0.067 mg/ml, New England Nuclear Corp., Boston, Mass.) released from cells labeled with a ^{14}C-amino acid mixture. Specimens of medium were mixed with equal volumes of cold 10% TCA. After 30 min in an ice bath, precipitates were pelleted by centrifugation at 1,500 g for 30 min at 4°C. The precipitates were washed three times in cold 5% TCA, and allowed to dry overnight at room temperature, before being dissolved in a small volume of Soluene 100 (Packard Instrument Co. Inc., Downers Grove, Ill.) and counted in a toluene base scintillant (Omnifluor, New England Nuclear Corp.) with an Ansitron 2 liquid scintillation spectrometer (Picker Corporation, White Plains, N.Y.) at an efficiency near 85%.

Monolayers of L cells were fixed for 5 min at 4°C in situ in a mixture of 1.5% acrolein (Eastman Kodak Co., Rochester, N.Y.) and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.18 M sucrose. The cells were dislodged and suspended in a fresh acrolein-glutaraldehyde mixture for 5 min. They were then washed five times in buffer, and postfixed for 1 h at 4°C in a solution of 1% osmium tetroxide in buffer. After subsequent washing in water, the cells were kept overnight at 4°C in a 2% solution of uranyl acetate in water. They were then washed again, dehydrated in graded ethyl alcohols, and embedded in Epon. After sectioning, the grids were examined in a Siemens Elmiskop 1.

RESULTS

The chlorides of calcium, manganese, magnesium, zinc, strontium, cadmium, barium, and copper were tested in Tris buffer for fusion-enhancing effect. Calcium (Ca^{2+}), previously shown (7, 12) to be a requirement for syncytium formation, was found to promote L cell fusion. However, a much greater enhancement of fusion occurred when cupric ion (Cu^{2+}) was incorporated into the medium. Only the cation component of cupric chloride was responsible for fusion enhancement, cupric acetate giving an equally marked effect. None of the other cations, either as chloride or acetate, had any stimulating effect. Calcium ion (1.8×10^{-3} M) in BSS allowed minimal syncytium formation with L cells but not with Vero cells. In the presence of increasing concentrations of Cu^{2+}, however, Vero cell fusion progressed to higher levels than fusion of L cells (Fig. 1). Treatment with 10^{-4} M zinc, cadmium, or copper chloride for 2 h at 37°C in Tris buffer greatly reduced the viability of all cultures examined after 24 h by the trypan blue exclusion technique. However, syncytia which had been formed with Sendai virus in the presence of Cu^{2+} remained viable for 48 h beyond the removal of both virus and Cu^{2+}, although mononucleated cells in the same preparations had died. This finding sug-
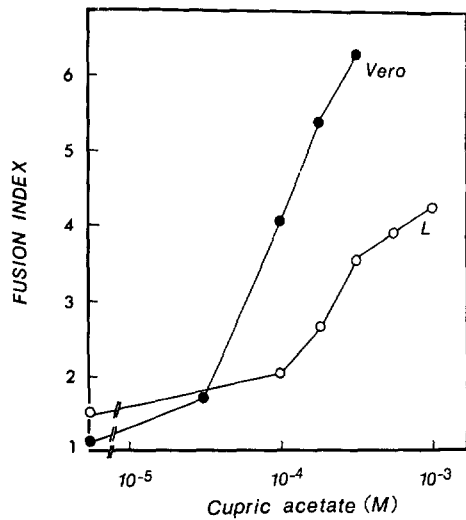
FIGURE 1 Effect of Cu²⁺ on fusion of L and Vero cells. Vero and L cell monolayers were challenged with 800 and 3,200 HAU of BPL-inactivated Sendai virus, respectively.

gested that with sublethal concentrations of Cu²⁺ relatively pure populations of viable polykaryons might be obtainable. This possibility is under further investigation.

