

MANGANESE STIMULATES ADHESION AND SPREADING OF MOUSE SARCOMA I ASCITES CELLS

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ABSTRACT

Adhesion of Sarcoma I cells (SaI) to untreated or to serum-treated glass was examined by layering ^{51}Cr -labeled cells on the substrate for 20 min at 34°C and determining the glass-bound radioactivity after the monolayers were rinsed. Adhesion to untreated glass proceeded in sodium chloride-imidazole-potassium medium (SIK) without added divalent cations, whereas SaI adhered maximally to the serum-coated substrate only in the presence of 50 μM or more Mn. Divalent Mg, Ca, Co, Ni, or Zn were inactive or minimally active. Mn-stimulated adhesion was sharply temperature dependent, reversible upon removal of Mn, and inhibited by Ca as well as by cytochalasin B, vinblastine, or tetracaine. Adhesion of SaI in SIK did not ensue when cells or the coated substrate were pretreated with Mn and washed in SIK before the adhesion assays. Microscope observations showed that Mn induced the formation of cell processes, ruffles, and veils, and that SaI spread on the uncoated or serum-coated substrate when exposed to Mn. Cells withdrew veils and processes and rounded up when postincubated in Mn-free medium. Formation of cell processes and spreading was inhibited by cytochalasin B, vinblastine, or tetracaine. Manganese-induced adhesion seems to require the participation of microtubules and microfilaments and may be mediated by an effect of Mn on Ca fluxes. The results support the role of cell processes and spreading in cell-to-substrate adhesion.

INTRODUCTION

Studies of cell-to-substrate adhesion are pertinent to cell movement and to the morphology and growth pattern of normal and transformed cells in culture (1, 8, 50, 51).

Cell-to-substrate adhesion has been examined by layering a cell suspension over the substrate and estimating the numbers of attached or free cells after certain distractive procedures are applied. Features of the adhesion include: (a) temperature dependence (10, 16, 28), (b) inhibition by serum or plasma (10, 28, 40, 46), (c) requirement for a divalent cation, often satisfied by Mg, with possible substitution by other metals (2, 7, 17, 39), (d) inhibition by sulfhydryl reagents (20) or by cytochalasins (45). Similar features have also been

reported for cell spreading (32, 33, 40). The present paper shows that manganese reversibly stimulates both the adhesion and spreading of Sarcoma I cells (SaI) on a glass substrate.

MATERIALS AND METHODS

Media

Except when otherwise noted the medium used was sodium chloride-imidazole-potassium (SIK), prepared as in reference 32. In experiments with different metal ions, NaCl-Tris-K (STK) was used (32). Gey's and bicarbonate-free Dulbecco's media were obtained from Grand Island Biological Co., Grand Island, N. Y. and buffered with 10 mM Tris. The pH of the different media was adjusted to 7.1-7.3.

Chemicals

Gelatin ("purified pigskin") was purchased from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Cytochalasin B was obtained from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y. and dissolved in dimethylsulfoxide (DMSO) at 5 mg/ml. Vinblastine sulfate was obtained from Eli Lilly & Co., Indianapolis, Ind., colchicine from Sigma Chemical Co., St. Louis, Mo., and tetracaine from Schwarz/Mann, Orangeburg, N. Y., respectively. Ethylene glycol bis(β -aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) was purchased from Sigma Chemical Co. The metal salts used were of analytical reagent grade: divalent Ca, Mg, Mn, Ni, or Co, as chlorides; Zn²⁺ as sulfate. Na₂⁵¹CrO₄ in aqueous solution was obtained from Amersham/Searle Corp., Arlington Heights, Ill.

Animals

B₆AF1 female mice 15–20 g body weight were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Ascites Tumor

SaI was obtained from Dr. M. Phillips of New York University and weekly transferred intraperitoneally in B₆AF1 mice. The early history of the tumor is summarized in reference 3. Cells used in the experiments were collected between 4 and 7 days of tumor transfer. Animals were killed with chloroform and 1 ml of Gey-Tris injected into the peritoneal cavity. Cell suspensions were aspirated, centrifuged (5 min, 1,000 rpm, 4°C), and resuspended in Gey-Tris at 3×10^6 per ml.

⁵¹Cr Labeling of SaI Cells

1 ml of cell suspension was incubated with 100 μ Ci of Na₂⁵¹CrO₄ dissolved in 0.1 ml of 0.85% NaCl, for 40 min at 37°C with intermittent agitation. Cells were washed three times by centrifugation in Gey-Tris and resuspended in 1.5 ml SIK. Aliquots of the suspension were diluted 1:1 with SIK to which were added divalent cations or drugs. Cell suspensions were kept at 4°C until the adhesion assays performed within 30 min.

Coating of the Cover Slips

9 \times 22 mm cover slips (Bellco Glass, Inc., Vineland, N. J.) were used either untreated or were coated with serum or with gelatin solutions in 0.85% NaCl. Mouse serum was heat inactivated for 30 min at 56°C and stored at –20°C. Cover slips were placed horizontally and covered with 0.1 ml of the serum or gelatin solutions. After 30 min at 20°C the

cover slips were thoroughly rinsed in two baths (100 ml each) of 0.85% NaCl, drained, and immediately used in the adhesion assays without being allowed to dry.

