Surface Components Involved in Virally Mediated Membrane Changes

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1. Virally mediated permeability changes are not brought about by neuraminidase or other enzyme. They are not mimicked by local anaesthetics such as benzyl alcohol or dibucaine. 2. Virus induces an increase in the exchange rate of membrane-bound Ca^{2+} . The temperature-dependence, and sensitivity to concanavalin A, of the permeability change and of Ca^{2+} exchange are similar. 3. It is concluded that an increase in the exchangeability of membrane-bound Ca^{2+} constitutes an early and specific event after the attachment of Sendai virus to cultured cells.

The effects of Sendai virus on cultured cells constitute a useful system for studying certain aspects of plasma membranes, as well as of events involved in viral infection. The nature of the surface components that are initially involved is as yet unclear. It has been shown that the permeability changes (Klemperer, 1960*b*; Pasternak & Micklem, 1973, 1974*a,b*) are not due to an alteration in phospholipid turnover (Falke *et al.*, 1967; Elsbach *et al.*, 1969; Pasternak & Micklem, 1974*a*). It is possible that some of the permeability changes underlie the haemolytic action of paramyxoviruses (Klemperer, 1960*b*), and are related to their neuraminidase activity (Klemperer, 1960*a*). The ability of exogenous neuraminidase, as well as of other enzymes, to elicit the permeability changes has accordingly been tested; the results are described in the present paper.

Another approach is to compare the effect of virus with that of cationic local anaesthetics, since interaction of the latter with surface membranes is well documented, as is their ability to cause permeability changes in a number of systems (Seeman, 1972; Papahadjopoulos, 1972). Benzylamine and dibucaine have been studied and their effects are reported below.

Finally the effect of Ca^{2+} in preventing the virally mediated permeability changes (Klemperer, 1960*b*; Pasternak & Micklem, 1974*a*; Pasternak *et al.*, 1976) has been examined. It appears that cells pre-labelled with $^{45}\text{Ca}^{2+}$ lose radioisotope in a temperature-dependent manner and that this loss is accelerated by virus. A possible sequence of events after the addition of Sendai virus to cultured cells is presented.

Experimental

Materials

Sendai virus and Lettree cells were obtained as previously described (Pasternak & Micklem, 1974*a*). Neuraminidase (Sigma, type V from *Cl. perfringens*)

was given by Dr. G. B. Robinson, Dept. of Biochemistry, University of Oxford; dibucaine (Nupercaine; CIBA) was given by Professor H. P. Rang, Dept. of Pharmacology, St. George's Hospital Medical School, London; it was dissolved in ethanol and further diluted in water. The highest concentration of ethanol present (4%, v/v) was without effect on leakage, passive uptake or facilitated uptake.

Methods

The relation between neuraminidase activity and leakage was tested as follows. A mouse bearing ascitic Lettree cells was injected with [$1\text{-}^3\text{H}$]glucosamine hydrochloride (100 μCi ; 0.03 μmol) and cells were removed 48 h later. The washed cells were incubated with [$\text{Me-}^{14}\text{C}$]choline (0.4 $\mu\text{Ci}/\text{ml}$) for 30 min at 37°C, washed and exposed to Sendai virus or neuraminidase in phosphate-buffered saline (Dulbecco & Vogt, 1954), pH 5.5. Cells were sampled over 30 min at 37°C, centrifuged (5000*g*-min) and the pellets extracted with cold 5% (w/v) trichloroacetic acid. The rate of release of ^{14}C (from trichloroacetic acid-soluble material) is taken as a measure of leakage (predominantly of phosphoryl- ^{14}C]choline; Pasternak & Micklem, 1973), and the rate of release of ^3H from trichloroacetic acid-insoluble material (containing bound [^3H]glucosamine and [^3H]sialic acid) as a measure of sialic acid hydrolysis, after the rate of ^3H release in the absence of virus of neuraminidase has been subtracted.

