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Solubilization of Initial Attachment Site Activity for Avian Tumor Viruses with Lithium Diiodosalicylate (39637)[1]

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Introduction. Since membrane constituents are frequently denatured during isolation, preservation of their biologic activity after solubilization provides a unique opportunity to study cell surface components *in vitro*. This report describes the solubilization of a cell surface attachment site for avian tumor viruses (ATV) from chicken embryo fibroblast (CEF) plasma membranes.

Attachment to the cell membrane by a virus must be the first event in cellular infection. The Rous sarcoma virus genome (RSV), with its Rous-associated virus envelope RSV(RAV-1) is the model we have used to investigate the early phases of ATV-cell interactions. Initial attachment is dependent, in the absence of polycations, upon intact viral surface glycoproteins (1). Interactions of the viral envelope and cell surface also determine the host range and interference patterns of the individual ATV subgroups (2). Cellular resistance to each virus subgroup is controlled by autosomal genes (3), and susceptibility is dominant. Chicken embryo fibroblasts resistant to a particular ATV subgroup are still able to adsorb that virus (4); resistance is dependent upon later surface events which may correspond to viral penetration (2). In order to gain insight into the cell surface events which influence the success of infection and determine some aspects of membrane structure, methods were sought which would solubilize the initial attachment site for ATV, while preserving its activity. Marchesi and Andrews (5) have reported that lithium diiodosalicylate would solubilize the principal glycoproteins of the human erythrocyte and preserve their influenza-binding activity. This compound was therefore utilized to solubilize attachment site activity for ATV from CEF plasma membranes.

Methods and materials. Reagents. Ribonuclease-free sucrose was obtained from Schwartz-Mann, Orangeburg, N.Y. All sucrose solutions (w/w) were prepared in TEN buffer (0.01 *M* Tris, 0.1 *M* $NaCl_2$, and 0.001 *M* EDTA, pH 7.4) and sterilized by filtration. Sodium [^{3}H]borohydride (490 mCi/mmole) was purchased from Amersham-Searle; [5-^{3}H]uridine (5 Ci/mmole) and [^{3}H]glucosamine hydrochloride (5–15 Ci/mmole) were from New England Nuclear, Boston, Mass. Lithium 3,5-diiodosalicylate (LIS) was obtained from Eastman Organic Chemicals, Rochester, N.Y. or synthesized according to Marchesi and Andrews (5); each preparation of LIS appeared equally active.

Cell culture and media. Tissue culture media and calf sera were purchased from Grand Island Biological Company, Grand Island, N.Y. Primary chicken embryo fibroblast cultures were prepared as previously described (1, 6) from 9-day-old gs- chick embryos obtained from SPAFAS, Inc., Rockford, Ill. Cells were passed 3 to 5 days after initial seeding and grown to confluence either for use in binding assays or for preparation of plasma membrane fractions. Secondary cells more than 7-days-old were not used. In selected experiments, secondary CEFs were treated sequentially with pyridoxal phosphate (Sigma) and sodium [^{3}H]borohydride according to Rifkin *et al.* (7) or grown in the presence of 50 μCi/ml of [^{3}H]glucosamine for 48 hr before collection.

Virus preparation and purification. Bryan strain Rous sarcoma virus and Rous-associated viruses RAV-1 and RAV-2 were used

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to prepare pseudotype RSV(RAV-1) and RSV(RAV-2) as described elsewhere (1). Supernatant fluid from infected cultures was replaced with media containing 20 μCi/ml of [^{3}H]uridine and harvested at 12-hr intervals. The virus-containing supernatant fluid was centrifuged to remove cell debris and concentrated by centrifugation over a discontinuous 20/60% sucrose gradient as previously described (8). Focus-forming units (FFU) were measured by established procedures (6).