Cu²⁺ in BSS at 5×10^{-4} M resulted in rapid killing of L cells. A concentration of 10^{-5} M had no lethal effect. Intermediate levels of the drug resulted in delayed killing which was manifest after 24 h, after which growth resumed (Fig. 2). 60-min exposure to a toxic concentration of Cu²⁺ (2.5×10^{-4} M) achieved a significant delayed killing effect in L cells (Fig. 3). Stained preparations of monolayers treated with 2.5×10^{-4} M Cu²⁺ for 15, 60, and 120 min, respectively, showed increasing loss of distinct cell boundaries, progressive cytoplasmic vacuolization and, subsequently, nuclear pyknosis. With 10^{-3} M Cu²⁺, cells exhibited very diffuse, sometimes fragmented cytoplasm by 2 h, with virtually indistinguishable cell borders.

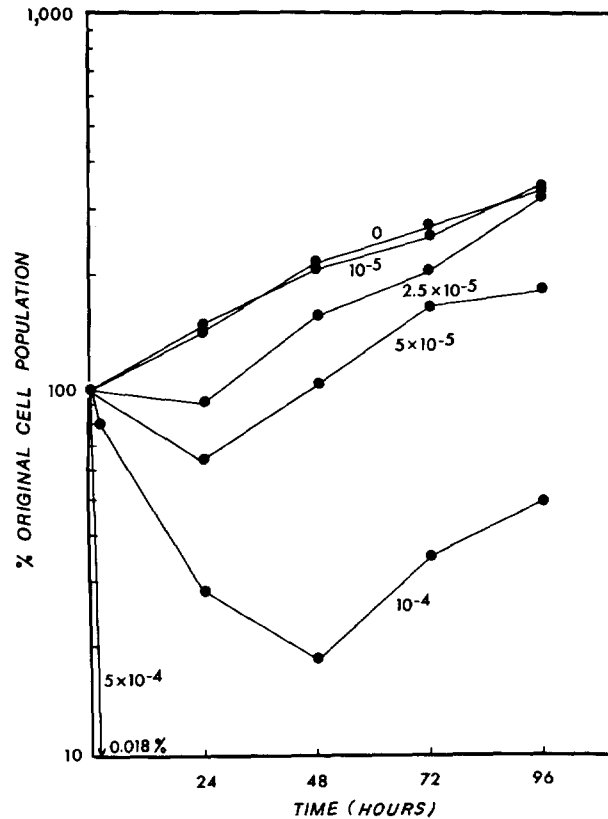To determine whether a depletion of macro-

FIGURE 2 Effect of different Cu²⁺ concentrations on L cell viability. Replicate monolayers were exposed to different concentrations (M) of cupric acetate in BSS for 2 h at 37°C, then rinsed with BSS and refed with MEM supplemented with 10% newborn calf serum. Viability counts were performed immediately after the removal of Cu²⁺, and at subsequent 24 h intervals.
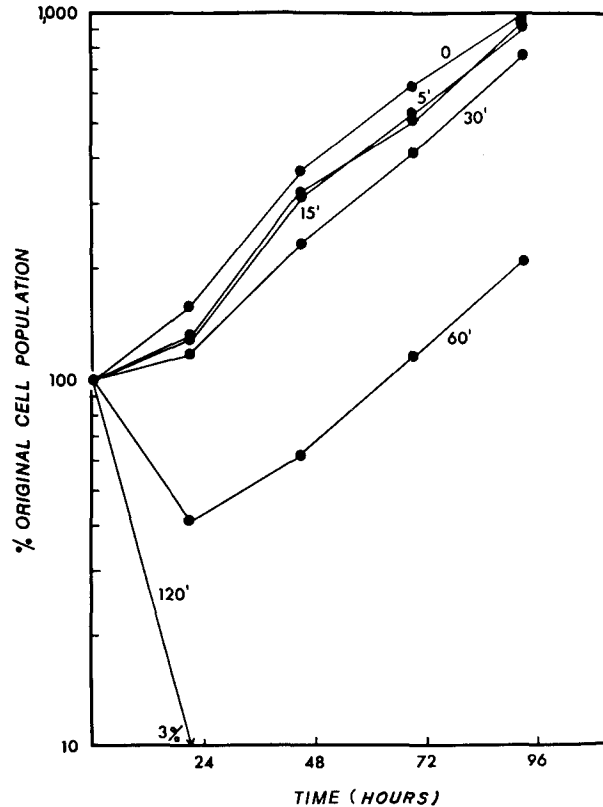
FIGURE 3 Effect of different times of exposure to 2.5×10^{-4} M Cu^{2+} on L cell viability. Replicate monolayers were exposed to 2.5×10^{-4} M cupric acetate in BSS for different periods before washing in BSS, and refeeding with MEM supplemented with 10% newborn calf serum. Viability counts were performed at 24 h intervals.