Adhesion Assays

50 μ l of ⁵¹Cr-labeled SaI were dispensed on untreated cover slips and incubated for 20 min at 34°C. The cover slips were rinsed by immersion (six times) in each of three separate beakers containing 100 ml SIK, and drained. Radioactivity associated with the cover slips was measured in a gamma counter. Control experiments showed that washing in SIK as opposed to SIK with 1 mM Mn made no difference in the counts. In every experiment labeled SaI suspensions were centrifuged to determine release of label from cells. Counts in the supernates showed that between 10 and 30% of the counts were released after SaI were incubated for 30 min at 20° or 34°C. The causes of the variability could not be determined. All data were corrected for the release of ⁵¹Cr. Control experiments also showed that ⁵¹Cr leakage of adherent cells was similar to that in suspension. Additional experiments were designed to study the detachment of SaI previously attached to the substrate. In these experiments after the adhesion period and rinse, the monolayers were reincubated with 0.1 ml media for 20–40 min at 34°C. Controls for leakage were incubated for these additional periods.

Microscope Observations

After the last rinse, cell monolayers were fixed in 2% glutaraldehyde in 0.06 M phosphate-buffered saline pH 7.2. Wet mounts were examined and photographed at \times 400 and 1,000 magnification with a phase-contrast microscope.

RESULTS

The Basic Phenomenon

Approximately half of the cell-associated counts remained bound to untreated cover slips overlaid with ⁵¹Cr-labeled SaI in SIK, incubated for 20 min at 34°C, and rinsed. Table I shows that, in agreement with earlier work, washed cells adhered to naked glass in serum-free medium without any added divalent cations (40) whereas few cells adhered to cover slips pretreated with serum and thoroughly rinsed. In addition, 0.1 mM Mn added to the cells did not change ⁵¹Cr counts bound to untreated glass, while Mn markedly stimulated adhesion of SaI to the serum-coated cover slips. The present paper examines several features of this Mn-induced cell-to-substrate adhesion.

TABLE I
Effect of Mn on the Adhesion of SaI to Uncoated or to Serum-Coated Cover Slips

Treatment of cover slips	Adhesion medium	
	SIK	SIK with 0.1 mM Mn
None	52.8 ± 3.5 (14)*	54.3 ± 4.8 (13)
Serum†	1.6 ± 0.33 (7)	30.5 ± 5.9 (9)

* Adhesion in per cent of cell-associated counts initially layered on cover slips. Figures are mean ± standard error (number of experiments in parenthesis). Two to three cover slips used in each experiment.

† In this and in the following tables cover slips were coated with 6.7% mouse serum and adhesion was performed as in Materials and Methods.

Time-Course

Fig. 1 shows the course of the binding of SaI to untreated glass in SI MgK medium, to serum-treated cover slips in the same medium, and to serum-treated cover slips with 1 mM Mn added to SI MgK. Adhesion to the coated substrate in SI MgK was low even at the maximum time period examined of 30 min. It is also seen that near maximal adhesion occurred at 20 min; this was the incubation period used in further experiments. It would have been preferable to estimate initial rates of adhesion rather than adhesion capacity, but such a procedure would prohibitively limit the number of samples that could be assayed in any one experiment.

Dose Response of Mn

Fig. 2 again shows that Mn did not affect adhesion of SaI to untreated cover slips, while cells layered on serum-coated glass adhered in proportion to the Mn concentration added to the medium. Significant adhesion was obtained with 50 μ M Mn in SIK.

Temperature Dependence

Fig. 3 shows that in agreement with previous reports (10, 28) appreciable adhesion to uncoated glass in SIK Mn occurred even at 2°C. Near maximal adhesion occurred at 20° and 34°C with only a minor increment between 20° and 34°C. In contrast, Mn-stimulated adhesion to serum-coated

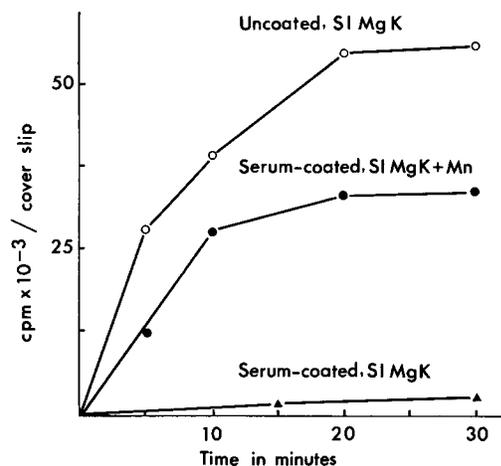


FIGURE 1 Time-course of the adhesion of SaI to uncoated or to serum-coated glass. Media for adhesion were SI MgK, with or without 1 mM Mn. In this and the following figures, unless otherwise noted, 6.7% mouse serum was used to coat the cover slips and adhesion was performed as described in Materials and Methods.