Binding assay. Viral attachment to CEF plasma membranes were measured as reported elsewhere (1). Briefly, 200 μg (protein) of CEF plasma membrane was incubated with labeled ATV in TEN buffer at 4° for 25 min. These samples were then adjusted to 45% sucrose, layered upon a 60% sucrose cushion, and overlaid with 40 and 10% sucrose. This mixture was centrifuged at 90,000*g* for 20 min. Fractions were collected from the bottom, and each tube was assayed for acid precipitable counts per minute. CEF membranes and membrane-bound virus migrate to the 10/40% interface, while unbound virus remains with the 45% sucrose. This procedure is highly reproducible. Binding is dependent upon virus concentration, membrane protein concentration, and the presence of intact viral glycoproteins (1). The soluble attachment site material was assayed by incubating the labeled virus in the presence of fractions extracted (see below) from CEF membranes for 30 min at 4°, then adding intact CEF plasma membranes, and measuring attachment as above. Activity was quantified by the reduction in the amount of labeled virus bound after incubation with the LIS solubilized attachment site, compared to the amount bound after incubation in buffer but in the absence of the LIS solubilized site.

Solubilization of attachment site activity with LIS. CEF plasma membranes were prepared by a modification (1) of the procedure of Boone *et al.* (9). Approximately 5–10 mg of CEF plasma membranes (10- to 20-fold enriched for plasma membrane markers) was suspended in 0.3 *M* buffered LIS (0.05 *M* Tris, 0.1 *M* NaCl, pH 7.4). After being stirred at room temperature for 15 min, the supernatant fluid from this mixture was extracted with phenol, dialyzed, lyophilized, and washed with ethanol as described by Marchesi and Andrews (5). The pellet from the ethanol wash was dialyzed against TEN buffer, and particulate material was removed by centrifugation at 10,000*g* for 15 min. This material was then assayed for the capacity to inhibit virus attachment to plasma membranes. For selected studies, membranes were prepared from labeled CEFs or human erythrocyte ghosts and extracted as above.

Analytical procedures. The method of Lowry *et al.* (10) was used to assay protein with bovine serum albumin as standard. Neutral sugar was assayed according to Dubois *et al.* (11) with galactose as standard, and sialic acid was quantified according to Warren (12).

Results. Glycophorin (5) solubilized from human erythrocytes with LIS will bind influenza virus *in vitro*. Accordingly, we examined an LIS extract of CEF plasma membranes to determine whether this extract would interact with virus *in vitro* to impair subsequent virus binding by CEF plasma membranes. RSV(RAV-1) was incubated either with CEF LIS extract or buffer and then exposed to CEF membranes to assay viral attachment as in Methods and Materials (Fig. 1). Attachment was consistently reduced by the chick-cell LIS extract (Fig. 1). A representative study (Fig. 1) shows a 55% reduction in RSV(RAV-1) binding after incubation with 19 μg of CEF LIS extract. Attachment of RSV(RAV-2) (not shown) was inhibited to a similar degree by the LIS extract. Since the number of virus-binding sites is not known, conditions are regulated so that attachment is linear with respect to both membrane concentration and time. A general relationship between the percentage of depression of virus binding and the amount of LIS extract used can be seen (inset to Fig. 1). Inhibition appears linear to 10 μg (neutral sugar), and, then, the blocking efficiency per microgram of extract decreases. Activity varied with individual extracts, and the percentage of blocking per microgram of neutral sugar ranged from 2 to 5%. In several separate studies, LIS extracts of plasma membranes obtained from between 2 and 6 × 10^7 CEFs would

