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AVIAN TUMOR VIRUS INTERACTIONS WITH CHICKEN FIBROBLAST PLASMA MEMBRANES

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A method is described which will rapidly measure the binding of avian tumor viruses (ATV) to plasma membrane receptors. With this procedure it may be shown that Rous sarcoma virus pseudotypes bind to protease-labile, heat-stable structures on the surface of chicken embryo fibroblast (CEF) plasma membranes. The binding sites for ATV subgroups A and B appear distinct, and membranes from genetically resistant CEF bind as well those of sensitive CEF.

Attachment to the cell surface must precede the later stages of viral infection, such as viral eclipse and penetration. Binding is mediated by cellular receptors, and precedent for specific viral receptors exists in many systems (notably, myxoviruses, piconaviruses, and adenoviruses) (1–5). Although the presence of receptors is a necessary antecedent to viral infection and ultimate replication, receptors alone will not guarantee successful viral production; for example, influenza virus binds but does not replicate within the mature erythrocyte. On the other hand, the presence of a receptor often determines the replicative potential of the cell toward polio virus (6).

The precise role of the initial attachment site for Avian tumor viruses (ATV) has not been delineated, but the control of the host range of ATV does appear to depend upon the existence of specific receptor sites for each viral subgroup (7). Genetic susceptibility is dominant over resistance, but viruses adhere to the surface of resistant cells and sensitive cells equally well, and it appears that penetration is blocked in resistant chick cells (8, 9). Several provocative studies with ATV indicate that initial absorption may be directly related to effective viral penetration. The following data support this: (1) the R_1 blood group antigen of chicken erythrocytes is associated with susceptibility to RAV-2 (10); (2) polycation treatment of cultured chick fibroblasts increases infectivity of viral subgroups B, C, D, and E, and this correlates with increased absorption of the virus at the cell surface (11); (3) Hanafusa and Hanafusa have shown that enhancement of secondary infection by RAV-2 of cells previously infected with RAV-1 is due to increased absorption of the challenge virus (12); (4) finally, removal of virus glycoprotein spikes enzymatically

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(13) or genetically (14) renders the virus noninfectious, presumably by preventing absorption.

This study sought to define the participation of the initial attachment site in ATV infection, therefore, experiments were designed which separate absorption from penetration. It was reasoned that virus binding to purified plasma membrane preparations at low temperature would eliminate the participation of intracellular structures, thereby isolating the attachment step for characterization. The interaction of plasma membranes with viruses has previously been used in the studies of influenza and enteroviruses (3, 15–17). Our assay quantifies the temperature-independent step of viral attachment to the plasma membrane, using this assay we have found: (1) that binding activity for Rous sarcoma pseudotypes is intact in resistant cells; (2) that virus absorption involves attachment to a heat-stable membrane site which may be removed by proteolytic digestion of the cell surface; and (3) that different cell surface binding sites are necessary for the absorption of viral subgroups A and B.

EXPERIMENTAL PROCEDURE

Materials

Tissue culture media and calf serum were purchased from Grand Island Biological Company (GIBCO). Bromelain (E.C. 3.4.4.24), lactoperoxidase (E.C. 1.11.1.7), neuraminidase (E.C. 3.2.1.18) from Clostridium perfringens), pronase (from Streptomyces griseus), ribonuclease A, trypsin (bovine pancreas), and bovine submaxillary mucin were purchased from Sigma Chemical Co. Deoxyribonuclease (E.C. 3.1.4.5 from bovine pancreas) was obtained from Worthington Biochemicals. ^{125}I, (5–15 Ci/mmole), [5-^{3}H] uridine (5 Ci/mmole), and ^{3}H-L-fucose (5 Ci/mmole) were obtained from New England Nuclear. All sucrose solutions (wt/wt) were made up in TEN buffer (0.01 M Tris, pH 7.4,0.001 M ethylenediamine-tetracetic acid, and 0.1 M NaCl).

Analytical Procedures

The procedure of Lowry et al. (18) was used to measure protein with bovine serum albumin as standard. Sialic acid was assayed by the thiobarbituric acid method of Warren (19), and neutral sugar quantified according to Dubois (20) with galactose as a standard.

Cell Culture

Primary C/O chick embryo fibroblast (CEF) cultures are prepared by established procedures (21) from 9-day old (Marek and COFAL negative) chick embryos obtained from SPAFAS, Rockford, Ill. These are maintained and propagated in M-199 supplemented with 6% calf serum. Unless otherwise noted, cells are subcultured once and grown to confluence before use in binding studies. Other cell types treated in a similar fashion included human embryonic kidney (HEK) and human urothelium cells (22). Genetically resistant C/A and C/B CEF were generously provided by Dr. Peter Vogt.