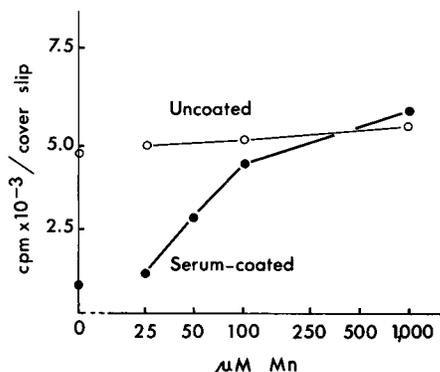


FIGURE 2 Relationship of concentrations of Mn (log scale) to adhesion of SaI to uncoated or serum-coated cover slips. Curves drawn as best fit by eye.

glass was negligible at 2°C, minor at 20°C, and sharply increased at 26° and 34°C. Adhesion to coated glass in the absence of Mn was less than 2% of the maximal possible counts at all temperatures (not shown in Fig. 3). In other systems, cell adhesion to serum- or plasma-coated glass was also sharply temperature dependent (10, 16, 28).

Requirement for Divalent Cations

It has been reported that adhesion of cells to serum- or to plasma-coated glass is dependent on

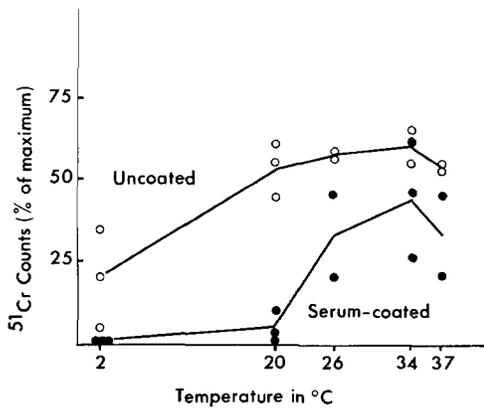


FIGURE 3 Temperature dependence of the adhesion, in SIK with 0.1 mM Mn, of SaI cells to uncoated or serum-coated cover slips.

Mg, although certain other cations can be substituted for Mg (2, 17). Table II shows the effect of divalent cations on the adhesion of SaI to serum-coated glass. It can be seen that, of the ions tested, only Mn markedly increased cell-to-substrate adhesion. The other cations were only minimally active or inactive. Thus, in the SaI system, Mn stimulates cell adhesion in a rather specific way and does not simply substitute for Mg.

Effect of Ca or Mg on the Mn-Stimulated Adhesion

Fig. 4 shows that Ca added to the medium inhibited the effect of Mn in a linear log-dose response fashion. In contrast, similar concentrations of Mg were clearly less inhibitory. It is of interest that Ca was more inhibitory than Mg in the uptake of Mn by erythrocytes (43). The inhibition by the divalents may explain why more Mn is needed to induce adhesion in media more complex than SIK. Thus, in a typical experiment, adhesion of SaI in SIK, Gey, or Dulbecco with 1 mM Mn resulted in, respectively, 3,810 \pm 173, 1,602 \pm 154, or 1,462 \pm 295 cpm bound to the serum-coated coverslips. With no Mn added, the counts were, respectively, 72 \pm 25, 178 \pm 12, or 120 \pm 5 (means \pm standard error of quadruplicate determination).

Role of Serum Concentration

In the previous experiments, the concentration of serum used to coat the glass substrate was 6.7% (vol/vol). The following experiments examined

TABLE II
Effect of Divalent Ions on Adhesion of SaI to Serum-Coated Cover Slips

Additions to the medium*	Adhesion†
Exp. A	
None	218 \pm 11 (4)
0.2 mM Mn	2,329 \pm 310 (4)
2.0 mM Mg	356 \pm 31 (4)
2.0 mM Ca	286 \pm 24 (4)
2.0 mM Mg, 2 mM Ca	354 \pm 28 (4)
Exp. B	
None	74 \pm 47 (4)
0.1 mM Mn	4,476 \pm 391 (4)
0.1 mM Co	152 \pm 19 (4)
0.1 mM Ni	128 \pm 14 (4)
0.1 mM Zn	202 \pm 11 (4)

* Medium was SIK in exp. A and STK in exp. B.

† Adhesion in counts per minute per cover slip \pm standard error (number of samples in parenthesis).

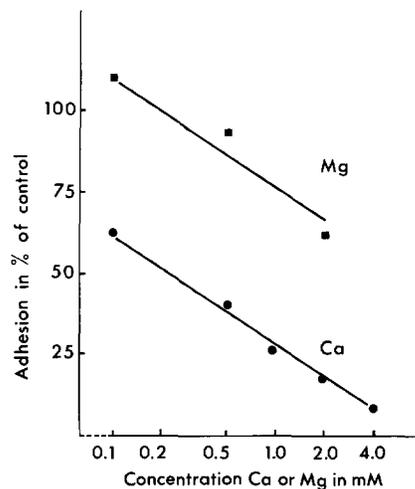


FIGURE 4 Effect of Ca or Mg on the Mn-dependent adhesion of SaI to serum-coated glass. Control adhesion (100%) was performed in SIK with 0.1 mM Mn. Curves were drawn as best fit by eye.