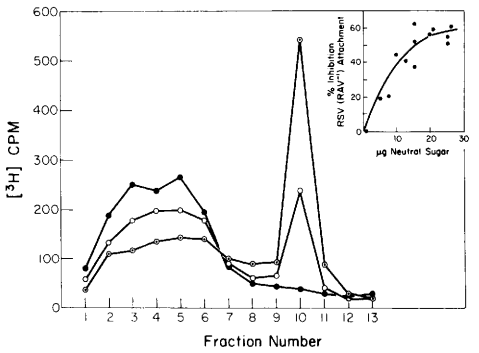

FIG. 1. Inhibition of RSV(RAV-1) binding to CEF membranes. Labeled RSV(RAV-1) (5×10^5 FFU, approximately 1700 cpm) was incubated either with 19 μg (neutral sugar) of CEF LIS extract for 30 min at 4° (○——○), or in TEN buffer (⊙——⊙). CEF plasma membrane (200 μg of protein) was then added to each, and the incubation continued for an additional 30 min at 4°. To assess the amount of attachment, RSV(RAV-1) (●——●) was also incubated in TEN buffer for the same time and then placed on the gradient without membranes. Virus binding to the membranes was assayed by discontinuous sucrose gradient centrifugation as in Methods and Materials. Inset: RSV(RAV-1) was incubated with increasing concentrations of CEF LIS extract for 30 min at 4°. Inhibition of virus binding of CEF plasma membranes was measured by comparing the binding to that of RSV(RAV-1) incubated in TEN buffer. The curve is a computer derived least squares plot weighted to pass through zero.

retard the binding of 5×10^5 FFU of RSV(RAV-1) by approximately 50%. The individual extracts had approximately equal amounts of protein and neutral sugar, and membranes from 3×10^6 CEFs yielded about 1 μg (neutral sugar) of LIS extract. The inhibitory activity remained with the supernatant fluid after centrifugation at 100,000*g* for 30 min, and is recovered in the aqueous phase after chloroform–methanol (3:1) extraction. Preincubation of RSV(RAV-1) with LIS extract also reduced the infectivity of the virus. RSV(RAV-1), 5×10^6 FFU, was incubated with 50 μg of LIS extract or in TEN buffer at 4° for 60 min and then titered in a standard FFU assay to determine infectivity. After 1 week, the total number of foci present at two dilutions (10^{-3}, 10^{-5}) were counted; at both dilutions, there was a 50% reduction in the number of foci with the LIS extract-treated virus compared to virus incubated in TEN buffer alone.

Evidence for a direct interaction between unlabeled RSV(RAV-1) and a LIS extract of CEF membranes is shown in Fig. 2; CEFs were labeled either on their surface with sodium [^{3}H]borohydride (7) or endogenously by growth in [^{3}H]glucosamine-containing media. Membranes prepared from each of these labeled cell preparations were then extracted with LIS as in Methods and Materials. The labeled LIS extracts were then incubated with unlabeled RSV(RAV-1) or with media, and these mixtures (labeled LIS extract ± RSV(RAV-1) were sedimented in discontinuous sucrose gradients. A peak of radioactivity (1–5% input counts) migrates with the RSV(RAV-1) (arrow), and much less radioactivity migrates to this region when sedimented after incubation in the absence of this virus. Nonspecific trapping of counts was eliminated since sedi-

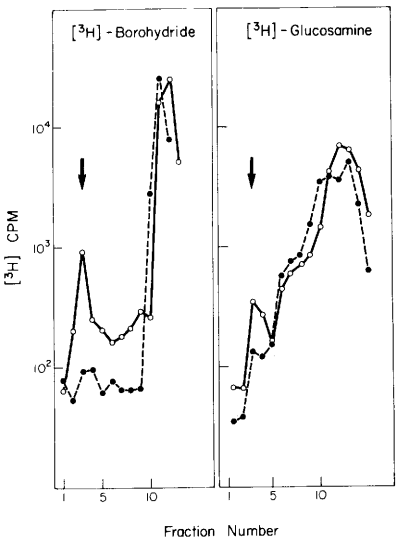

FIG. 2. Direct attachment of labeled LIS extract to RSV(RAV-1). CEF were labeled by sequential exposure to pyridoxal phosphate and sodium [^{3}H]-borohydride (7) (left) or by growth in media containing 50 μCi/ml of [^{3}H]glucosamine (right). LIS extracts of the plasma membranes from these cells were mixed with unlabeled RSV(RAV-1) (○——○) 1×10^8 FFU or media (●-----●). These mixtures were incubated for 1 hour at 4° and sedimented on a discontinuous 20/60% sucrose gradient at 100,000*g* for 2.5 h; the gradient was collected from below, and the ethanol precipitable radioactivity in each fraction was measured. The migration of virus alone is indicated by the arrow. The labeled material migrating above fraction 9 represents material which failed to enter the gradient.