Leakage of radioisotope from cells preincubated with all radioisotopes was measured as previously described (Pasternak & Micklem, 1974*a*); displacement of $^{45}\text{Ca}^{2+}$ from cells preincubated with $^{45}\text{CaCl}_2$ (5 Ci/ml; 0.1 mM) was measured in the same way. Uptake of radioisotope by cells was measured as follows. Cells and label, in a final volume of 0.2 ml, were incubated with virus, dibucaine or other additive and the uptake was 'quenched' at various times by the addition of 10 ml of phosphate-buffered

saline. The suspension was centrifuged (5000g-min) and the pellet assayed for radioactivity as previously described (Pasternak & Micklem, 1974a).

Results

Effects of neuraminidase on leakage

Sendai virus possesses a neuraminidase (Frankel-Conrat, 1969, p. 103) which causes cleavage of certain sialic acid groups during the initial membrane modification (Table 1). The addition of bacterial neuraminidase to Lettree cells, such that an equivalent amount of sialic acid groups is released, has no effect on leakage (Table 1). Subject to the possibility that viral neuraminidase and bacterial neuraminidase are attacking different surface molecules, which is unlikely, the results of Table 1 indicate that leakage is not due to the action of viral neuraminidase.

It is possible that leakage is caused by virally activated cell proteinases. This has not been tested directly, but the addition of trypsin (Flow Laboratories Ltd., Irvine, Ayrshire KA12 8NB, U.K.; 50 µg/ml) to Lettree cells (8×10^6 /ml) does not cause any leakage from prelabelled cells. Neither does the addition of phospholipase C from *Bacillus cereus* (Makor Chemicals, Jerusalem, Israel), which is non-cytolytic, although in this case any enzymic modification that might have occurred was below the limit of detection (less than 5% of total phosphatidylcholine hydrolysed by 50 µg of phospholipase C/ml).

Effect of local anaesthetics

The ability of local anaesthetics to mimic the effect of Sendai virus in stimulating passive diffusion concomitantly with inhibiting facilitated diffusion was tested. Passive diffusion was measured by leakage of intracellular metabolites such as phosphorylcholine or 2-deoxyglucose 6-phosphate (Pasternak & Micklem, 1973, 1974a) as well as by uptake of choline at high concentration (Pasternak & Micklem, 1974b; Pasternak *et al.*, 1976). This allows one to assess any lysis that accompanies, or results from, changes in permeability; thus an increase in leakage is likely to be due to lysis or other cell damage, unless it is accompanied by an increase in passive uptake. As shown in Table 2, the latter criterion is fulfilled by Sendai virus but not by dibucaine. Hence the apparent inhibition of facilitated uptake by dibucaine is likely to be due merely to its lytic action, as is the increased leakage (Table 2). Measurement of volume changes with a Coulter Counter confirms that cells in the presence of 1 mM-dibucaine (but not of virus) begin to lyse.

An effect opposite to that of dibucaine, namely stimulation of passive uptake without concomitant

inhibition of facilitated uptake, is seen with certain batches of Sendai virus. Since biological activity, e.g. ability to cause cell-cell fusion, is normal in such batches, it is possible that stimulation of passive uptake is a better criterion of a true permeability change and is more closely related to infectivity than is inhibition of facilitated uptake. Moreover, the latter activity gradually appears on repeated freezing and thawing. It thus resembles haemolytic activity, which is absent from 'early harvested' Sendai virus, but appears on freezing and thawing (Homma *et al.*, 1976), and supports the notion (Homma *et al.*, 1976) that haemolytic activity is not an essential component of infective virus. Another difference between dibucaine and Sendai virus is seen in the effect of Ca^{2+} ; virally mediated changes only are reversed (Table 2), as might be expected if the action of dibucaine is essentially lytic.

One may conclude that the effects of dibucaine and Sendai virus on Lettree cells are different. Benzyl alcohol at concentrations up to 1 mM is without effect on Lettree cells; at 10 mM some stimulation of leakage occurs.

Local anaesthetics such as dibucaine, which weaken membranes at high concentration, stabilize membranes at lower concentrations (Seeman, 1972). In the case of membrane weakening by Sendai virus, however, the addition of dibucaine at concentrations between 0.001 and 0.1 mM caused no relief of the permeability changes (Table 2).