Virus Preparation

Rous sarcoma virus pseudotypes RSV (RAV-1) and RSV (RAV-2) (a gift from Dr. H. Hanafusa) are seeded onto 100-mm tissue culture plates with 4×10^{6} freshly subcultured chick embryo fibroblasts. Focus-forming units (FFU) are determined according to Rubin (23). Between 5 and 7 days after infection as determined by focus formation, the medium is harvested and replaced with 5 ml of Scherer's maintenance medium with

2% calf serum and 10 μCi/ml of [^{3}H] uridine. This culture medium is harvested and replaced every 24 hr for the life of the monolayer. All virus stocks are stored at −70°C. Viral glycoproteins are labeled with ^{3}H-D-glucosamine (25 μCi/ml) in the same manner. In certain experiments, [^{3}H-uridine-labeled RSV (−) (provided by Dr. H. Hanafusa) was prepared in a similar manner.

Viral Purification

Culture media containing RSV (RAV-1) and RSV (RAV-2) is centrifuged at 10,000 × g for 5 min to remove cellular debris. The supernatant is filtered through a Nalgene 0.45 micron filter and concentrated 10:1 in an AMICON ultrafilter (UM 100). The concentrated virus is layered on top of a discontinuous 20/60% sucrose gradient and centrifuged at 90,000 × g for 2.5 hr. The 20/60% sucrose interface is collected and assayed for acid-precipitable [^{3}H] uridine or [^{3}H] glucosamine and FFU, and stored in M-199 with 2% calf serum at −70°C. This method of purification concentrates virus 25–40-fold.

Membrane Purification

Membranes are prepared by a modification of the procedure of Boone et al. (24). Secondary CEF monolayers are washed with cold PBS and harvested after incubation with 0.05% trypsin, 0.5 mM EDTA in PBS, pH 7.0, for 5 min at 37°C. The cells were washed, and then resuspended in hypotonic buffer (20 mM Tris-1 mM EDTA, pH 7.8) at a concentration of 2×10^7 cells per ml and allowed to swell for 20 min. One-tenth volume of 30 mM $MgCl_2$-0.15 M NaCl, pH 7.8, is then added for 5 min before homogenizing the cells with 25–30 strokes in a stainless steel Dounce homogenizer (0.002-inch clearance) until fewer than 5% intact cells are observed by phase-contrast microscopy. Unruptured cells and nuclei are removed by centrifuging the mixture at 1,000 × g for 4 min, and the supernatant fluid is then centrifuged at 90,000 × g for 25 min. This pellet is resuspended in 4.0 ml 45% sucrose and layered on 1 ml of 60% sucrose. The discontinuous gradient is completed with 3.0 ml of 40% sucrose and 3 ml of 10% sucrose. The gradient is centrifuged at 90,000 × g for 20 min and the plasma membrane fraction collected from the 40/10% sucrose interface (density = 1.16 g/cm^3). Lactoperoxidase-catalyzed iodination is performed by the method of Hubbard and Cohn (25), and fucose incorporation determined according to Atkinson and Summers (26). ATPase measurements were done on cells that were homogenized without exposure to Mg^{++} with added dithiothreitol (kindly performed by Dr. J. Sheppard of the Department of Genetics).

Membrane Characteristics

Purified chick embryo fibroblast plasma membranes (CM) used in this assay are 10–17 times increased for plasma membrane markers (Table I). These markers are: the incorporation of ^{3}H-L-fucose into CEF glycoprotein, and the lactoperoxidase-catalyzed iodination of CEF surface with ^{125}I. In addition, Na-K-activated ATPase was determined to be 10-fold increased in the purified membrane fraction (27).

When the distribution of total cell protein is followed during the plasma membrane isolation procedure, $6–8 \times 10^7$ secondary CEF are found to yield 1 mg of gradient-purified membrane protein. Electron microscopy reveals typical bilamellar membrane sheets and vesicles relatively free of cytoplasmic contamination. The bouyant density (1.16 g/cm^3) of the membranes corresponds with that of the HeLa cell plasma membrane fraction isolated by Boone et al. (24). Our procedure yields two discrete membrane bands

TABLE I. Purification of CEF Plasma Membranes

Experiment no.	Labeling procedure	cpm/mg cell protein / cpm/mg membrane protein	RSA
1	[^{3}H] fucose	200 / 2,837	14
2	[^{3}H] fucose	537 / 5,429	10
3	[^{125}I] lactoperoxidase	1,790 / 30,300	17
4	[^{125}I] lactoperoxidase	1,292 / 17,400	12

Membranes were labeled using either ^{3}H-L-fucose incorporation (experiments 1 and 2) or lactoperoxidase-catalyzed iodination (experiments 3 and 4). In each instance acid-precipitable radio activity per milligram of protein was determined in washed cells and membranes isolated therefrom. RSA, the relative specific activity, is the reciprocal of the cell specific activity divided by that of the membrane fraction.

at the 10/40% sucrose interface similar to those described by Perdue (28) which are routinely harvested and assayed together.