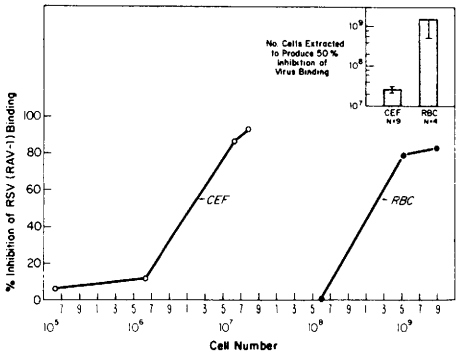

FIG. 3. Comparison of activity extracted from CEF membrane and human erythrocyte ghosts. CEF plasma membranes and human erythrocyte ghosts were extracted with LIS as in Methods and Materials. RSV(RAV-1) (5×10^5 FFU, approximately 1500 cpm) was incubated with these extracts or in TEN buffer for 30 min at 4°. CEF membrane (protein), 200 μg, was added and the incubation continued for 30 min at 4°. Binding of the virus to membranes was assayed by sucrose gradient sedimentation as described in Methods and Materials. The percentage of inhibition of RSV(RAV-1) binding is plotted against the number of cells required to produce the amount of extract giving that degree of inhibition. Inset: The number of cells to produce 50% inhibition of RSV(RAV-1) binding to CEF membranes. The number of separate studies and SEM is shown.

mentation of the labeled LIS extract with polio virus (3×10^6 $TCID_{50}$) did not reveal a similar peak at the 20/60% sucrose interface.

Glycophorin (5) prepared by LIS extraction of human erythrocyte ghosts was compared to a CEF LIS extract to determine the relative capacity of each to inhibit ATV attachment to CEF membranes. As illustrated in Fig. 3, glycophorin did not antagonize RSV(RAV-1) attachment as well as LIS extracts of CEF. Membranes from approximately 200 erythrocytes must be extracted to produce the activity of a single CEF. Other studies (not shown) indicate that labeled glycophorin did not bind directly to RSV(RAV-1).

Discussion. Previous studies [(1); Moldow, C. F., manuscript submitted for publication] indicated that virus binding to CEF plasma membranes is a sensitive measure of the initial attachment of ATV to the CEF surface. The data presented show that soluble LIS extracts of CEF plasma membrane reduce the attachment of at least two RSV pseudotypes to fresh CEF plasma membranes. RSV(RAV-1) incubated with the LIS extract is also less effective in transforming CEF than control virus, and evidence is presented which indicates that constituents of the LIS extract may bind directly to RSV(RAV-1) *in vitro*. We infer that some component of the LIS extract is binding to the ATV surface glycoproteins, preventing subsequent binding of the virus to its membrane attachment site. Since the viral RNA remains resistant to RNase (Moldow, C. F., unpublished observation) and infectious virus is still present after incubation with the CEF LIS extract (albeit at reduced levels), virus disruption by the LIS extract appears an unlikely alternate explanation.

Human erythrocyte membranes bind ATV poorly (1), yet, an LIS extract of these membranes interferes with ATV attachment to CEF membranes. Although precise quantitation is not possible, it appears that many more erythrocytes must be extracted to produce activity equivalent to that present on CEF. This difference is quantitatively similar to that reported for attachment site activity solubilized by trypsinization of CEF (Moldow, C. F., manuscript submitted for publication). Direct binding of LIS extract from labeled ghosts to ATV has not been demonstrated, and the erythrocyte extract may interfere with binding by an alternate mechanism. Quantitative and qualitative comparison of the activity present in various cells will ultimately require identification and purification of the attachment site activity solubilized from the CEF.

Summary. Extraction of CEF plasma membranes with LIS solubilizes membrane components which interact directly with ATV *in vitro*, antagonizing ATV binding to CEF plasma membranes and reducing the transforming capacity of these viruses. This activity is specific to CEF; approximately 200 human erythrocytes must be extracted with LIS to produce the activity equivalent to that extractable from a single CEF.

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