Mn-induced adhesion at different concentrations of serum. Fig. 5 shows that as little as 0.01% serum applied to glass detectably inhibited adhesion of SaI in SIK. Maximal inhibition was obtained with 0.1% serum and above. With 0.1 mM Mn in the medium the relationship of adhesion to serum concentration was more complex. As the concentration of serum was increased to 0.5%, adhesion was proportionately reduced. However, more cells ad-

hered with Mn than in its absence. Between 1 and 5% serum, adhesion increased and then leveled off between 5 and 100%. This biphasic response was confirmed in three other separate experiments and is compatible with the presence in serum of both an adhesion promoter and inhibitor.

SaI Adhesion to Glass Coated with Gelatin

Blood plasma has been reported to contain a β_1 globulin with high affinity for Mn (13). It was therefore of interest to determine whether a coat different from serum would permit the Mn-mediated adhesion. Gelatin was chosen because it is known to inhibit the adhesion of erythrocytes to glass (31). Fig. 6 shows that adhesion in SIK was entirely inhibited when the cover slips were pre-

treated with 20 $\mu\text{g}/\text{ml}$ or more gelatin. However, in the presence of Mn the inhibition by gelatin was partially reversed and adhesion leveled off between 50 and 1,000 $\mu\text{g}/\text{ml}$ gelatin. Therefore Mn induced adhesion not only to serum- but also to gelatin-coated cover slips.

Site of Action of Mn

The following experiments ask whether Mn irreversibly binds or in some way modifies the cell or the substrate. SaI were pretreated for 15 min at 34°C in SIK alone or with 0.1 or 1.0 mM Mn. The cells were then twice washed by centrifugation in SIK, resuspended in the SIK, and layered on serum-coated cover slips. Cover slip-associated radioactivity was negligible (20, 12, or 22 cpm, respectively, average of triplicates). In contrast, when other cell aliquots were preincubated in SIK or in SIK with 1 mM Mn, washed, and layered in SIK-1 mM Mn, the counts were, respectively, 545 and 520 per min. Thus the cells preincubated with Mn and washed did not bind irreversibly as shown by their inability to adhere to the substrate in the absence of Mn, whereas after similar preincubation, SaI did adhere when postincubated in the presence of Mn.

In other experiments uncoated and serum- or gelatin- (100 $\mu\text{g}/\text{ml}$) coated cover slips were incubated for 20 min at 34°C with SIK alone or with 2.0 mM Mn. The cover slips were rinsed and overlaid with SaI in SIK. Adhesion was less than 5% of that obtained when Mn was present in the adhesion medium. Thus there was no binding of Mn to the substrate that would survive washing.

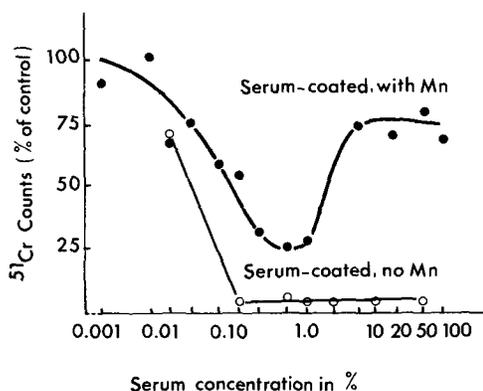


FIGURE 5 Relationship of serum concentration (log scale) used to coat cover slips to the adhesion of SaI in SIK alone or with 0.1 mM Mn. Control adhesion (100%) was the adhesion of SaI to uncoated glass in SIK. Curves drawn as best fit by eye.

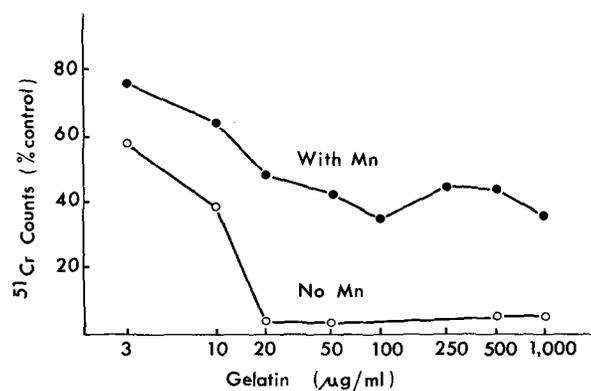


FIGURE 6 Relationship of gelatin concentration (log scale) used to coat cover slips to the adhesion of SaI in SIK alone or with 0.1 mM Mn. Control adhesion (100%) was the adhesion of SaI to uncoated glass in SIK.

The results, however, do not rule out a reversible interaction of Mn either with the cell surface, the substrate protein coat, or with both.