molecular components accompanied treatment of cells with sublethal concentrations of Cu^{2+}, the proteins of replicate, growing monolayers of L cells were prelabeled with ^{14}C-amino acid mixture over a 48-h period. Varying concentrations of Cu^{2+} were then added to the monolayers, and samples of medium were removed at different times and radioactivity was determined. Exposure to 5×10^{-4} M or 10^{-3} M Cu^{2+} resulted in a rapid leakage of labeled cellular protein followed by cell death (Fig. 4). More moderate concentrations of drug promoted leakage of protein after 60 min, and proved lethal only after a subsequent 24 h of incubation.

For both FL and L cells in Tris buffer, Cu^{2+} had a far greater stimulatory effect than Ca^{2+}. Increasing fusion indices were observed with increasing concentrations of Cu^{2+} to 2.5×10^{-4} M and of Ca^{2+} to 5×10^{-2} M (Fig. 5). Dilutions of virus in BSS alone and in BSS containing 2.5×10^{-4} M Cu^{2+} were used to challenge replicate

monolayers of FL and L cells, respectively (Fig. 6). At equivalent concentrations of virus, FL cells in the absence of Cu^{2+} were always fused to higher levels than were L cells in the presence of 2.5×10^{-4} M Cu^{2+}.

Little or no syncytium formation occurred when a Sendai virus inoculum in Tris buffer was removed from L cells after varying periods, and Cu^{2+} subsequently added to the end of a total 2-h incubation. Similarly, pretreatment with Cu^{2+}, followed by the addition of Sendai virus in Tris buffer, yielded little cell fusion. However, if both virus and Cu^{2+} were present simultaneously for at least 40 min, then cell fusion progressed as well thereafter as in the continued presence of both reagents (Fig. 7).

Concentrated Sendai virus treated for 30 min at 37°C with 5×10^{-4} M Cu^{2+} in Tris buffer, and washed free of unbound Cu^{2+}, was incapable of inducing significant levels of L cell fusion in the absence of added Cu^{2+}. Viral neuraminidase,