Displacement of Ca^{2+}

When Lettree cells are incubated with 0.1 mM- $^{45}\text{CaCl}_2$ at 37°C, radioisotope is fixed to cells. The amount remaining after three washes in phosphate-buffered saline is equivalent to a concentration of

Table 1. *Effect of Sendai virus and neuraminidase on Lettree cells*

Cells labelled by incorporation of [^3H]glucosamine *in vivo*, followed by uptake of [^{14}C]choline *in vitro*, were exposed to Sendai virus or neuraminidase and initial rates of ^3H and ^{14}C release were measured as described under 'Methods'. Leakage of ^{14}C by control cells was 0.25% of total in cells/min; loss of ^3H by control cells was 2.9% of total bound to cells/min. HA, Haemagglutinating units.

Addition	Concentration	Leakage of	Hydrolysis of
		[^{14}C]choline metabolites	bound [^3H]sialic acid
		1.0	0.0
Virus	(HA units/ml)		
	7	5	0.3
	70	12	1.2
	(µg/ml)		
Neuraminidase	10	1.0	0.5
	100	1.5	1.2

Table 2. Effect of Sendai virus and dibucaine on permeability changes in Lettree cells

Uptake was measured by exposure of cells in the presence of virus or dibucaine to 2-deoxy³H]glucose (1–10 μ Ci/ml; <0.3 μ M) (facilitated uptake) and [*Me*-¹⁴C]choline (0.1–0.5 μ Ci/ml; 20–50 mM) (passive uptake). Leakage was measured by preincubating cells for 30 min in the presence of 2-deoxy³H]glucose or [*Me*-³H]choline and treating the washed cells with virus or dibucaine. Other details were as described under 'Methods'. The rates of uptake and leakage were calculated from data obtained over a 20 min period of incubation. The initial rate of facilitated uptake of ³H by control cells was 12000–120000 d.p.m./min per 10⁶ cells; the initial rate of passive uptake of ¹⁴C by control cells was 6–23 d.p.m./min per 10⁶ cells. The initial rate of leakage of deoxy³H]glucose metabolites by control cells was 2% of total in cells/min; initial rate of leakage of [*Me*-³H]choline metabolites was 0.3% of total in cells/min. N.D., Not determined; HA, haemagglutinating unit.

Addition	Concentration	Relative rate		
		Facilitated uptake	Passive uptake	Leakage
—	—	100	100	100
Virus	10 ⁻⁵ HA unit/cell	34	330	190*
Virus	10 ⁻⁵ HA unit/cell	90	130	80*
+Ca ²⁺	+2 mM			
Dibucaine	0.2 mM	90	100	200*
	1.0 mM	61	97	250*
	2.2 mM	14	82	N.D.
+Ca ²⁺	2.2 mM + 2 mM	9	82	N.D.
Virus	10 ⁻⁵ HA unit/cell	N.D.	N.D.	370†
Virus	10 ⁻⁵ HA unit/cell			
+dibucaine	+1 μ M	N.D.	N.D.	540†
+dibucaine	+10 μ M	N.D.	N.D.	400†
+dibucaine	+0.1 mM	N.D.	N.D.	430†

* Preincubation with deoxy³H]glucose.
† Preincubation with [*Me*-³H]choline.

Fig. 1. Effect of Sendai virus on ⁴⁵Ca²⁺ exchange and leakage by Lettree cells

Cells, preincubated as described in the legend to Table 3, were incubated at 27°C. The amount of ⁴⁵Ca or ³H released is expressed as a percentage of the total present in the cells at zero time. ●, ³H release with virus; ○, ³H release without virus. ■, ⁴⁵Ca release with virus; □, ⁴⁵Ca release without virus.

10 mM-EDTA in the washing medium. It therefore represents a true uptake of Ca²⁺ by certain cellular components. These are unlikely to be within the cell, since normal concentrations of intracellular Ca²⁺ are generally in the range of 100–10 nM (Rasmussen, 1970; Borle, 1973). It is therefore likely to represent calcium bound to the outside of the cell. A similar conclusion has been reached with regard to Ca²⁺ taken up by mouse lymphocytes during incubation at 37°C (Freedman *et al.*, 1975). Such conclusions are supported by the observation that during immune cytolysis of ⁴⁵Ca²⁺-labelled Lettree cells (when more than 50% of the intracellularly accumulated [⁵¹Cr]chromate is released; Pasternak & Micklem, 1973), no loss of ⁴⁵Ca²⁺, over that occurring in the absence of antiserum or complement (see below), takes place. The uptake of ⁴⁵Ca²⁺ is increased only slightly by the presence of virus, which confirms an earlier report on the failure of Ca²⁺ to enter cells made 'leaky' by virus (Pasternak & Micklem, 1974a) and strengthens the supposition that uptake reflects binding to the cell surface.