Viral Binding Assay

Purified and labeled virus (5×10^5 FFU, 1.5×10^3 cpm [^{3}H] uridine) is incubated with gradient purified plasma membranes (CM) (200 μg membrane protein) in a 1.2 ml reaction mixture containing TEN buffer at 0°C with constant shaking for 25 min. At the end of the incubation period, sucrose is added to give a final sample concentration of 45%. The sample is layered onto 1 ml of 60% sucrose, and overlaid as described above. The gradient is centrifuged for 20 min at 90,000 × g and fractions collected which may be assayed for viral infectivity (FFU) or acid-precipitable [^{3}H] uridine or [^{3}H] glucosamine. In all experiments, a viral control (virus incubated without membranes), is included. The percentage of viral radioactivity bound to membranes is calculated using the following formula:

$$\% \text{ virus bound} = \frac{\text{cpm in membrane peak}}{\text{total cpm gradient}} - \frac{\text{cpm virus control peak} \times 100.}{\text{total cpm gradient}}$$

RESULTS

Characteristics of Plasma Membrane Binding

Upon incubation with CM, RSV (RAV-1) or RSV (RAV-2) associates with the plasma membranes and will comigrate with them through the gradient to the 40/10% interface; virus alone does not migrate through the gradient in appreciable amounts. With exposure to increasing concentrations of membranes, the amount of virus bound also increases (Fig. 1). The assay is highly reproducible, and identical curves are obtained whether the viral position is assayed as infectious FFU or TCA-precipitable [^{3}H] uridine or [^{3}H]-D-glucosamine labeled virus. Virus binding activity is temperature independent (between 4° and 37°C) and not affected by prior treatment of the membranes with ribonuclease A, deoxyribonuclease, or exposure of the membranes at 100°C for 5 min (Table II).

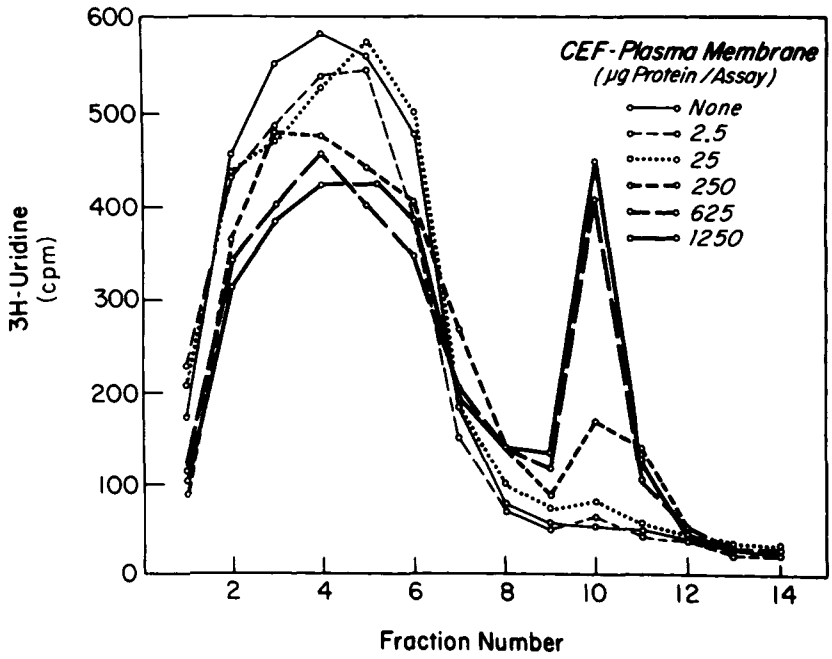

Fig. 1. Representative binding assay in which 5×10^4 FFU RSV (RAV-1) are incubated with increasing concentrations of CEF plasma membrane protein at 0°C for 25 min. Binding quantified as in text.