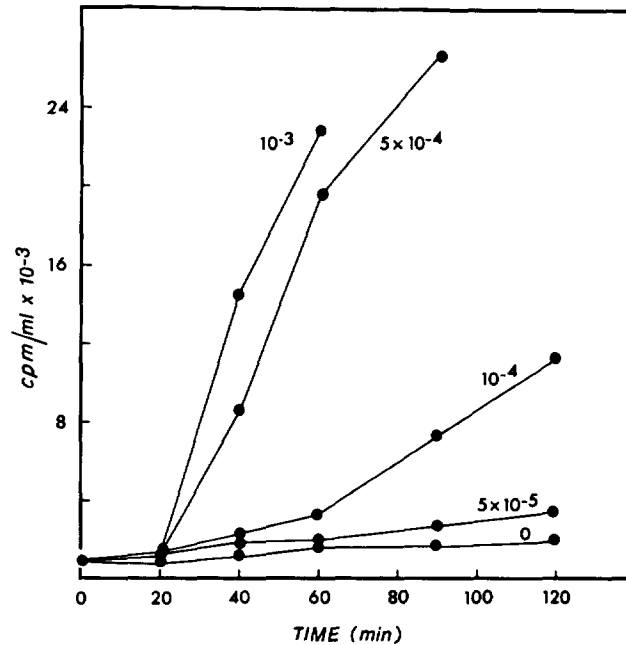FIGURE 4 Replicate growing monolayers of L cells in 30 ml flasks were continuously labeled over 48 h at 37°C with a ^{14}C-amino acid mixture. After an additional 4 h in nonradioactive medium, monolayers were exposed to varying concentrations (M) of cupric acetate in BSS. 0.5 ml samples were removed after different times and centrifuged at 2,000 g to pellet whole detached cells, after which the supernatants were transferred to fresh tubes and an equal volume of cold 10% TCA was added. Precipitates were processed for counting as described in Materials and Methods.
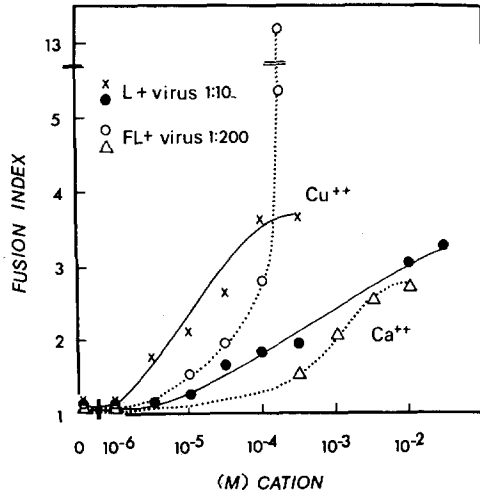

FIGURE 5 Comparison of effects of Cu^{2+} and Ca^{2+} on fusion of L and FL cells.

hemolytic, and hemagglutination (HA) activities were quantitatively unaltered by the Cu^{2+} treatment.

Thin sections of L cells which had been treated with Cu^{2+}, either with or without Sendai virus, were examined electron microscopically in an attempt to correlate ultrastructural changes with increased susceptibility to fusion. Virus particles attached to microvilli were occasionally observed in preparations which had been exposed to Sendai virus alone and in which little or no cell fusion had occurred. By contrast, when 2.5×10^{-4} M cupric chloride was incorporated in the viral inoculum, many polynucleated cells were visible, but no viral particles were seen at the cell surface. In L cell monolayers exposed to the same concentration of Cu^{2+} without virus, no fusion occurred; but individual cells displayed an apparent decrease in the number of microvilli and concomitant appearance of partly "bald" areas (Fig. 8). These areas are defined, and may be recognized as smooth cell surface contours without evident microvilli or microspikes, ridges, or processes. In addition, cupric ion alone often induced the formation of intracytoplasmic vacuoles which were visible by light microscopy in fixed preparations as well as in living cells examined at higher magnification in phase contrast. Electron

microscopy revealed, in addition to the afore-mentioned vacuoles, numerous vesicles on or adjacent to the cell membrane, which were apparently extruded or in the course of extrusion from the cell cortex. These vesicles ranged from 100 to 200 nm in diameter, were completely enclosed, and invariably contained partly electron-transparent material (Fig. 8). A few such

vesicles were occasionally observed in untreated monolayers but to an extent not more than 10% of that seen in treated cells.

DISCUSSION

The experiments described here indicate that Cu^{2+} is a far more potent enhancer of polykaryon formation than Ca^{2+}, and suggest that the fusion-stimulating effect of Cu^{2+} may be related to its cytotoxicity. Of all divalent cations tested, calcium has been reported to be the most potent in enhancing cell fusion (7, 12). However, Cu^{2+} has not heretofore been examined for this property, an omission which may be due to the well-known toxic effect of this ion both in vivo and in vitro. Mauersberger and Finsterbusch-Zorn (5) and Mauersberger and Zorn (6) treated HeLa, KB, and L cells with copper sulphate at concentrations ranging from 5×10^{-5} M to 10^{-3} M and found that L cells were the most resistant of all cells tested to the toxic effects of the cation. They also showed that the addition of serum to the Cu^{2+}-containing medium had a protective effect, which was interpreted to mean that Cu^{2+} normally binds to cellular sulfhydryl (SH) groups, but that exogenously added protein may compete for free copper ion. Nayak and Mahelkar (8) tested the effect of various Cu^{2+} concentrations on the growth of L cells in tissue culture, and reported that when as little as 1.5×10^{-5} M Cu^{2+} was incorporated into the medium, cell viability decreased by 50% in the first passage, and by an additional 25% in the second passage.