Removal of SaI Attached to Serum-Coated Glass

The following experiments tested whether the Mn-induced adhesion was reversible, i.e., whether SaI that had adhered could be removed from the substrate by further incubation in Mn-free medium. SaI were allowed to adhere to serum-coated cover slips at 34°C in SIK with 1 mM Mn. After 20 min the cover slips were rinsed. Some of these were not further treated; others were postincubated in SIK, or in SIK with 1 mM Mn for an additional 20 min at 34°C. A few cover slips were incubated in SIK for 40 min. All of the cover slips were rinsed and counted as in the standard procedure. Table III shows that post-incubation for 20 min in the absence of Mn reduced the glass-associated counts to about 30% of the control. This reduction did not occur in the presence of Mn. These findings were confirmed by microscope observations of the cover slips. In the absence of Mn fewer cells remained attached to glass, indicating that the reduction in the counts (Table III) is not due to more extensive leakage of ⁵¹Cr from SaI. EGTA, 0.1 mM, added to SIK in the postincubation, removed 85% of the counts after 20 min (not shown in Table III). EDTA has been used to differentially remove leukocytes from glass bead columns after the cell had attached in the presence of plasma (34).

Effect of Inhibitors on Mn-Mediated Adhesion of SaI

It has been shown that cytochalasins but not colchicine reduce cell-to-substrate adhesion (45). The effect of these and other drugs was also examined in the SaI system. Table IV shows that cytochalasin B and vinblastine markedly inhibited Mn-induced adhesion of SaI to glass. In contrast, only high concentrations of colchicine were effective. Table IV also shows that as little as 0.5 mM tetracaine was markedly inhibitory.

Microscope Observations

The quantitative measurements of SaI adhesion were supplemented by light microscope examination. After the adhesion assay cells were fixed in

TABLE III
Removal of SaI after Mn-Mediated Adhesion to Serum-Coated Substrate

Adhesion step*	Postincubation†	Adhesion‡
<i>min</i>		
20	none	3,721 ± 138 (4)
20	20 min in SIK	1,260 ± 214 (4)
20	20 min in SIK 1 mM Mn	3,493 ± 77 (4)
40	none	3,683 ± 170 (4)

* Adhesion in SIK with 1 mM Mn, 34°C.

† Postincubation at 34°C.

‡ Adhesion in counts per minute per coverslip ± standard error (number of samples in parenthesis).

glutaraldehyde as indicated in Materials and Methods, rinsed, wet mounted, and examined. When washed SaI adhered for 20 min at 34°C to untreated glass, over 95% of the cells were found to be rounded, quite refractile, and showed no cell processes on the glass (at × 1,000 magnification). Only a few cells showed small, gray, fingerlike, or stubby processes (Fig. 7 a). In the presence of 1 mM Mn, SaI on uncoated glass appeared markedly different. They were flatter and less refractile (Fig. 7 b). About 50% were rounded, with short processes or narrow veils often at one pole of the cells. The remaining were oval or polygonal, and showed broader veils all around or along part of the circumference; the veils were up to half of the cell diameter. In some cells the processes were branched and their tips had a ruffelike structure, more phase dense than the bulk of the cell process. Thus, while Mn did not increase glass-associated ⁵¹Cr counts (Table 1, Fig. 2) it did markedly affect the morphology of the cells on uncoated glass inducing the formation of cell processes and veils.

We also examined the appearance of SaI that adhered to serum-coated glass in the presence of different concentrations of Mn. In a typical experiment, with 50 μM Mn, more than 90% of SaI were rounded, but the cells were less refractile than those attached to uncoated glass in SIK. In addition, some 70% had short projections in a brushlike arrangement, unseen in the former cells. Some 20% of the cells had longer processes, often several per cell, with ruffles at their tips. Very few cells had aprons or veils all around their circumference. At 0.25 mM Mn, cells were less refractile

TABLE IV
Influence of Inhibitors on Mn-Mediated Adhesion of SaI to Serum-Coated Cover Slips

Drug	Concentration	Inhibition*
		%
Cytochalasin B ‡	10 µg/ml	72.6, 78.9
	20 µg/ml	52.4, 72.8, 84.4
	50 µg/ml	95.3
Vinblastine	6×10^{-6} M	40.9, 41.3, 50.6, 29.8
	2.5×10^{-5} M	59.3, 64.6
Colchicine	2.5×10^{-4} M	10.0, 15.8, 29.7
	1×10^{-3} M	29.7, 50.5
Tetracaine	5×10^{-4} M	93.5, 78.4
	2×10^{-3} M	97.8

* Each figure obtained in separate experiment with two to four cover slips for each concentration. Drugs were preincubated with SaI for 10–20 min at room temperature.

‡ Cytochalasin B was dissolved in DMSO at 5 mg/ml. DMSO at the concentrations used had no effect on the adhesion of SaI.

than in the preceding group. 0.75 of the cells had brushlike processes (Fig. 7 *c*); about half of the cells had veils that narrowly extended around the cell body, with a width about one-fifth that of the cell diameter. Processes and veils often projected at only certain points of the cell circumference and many had ruffles. At 1 mM, 0.75 of the cells were flattened and displayed more veils, processes, and ruffles than those at lower Mn concentrations. The appearance of the cells was similar to that of SaI on uncoated glass.