On incubation above 22°C, the ⁴⁵Ca²⁺ that is bound is rapidly displaced (Fig. 1), presumably by exchange with exogenous cations, as is to be expected from the fact that cells become labelled in the first place. The displacement is relatively non-specific, in that it occurs almost as rapidly in the presence of 5 mM-EDTA (sodium salt) as in the presence of

approx. 10 μ M (taking cell water as 80% of cell volume). The value remains constant during further washes, and is unaffected by the inclusion of

Table 3. *Effect of Sendai virus on $^{45}\text{Ca}^{2+}$ exchange and leakage by Lettree cells*

Cells were preincubated for 30 min in the presence of [$\text{Me-}^3\text{H}$]choline ($0.1\ \mu\text{M}$) and $^{45}\text{CaCl}_2$ ($0.1\ \text{mM}$), washed and exposed to virus (10^{-5} HA units/ml) and other additive as indicated. Rates of $^{45}\text{Ca}^{2+}$ exchange and leakage were calculated from data obtained over a 20 min period of incubation at 32° or 37°C , and are expressed relative to the rate shown by controls in the absence of virus. The initial rate of $^{45}\text{Ca}^{2+}$ exchange by control cells was 2.7–4.9% of total bound to cells/min; the initial rate of leakage of [$\text{Me-}^3\text{H}$]choline metabolites was 0.24–0.75% of total in cells/min. All values quoted are means of two or more experiments.

Addition	Concentration	Relative rate of $^{45}\text{Ca}^{2+}$ exchange		Relative rate of leakage	
		–Virus	+Virus	–Virus	+Virus
EDTA	—	100	119	100	480
	5 mM	90	124	86	800
Citrate	10 mM	112	155	104	420
Ca^{2+}	2 mM	123	127	120	160
Concanavalin A	20 $\mu\text{g/ml}$	93	93	120	160
Anti-Lettree serum*	6 mg of protein/ml	102	143	107	840

* Heated at 55°C for 30 min to destroy complement; in 5 mM-EDTA.

Fig. 2. *Temperature-dependence of $^{45}\text{Ca}^{2+}$ exchange and leakage by Lettree cells*

Cells, preincubated as described in the legend to Table 3, were incubated at the temperature indicated and sampled over appropriate time-periods. The rate of leakage (in terms of percentage released, as for Fig. 1), and approximate lag, were measured and are plotted as previously described (Pasternak & Micklem, 1973): ●, ^3H release with virus; ■, ^{45}Ca release with virus; □, ^{45}Ca release without virus.

2 mM- CaCl_2 (Table 3). In other words, $^{45}\text{Ca}^{2+}$ is displaced and is replaced by either $^{40}\text{Ca}^{2+}$ or Na^+ .

Exchange of $^{45}\text{Ca}^{2+}$ is stimulated by virus, an additional 30–40% of bound $^{45}\text{Ca}^{2+}$ being displaced (Fig. 1; Table 3). This corresponds very approximately to an additional 10^5 atoms of Ca^{2+} /cell. As with leakage (Pasternak & Micklem, 1974a), displacement of $^{45}\text{Ca}^{2+}$ is maximum in the presence of EDTA and virus; neither EDTA, citrate, anti-Lettree serum or concanavalin A on its own effects displacement, although concanavalin A prevents the virally mediated increase in exchangeability (Table 3). The effect of calcium itself is different. Although it prevents leakage, it has little effect on $^{45}\text{Ca}^{2+}$ exchange by virus. This is actually what one might predict in view of the fact that Ca^{2+} is required for cell-cell fusion.