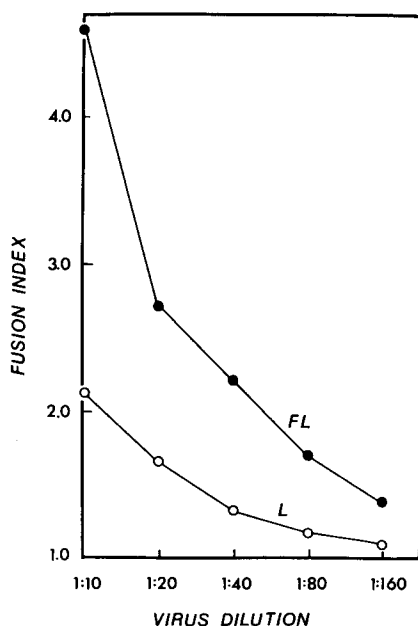Leakage of cellular protein after treatment with

Figure 6 Fusion of FL and L cells under optimal conditions. BPL-inactivated Sendai virus was diluted from a concentrate containing 81,920 HAU/ml.
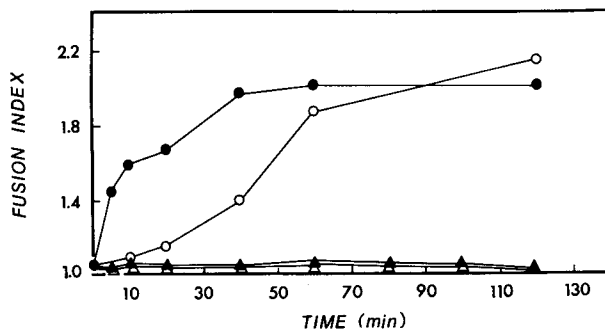