Observations were also made of SaI incubated on coated coverslips with 1 mM Mn in the presence of cytochalasin B, vinblastine, or tetracaine. In the presence of these inhibitors most cells were rounded, refractile, and the proportion of cells with processes and veils was reduced to some 10% of that of controls incubated in the absence of the inhibitors (Fig. 7 *d* and *e*).

We have shown (Table III) that SaI induced to adhere to coated cover slips, in the presence of Mn, detached when postincubated in SIK. In order to determine whether SaI rounded up before their detachment or detached in the spread configuration the following experiment was performed. SaI were attached to coated cover slips in the presence of 1 mM Mn, rinsed, postincubated in SIK for 2, 5, 10, or 20 min at 34°C, fixed in glutaraldehyde, and examined. Control cells fixed immediately after incubation with Mn showed characteristic cell flattening, processes, ruffles, and veils. After 2 min in SIK, SaI were more refractile and had thinner processes than the

controls. At 5 min most cells were rounded and refractile and had three to eight slender processes giving a spiderlike appearance (Fig. 7 *f*). At 10 min, only one-third of the cells had one to three slender processes with terminal ruffles. At 20 min most cells were rounded, refractile, showed no veils or ruffles, and less than 10% had thin, short, blunt processes left (Fig. 7 *g*). Similar observations were made on SaI that spread in SIK Mn on uncoated glass. After the reversal of spreading, SaI could once again be induced to spread by further exposure to Mn (Fig. 7 *h*).

These observations show that Mn induced spreading of SaI layered on uncoated cover slips. It induced both adhesion and spreading of SaI on serum-coated glass and the effects were proportional to the Mn concentration. Inhibitors of the adhesion also inhibited the spreading of the cells. SaI exposed to Mn and postincubated in SIK rounded up and retracted their processes and veils. Thus, both Mn-induced adhesion, as measured by the ^{51}Cr method, and SaI spreading, as observed microscopically, were reversible phenomena.

Adhesion of Mouse Erythrocytes

The experiments reported here and those of others (40) indicate that there are two components in the cell-to-substrate adhesion: the initial attachment, or "sticking," independent of divalent cations and relatively temperature insensitive; and the appearance of cell processes and veils—spread-