The temperature-dependence of Ca^{2+} exchange in the presence and absence of virus was examined. Figs. 1 and 2 show that in each case, the lag period and rate of displacement are similar to the lag period and rate of leakage of intracellularly accumulated

[^3H]choline caused by virus. In each case the rate has a Q_{10} (rate at $T+10^\circ\text{C}$ /rate at $T^\circ\text{C}$) of above 3 (3.3–5.6) above 22°C . Below that temperature, the plot of the rate of [^3H]choline leakage shows a discontinuity (Fig. 2a and Pasternak & Micklem, 1973).

Discussion

The present results indicate that the early stages of membrane modification by Sendai virus do not appear to be caused by viral neuraminidase (Table 1) or other enzyme. What then is the reason for the temperature-dependence (Pasternak & Micklem, 1973) of the permeability change? The finding that residual Ca^{2+} bound to the cell surface after repeated washing at room temperature is displaced (by exchange with exogenous cations) in an entirely similar temperature-dependent manner (Fig. 2), and that the exchange is accelerated by virus (Figs. 1 and 2; Table 3), seems relevant. Since the anionic groups to which residual Ca^{2+} is bound are likely to be predominantly phospholipid (Williams, 1975), the

Scheme 1. Virally mediated cell-surface changes

temperature-dependence of the exchange presumably reflects a change in the 'accessibility' of the phospholipid head groups. How this is related (a) to a change in the permeability properties of the cell and (b) to the effect of virus is not clear.

(a) It is difficult to envisage how an increase of less than 50% in the exchange rate of $^{45}\text{Ca}^{2+}$ by virus causes a permeability change of over 400% (Table 3). Perhaps there are two classes of calcium-binding site. Displacement of Ca^{2+} from the major site (achieved by incubation in the absence of virus) does not lead to increased permeability; displacement from the minor site (by incubation with virus) does. Against this hypothesis is the observation that preincubation of $^{45}\text{Ca}^{2+}$ -labelled cells in phosphate-buffered saline for 30 min at 37°C (during which time approx. 50% of bound $^{45}\text{Ca}^{2+}$ is released) results in a further virally mediated release of approx. 40%. In other words, virus displaces a constant percentage of surface-bound Ca^{2+} and this does not fit a 'two-site' hypothesis. Nor is a 'threshold' effect indicated. Thus the permeability changes (in the presence of virus) follow the same time-course as Ca^{2+} exchange (Fig. 1); that is, they are manifest long before the maximum Ca^{2+} displacement achievable in the absence of virus is reached. It is therefore unlikely that displacement of Ca^{2+} is a direct cause of the permeability changes. Rather it may be a consequence of some other virus-membrane

interaction that results in a concomitant triggering of passive permeability and increased $^{45}\text{Ca}^{2+}$ exchange.

That the permeability changes are related to Ca^{2+} binding is indicated by the fact that they are prevented by exogenous Ca^{2+} (1–2 mM) (Pasternak & Micklem, 1974a; Pasternak *et al.*, 1976). Moreover, the possibility that the permeability changes and calcium displacement are somehow involved in viral entry, which occurs by fusion of the viral envelope with the cell surface, lends support to the hypothesis of Poste & Allison (1973) about the role of Ca^{2+} in membrane fusion in general. However, it must be stressed that since viral entry, and at high cell concentrations intercellular fusion, each occurs in the presence of Ca^{2+} , the permeability changes should be regarded not so much as a trigger in itself, but rather as a reflexion (observable only in the absence of Ca^{2+}) of the actual destabilization event (Scheme 1).

(b) The molecular events that occur between viral attachment, which is instantaneous and occurs at 0°C , and the temperature-dependent changes, is unclear. Certainly virus does not displace Ca^{2+} at 0°C (Fig. 2) nor does exogenous Ca^{2+} prevent viral attachment (S. Knutton, D. Jackson, K. J. Micklem & C. A. Pasternak, unpublished work). In other words, the initial binding of virus, to neuraminidase-sensitive components (Fraenkel-Conrat, 1969, p. 184), merely alters the membrane so as to make it potentially 'leaky' and more susceptible to Ca^{2+} exchange, when the temperature is raised. Note that neither EDTA (Pasternak & Micklem, 1974a) nor citrate is able to elicit these changes (Table 3).