Figure 7 Fusion of L cells by inactive Sendai virus (1,600 HAU) in relation to cupric ions (2.5×10^{-4} M $CuCl_2$ in Tris buffer). ●, Virus and $CuCl_2$ added at 0 time, replaced with BSS at increasing intervals during 2 h incubation at 37°C. ○, Virus and $CuCl_2$ added together at 0 time, monolayers fixed in methanol after increasing periods of incubation at 37°C. ▲, Virus alone added at 0 time, replaced with $CuCl_2$ at increasing intervals during 2 h incubation at 37°C. △, $CuCl_2$ alone added at 0 time replaced with virus at increasing intervals during 2 h incubation at 37°C.
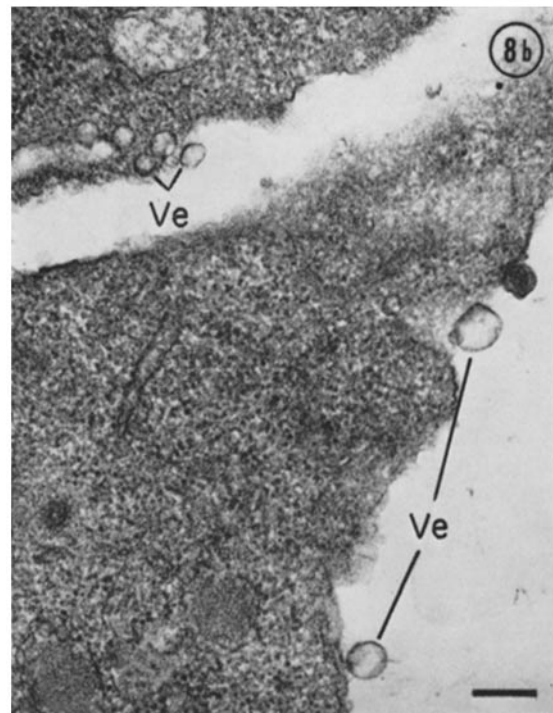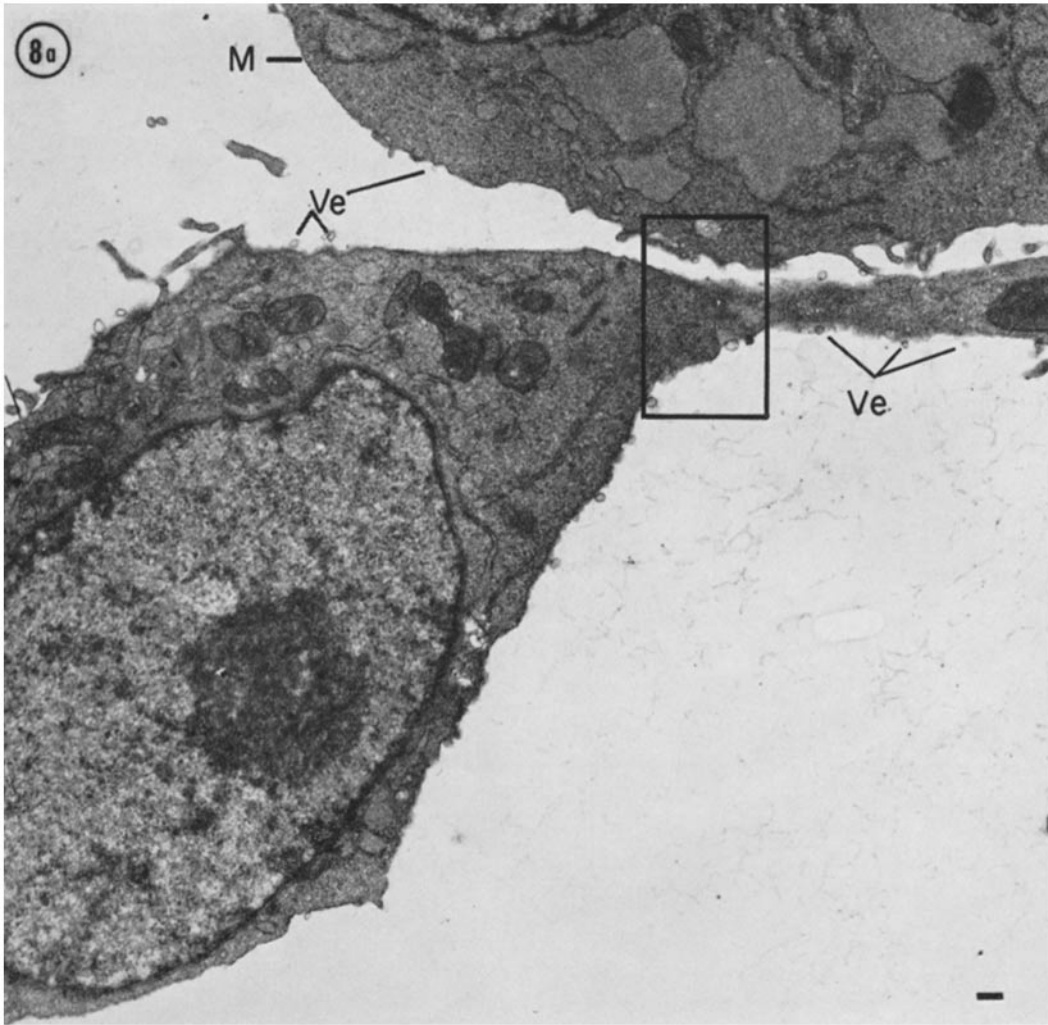
FIGURE 8   Fig. 8 a is an electron micrograph of L cells treated 1 h with 2.5×10^{-4} M $CuCl_2$, showing paucity of microvilli, vesicles (*Ve*), and "bald" areas on membrane (*M*). Fig. 8 b is an enlargement of the boxed area in Fig. 8 a. Bars, 200 nm. Fig. 8 a, $\times$ 14,250. Fig. 8 b, $\times$ 42,750.