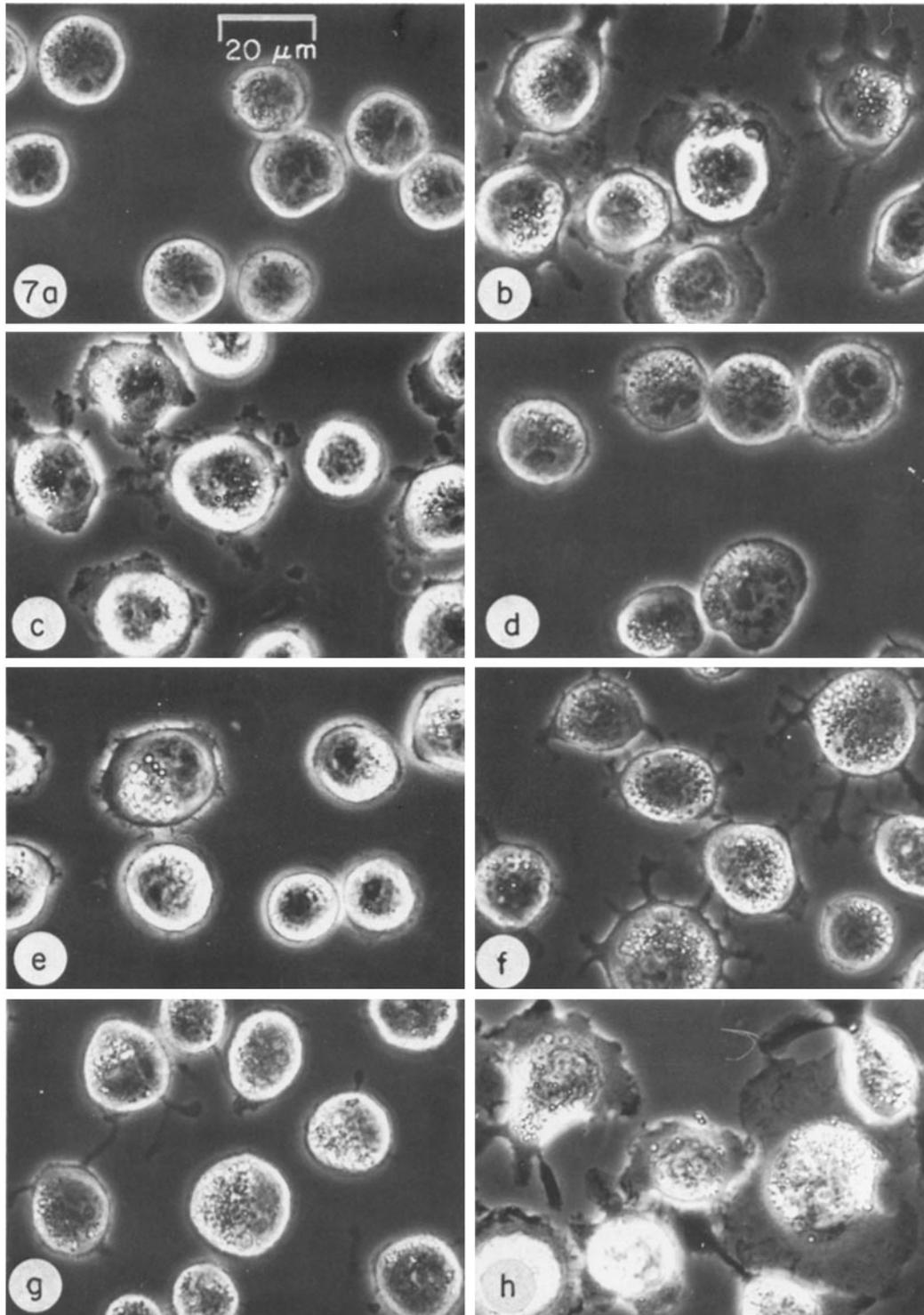


FIGURE 7 Phase-contrast micrographs of glutaraldehyde-fixed SaI. Bar equals $20\ \mu\text{m}$. Original magnification $\times 700$. (a and b) Cells layered on uncoated glass. (c-h) Cells layered on serum-coated glass. (a) SaI incubated in SIK for 20 min at 34°C . (b) SaI incubated under similar conditions with the addition of 1 mM Mn to the medium. (c) SaI treated with 0.25 mM Mn under similar conditions. (d and e) SaI treated with 1 mM Mn in the presence of $20\ \mu\text{g}/\text{ml}$ vinblastine or 1 mM tetracaine, respectively. (f and g) SaI incubated for 20 min in 1 mM Mn, rinsed, and postincubated for 5 or 20 min, respectively, in SIK alone. Fields selected to show cell processes. (h) SaI treated like cells shown in Fig. 7 g and further incubated for 20 min in 1 mM Mn.

ing—requiring divalent cations (Mn in the case of SaI) and more temperature sensitive. The former step is the main one involved in the adhesion to naked glass. Spreading or extensive spreading is not needed for adhesion because of the high affinity of the cell surface to naked glass. However, spreading is required for the adhesion to serum-coated surfaces, presumably because the stickiness of the cell surface to glass is inhibited by serum. We have shown that Mn markedly increased adhesion to serum-coated surfaces by increasing spreading, while the ion was not needed for sticking to naked glass. It was of interest to determine whether Mn would increase the adhesion of a cell unable to spread or to put out processes. We therefore examined the effect of Mn on the adhesion of mouse erythrocytes. It is known that washed red cells attach to naked glass and that this attachment is inhibited by serum (31, 46). Erythrocytes were labeled with ^{51}Cr , washed, and overlaid on uncoated or serum-coated cover slips for 20 min at 34°C. The cover slips were then rinsed and counted. In a typical experiment, 933 and 876 cpm were obtained on uncoated cover slips when the red cells were incubated in SIK, or in SIK with 0.2 mM Mn, respectively. Counts of serum-coated cover slips incubated with red cells in SIK, SIK with 1 mM Mn, 2 mM Mg, or 2 mM Ca were, respectively, 0, 8, 9, 1, and 0. Therefore, neither Mn nor Mg or Ca detectably enhanced the adhesion of mouse erythrocytes to serum-coated glass.

DISCUSSION

We have evaluated the effect of Mn on the adhesion of SaI to an uncoated or to a serum-coated glass substrate. Adhesion was measured by a radiometric procedure utilizing ^{51}Cr -labeled cells and the findings were supplemented by light microscope observations. The most significant results were: (a) Adhesion of SaI to the serum-coated substrate was far more stimulated by Mn than by other divalent cations examined, Ca, Mg, Co, Ni, or Zn. (b) Exposure to Mn increased the output of cell processes and veils, with cell flattening and spreading. (c) Mn-induced cell-to-substrate adhesion was sharply temperature dependent and was inhibited by Ca as well as by cytochalasin B, vinblastine, or tetracaine. (d) Mn-induced adhesion and spreading was reversible upon removal of the metal ion.

The attachment of SaI was examined both on untreated glass and on serum-coated glass. In the absence of Mn, SaI attached well to the former while adhesion to serum-coated glass was markedly

inhibited (Table I). With the technique used, adhesion to the naked substrate was not increased by Mn although the formation of cell processes and cell spreading was markedly stimulated. It is possible that if graduated distractive forces were applied it would be more difficult to detach SaI attached in the presence of Mn than in its absence. On the other hand, adhesion to a serum- or gelatin-coated substrate was strikingly increased by Mn in a dose-dependent fashion. Cell processes and spreading also paralleled Mn concentrations. We have previously shown that in micromolar concentrations Mn could favorably substitute for Mg in induced macrophage spreading while at higher concentrations Mn itself induced the spreading of the phagocytes without the need for other inducers (33). It is reasonable to assume that the action of Mn on SaI was similar to that on macrophages. Mg has also been shown to be the divalent required for the adhesion of other cells to serum- or plasma-coated glass (7, 39). Why didn't Mg stimulate adhesion of SaI? The answer seems to be that SaI are less adhesive cells as opposed to the highly adhesive macrophages and granulocytes. The specific stimulatory effect of Mn on adhesion and spreading is made more striking by the low adhesiveness of SaI. Our results therefore support the contention that the higher doses of Mn do not merely substitute for Mg but have an effect of their own (33).