Scheme 1 summarizes the present results. Recovery from membrane destabilization has been included to accommodate the observation that HeLa cells regain their initial permeability characteristics within 4 h after a 10 min exposure to Sendai virus (Pasternak *et al.*, 1976). Scheme 1 also accommodates the finding that the permeability changes and associated events are quite distinct from subsequent cell-cell fusion, as indicated by a differential sensitivity to EDTA and to cytochalasin B (Pasternak & Micklem, 1973, 1974a).

Some of the changes after viral attachment resemble those that occur during immune cytolysis of cells, and a connexion between the two processes has been suggested (Holmes *et al.*, 1969). Certainly cells do undergo permeability changes and transient swelling (Green *et al.*, 1959; Micklem & Pasternak, 1977) during immune cytolysis. The role of Ca^{2+} , however, is different. Although Ca^{2+} prevents detection of the virally mediated changes (Scheme 1), it is required for immune cytolysis (Muller-Eberhard, 1975). Moreover, virally mediated permeability changes do not result in lysis (Pasternak & Micklem, 1973). A closer analogy may be with the establishment of intercellular junctions. These too allow passive diffusion of cations and intracellular metabolites

to take place (Pitts, 1976), and the 'gates' through which this occurs are also closed by calcium (Rose & Lowenstein, 1975).

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References

- Borle, A. B. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1944-1950
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167-173
- Elsbach, P., Holmes, K. V. & Choppin, P. W. (1969) *Proc. Soc. Exp. Biol. Med.* **130**, 903-908
- Falke, D., Schiefer, H. G. & Stoffel, W. (1967) *Z. Naturforsch.* **B22**, 1360-1362
- Fraenkel-Conrat, H. (1969) *The Chemistry and Biology of Viruses*, Academic Press, New York
- Freedman, M. H., Raff, M. C. & Gomperts, B. (1975) *Nature (London)* **255**, 378-382
- Green, H., Barrow, P. & Goldberg, B. (1959) *J. Exp. Med.* **110**, 699-713
- Holmes, K. V., Klenk, H. D. & Choppin, P. W. (1969) *Proc. Soc. Exp. Biol. Med.* **131**, 651-657
- Homma, M., Shimizu, K., Shimizu, Y. K. & Ishida, N. (1976) *Virology* **71**, 41-47
- Klemperer, H. G. (1960a) *Virology* **12**, 495-498
- Klemperer, H. G. (1960b) *Virology* **12**, 540-552
- Micklem, K. J. & Pasternak, C. A. (1977) *Proceedings of the Coulter Conference, London, April 1975*, in the press
- Muller-Eberhard, H. J. (1975) *Annu. Rev. Biochem.* **44**, 697-724
- Papahadjopoulos, B. (1972) *Biochim. Biophys. Acta* **265**, 169-186
- Pasternak, C. A. & Micklem, K. J. (1973) *J. Membr. Biol.* **14**, 293-303
- Pasternak, C. A. & Micklem, K. J. (1974a) *Biochem. J.* **140**, 405-411
- Pasternak, C. A. & Micklem, K. J. (1974b) *Biochem. J.* **144**, 405-595
- Pasternak, C. A., Strachan, E., Micklem, K. J. & Duncan, D. (1976) in *Cell Surfaces and Malignancy* (Mora, P. T., ed.), pp. 147-151, U.S. Government Printing Office, Washington, D.C., Fogarty International Center Proceedings no. 28
- Pitts, J. D. (1976) in *Developmental Biology of Plants and Animals* (Graham, C. F. & Wareing, P. F., eds.), pp. 96-110, Blackwell Scientific Publications, Oxford
- Poste, G. & Allison, A. C. (1973) *Biochim. Biophys. Acta* **300**, 421-465
- Rasmussen, H. (1970) *Science* **170**, 404-412
- Rose, B. & Loewenstein, W. R. (1975) *Nature (London)* **254**, 250-252
- Seeman, P. H. (1972) *Pharmacol. Rev.* **24**, 583-655
- Williams, R. J. P. (1975) in *Biological Membranes* (Parsons, D. S., ed.), pp. 106-121, Clarendon Press, Oxford