Cu^{2+} may signify that cupric salts affect the integrity of the plasma membrane. Such a mode of action would be consistent with the protein-releasing and fusion-enhancing properties of this cation. The numerous extracellular vesicles seen by electron microscopy on the outer border of the plasma membrane of Cu^{2+}-treated cells may represent tiny sacs of leaked protein (Fig. 8 a). The 60-min incubation of L cells with 10^{-4} M Cu^{2+} which is necessary to achieve high levels of protein leakage (Fig. 4) corresponds well with the minimal time of exposure to 2.5×10^{-4} M Cu^{2+} needed for significant killing after 24 h (Fig. 3). In addition, the fact that moderate concentrations of Cu^{2+} (10^{-4}, 5×10^{-5} M) exert a delayed killing effect, yet are able to cause rapid protein leakage, indicates that cell death of the delayed type may occur only after large amounts of protein have escaped.

Cu^{2+}-induced damage to the cell surface may be caused by interference with the mechanisms of active transport across the plasma membrane. Bowler and Duncan (1) prepared microsomal fractions from rat brains, and found that 10^{-4} M Cu^{2+} inhibited both the Mg^{2+}-ATPase and the Na^+-K^+-Mg^{2+}-ATPase systems. Peters (14), working with a microsomal fraction of pigeon brain, observed that the addition of 10^{-5} M Cu^{2+} resulted in a 20% inhibition of membrane ATPase activity. Moreover, minute amounts of Cu^{2+}, when introduced into the subarachnoid space of pigeons, could induce convulsions and death. These effects, however, could be inhibited by co-incubation, before injection of Cu^{2+}, with 2, 3 dimercapto-1-propanol (BAL). Sahaphong and Trump (16), and Laiho et al. (4) have observed that organic mercurials capable of binding to plasma membrane SH groups have toxic effects on flounder kidney tubules and on Ehrlich ascites tumor cells. The foregoing observations offer further, albeit indirect, evidence of the ability of Cu^{2+} to bind to free SH groups at cell membranes.

Inspection of Fig. 5 may lead to the impression that low concentrations of Cu^{2+} promoted L cell but not FL cell fusion, despite the fact that FL cells fused readily in BSS while L cells did not. This is probably illusory and due to the fact that much more virus was required for fusion of L cells than for FL cells, as shown in Fig. 6, in which fusion indices for each cell type under optimal conditions have been plotted against dilutions of concentrated Sendai virus. At all dilutions, poly-

karyocytosis progressed to higher levels with FL cells than with L cells. Moreover, divalent cations and virus had to be present simultaneously at the membrane in order for fusion to occur. Pretreatment of cells with Cu^{2+} before addition of virus was ineffectual in promoting polykaryocytosis. However, adsorption of virus particles was not impeded under these circumstances, since hemagglutinin could be detected by hemadsorption on pretreated L cells, even though no fusion occurred. Likewise, treatment of the virus itself with Cu^{2+} did not promote fusion of normal cells in the absence of cation added at the time of viral adsorption.

In summary, we have shown that Cu^{2+} enhanced Sendai virus-mediated cell fusion through action on the plasma membrane in concert with virus particles. Sublethal concentrations of Cu^{2+} induced structural alterations in the membrane which were reflected in the development and extrusion of vesicles from treated cells. These changes themselves may somehow have facilitated the fusion process once virus was adsorbed. It is otherwise not clear what factors are involved in the requirement for the simultaneous presence of cation and virus at the cell membrane. In the absence of added Sendai virus, the same moderate concentrations of Cu^{2+} induced alterations of the cell surface and leakage of protein which were doubtless responsible for retardation of growth and delayed cytocidal effects. This was in contrast to the continued viability of the virus-Cu^{2+}-induced polykaryons.

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