The finding that Mn-stimulated adhesion to serum-coated glass paralleled an increased output of cell processes and extensions supports the conclusion of recent experimental and theoretical work that cell "probes" are needed to initiate and stabilize cell-to-substrate adhesion (19, 45, 48). These probes as well as the ensuing spreading would increase the area available for adhesion and make cell detachment more difficult. We have previously noted the correlation between features of cell-to-substrate "adhesion" and those for spreading of cells on glass (33). This conclusion is further supported by the finding that inhibitors of adhesion of SaI, namely cytochalasin B, vinblastine, or tetracaine (Table IV), also markedly reduced the output of cell processes and spreading.

Some Possible Mechanisms of Mn-Induced Adhesion

Establishment of cell processes and cell spreading is constrained by the opposing factors of cell surface adhesiveness to the substrate and cell re-

sistance to deformation (21). Increased adhesiveness to the substrate could result from Mn bound either to the substrate, to the cell surface, or to both. The finding that Mn induced the spreading of cells layered either on uncoated glass (or plastic) or on serum-coated substrate makes it unlikely that interaction with the substrate is relevant to adhesion and spreading. The peculiar dose response of serum (Fig. 5) was also found for the adhesion of leukocytes in experiments that did not involve Mn (10). Mn might interact with the surface of SaI to make it more "sticky." While this alternative cannot be excluded, it gains no support from the ineffectiveness of Mn in inducing adhesion of erythrocytes. It is therefore more likely that Mn induced spreading through increased cell deformability, a possibility that can be experimentally tested (47).

Morphological modulations of cell periphery, such as the formation of processes, pseudopodia, or cell spreading, may require the participation or the compliance of microtubules and/or microfilaments (18, 41, 50). Thus, spreading may involve the detachment and/or reattachment of actinlike filaments on the cell membrane (30), as well as the reassembly of microtubules. Inhibition of adhesion and spreading of SaI by cytochalasin B or by vinblastine points towards a role of both microfilaments and microtubules. However, the specificity and exact site of action of the drugs is not fully established (25, 38, 49). In agreement with previous results colchicine had a minor effect on adhesion at 0.25 mM, and only at 1 mM was partially inhibitory. We believe that the effect of such high doses is most likely nonspecific. Colchicine induces clear morphological changes on cultured cells (6, 9, 18), changes that may involve increased amoeboidism (6). In addition, cells attached and spread in a medium that contained 0.1 mM of the alkaloid (18). However, colchicine or vinblastine at low concentrations did inhibit cell-to-cell adhesion mediated by concanavalin A (5).

Since there is not enough information in support of a single hypothesis of the effect of manganese, it may be useful to mention some possible alternatives. One possible way to relate the effect of Mn to the function of microfilaments and microtubules is through an inhibitory action on calcium fluxes. The role of Ca in the contraction-relaxation cycle of muscle cells is well established (4) and Ca may fulfill a similar role in the motility of other

cells (29, 53). There is also evidence that *in vitro* microtubular reassembly is inhibited by Ca (44). Functional effects of Mn in several other systems are antagonized by calcium. Examples are Mn-inhibited neuromuscular transmission (27) and heart muscle excitation and contraction (24, 37, 52). More recently, Mn was found to reduce the uptake of Ca by chick embryo fibroblasts (29). These considerations suggest that Mn could induce adhesion and spreading of SaI by inhibiting Ca fluxes between as yet nonidentified compartments, with subsequent effect on contractile and microtubular proteins. This hypothesis is compatible with the inhibition by Ca of Mn-stimulated adhesion of SaI (Fig. 4). It should be emphasized that while the medium used for the adhesion studies contained no added Ca, traces of the metal could be present due to leakage from cells, contamination from chemicals, or from the substrate (reference 4, page 60). The inhibitory effect of tetracaine on Mn-stimulated adhesion may be explained by competition of the drug for Mn binding sites (35, 43). However, the effect of tetracaine could be related to some other action than on Ca binding sites. It is of interest that another local anesthetic, procaine, was recently shown to inhibit cell motility (5).

Alternatives to an inhibitory action of Mn are models based on a "positive" effect of the ion. It is known that Mn activates a wide range of enzymes (42) and has metabolic effects on isolated mitochondrial fractions (12), tissue slices (36), and perfused organs (14). Mn could act directly on the contractile machinery (26) or on the microtubular assembly. It has recently been reported that cyclic AMP increases cell-to-substrate adhesion (23). In view of the activation of nucleotide cyclases by Mn (11, 22), the possibility should be considered that the adhesion promoted by Mn is mediated through an increase in cyclic nucleotide concentration.

Further information is needed before a choice can be made between these and other mechanisms. However, the effect of Mn may provide a useful tool in the analysis of events involved in cell-to-substrate adhesion, cell spreading, and cell motility (1, 50, 51).

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