TABLE II. ATV Binding to CEF Plasma Membranes. Lack of Effect of Prior Treatment with DNAase, RNAase and Heat

Treatment of chick fibroblast plasma membranes	Binding % of control
Untreated	100
Membrane + DNAase	95–100
Membrane + RNAase A	95–105
Membrane heated to 100°C × 5 min	85–110

Membranes were exposed to 25 U of DNAase in the presence of $MgSO_4$ or 75 U of RNAase A for 1 hr at 37°C. Controls were aliquots of the same membrane fraction not exposed to enzyme. The values above represent the range of triplicate experiments. Identical results were obtained with RSV (RAV-1) or RSV (RAV-2).

Binding shows saturation with time and membrane protein concentration (Fig. 2). Saturating levels of virus have not yet been ascertained; however, exposure of 75 μg of purified membrane to 1×10^8 FFU of unlabeled RSV (RAV-1) reduced the subsequent binding of labeled RSV (RAV-1) (5×10^5 FFU) by 65%. Since saturating levels of virus could not be used, all subsequent binding studies were performed using time points and membrane protein concentration below the plateau indicated in Fig. 2.

The possibility that membrane fragments are trapping virus nonspecifically is eliminated by the following experiments. RSV (RAV-2) is treated with bromelain to

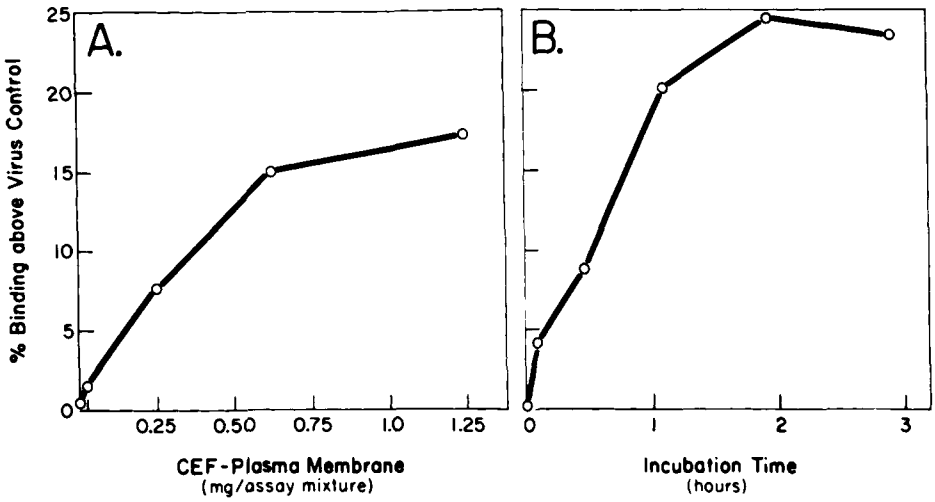

Fig. 2. Dependence of ATV binding upon membrane protein concentration and time. (A) Increasing concentration of plasma membrane (protein) incubated with 5×10^5 FFU RSV (RAV-1) for 25 min at 0°C. (B) 5×10^5 FFU RSV (RAV-1) were incubated with 400 μg of CEF membrane protein for varying time intervals. See text for binding assay details.

remove surface glycoproteins, rendering the virus noninfectious (13). Virus so treated fails to bind to chick plasma membranes (Fig. 3a), RSV (–), which has reduced surface glycoproteins (14), also fails to bind significantly to the plasma membrane fraction when compared to identical concentrations of RSV (RAV-1) (Fig. 3b). Finally, heat-killed virus binds very poorly. These experiments support the view that the virus binds to membrane receptors rather than being trapped by membrane vesicles.

Binding Specificity

The specificity of the interaction between ATV and the plasma membranes is studied with the gradient sedimentation procedure. Equal concentrations of membranes obtained from chick cells and "nonpermissive" mammalian cells are incubated with virus, and the degree of binding is measured (Table III). In all instances, the binding by the chick cell membranes is quantitatively greater than with the mammalian membranes. The level of binding observed with the nonpermissive membranes may represent nonspecific binding, or alternatively, be due to a reduction in the number or availability of sites. The membranes obtained from genetically resistant chick cells bind both viral subtypes as well as those of sensitive C/O chick cells. This provides confirmatory evidence that the site of initial attachment is not involved in genetic resistance.

Results from experiments designed to provide indirect evidence as to the nature of the chick cell receptor sites are illustrated in Table IV. Whole cells are incubated with enzymes of known specificity in an attempt to modify the binding activity of membranes subsequently prepared from these treated cells. Binding of RSV (RAV-1) to membranes is blocked by extensive pronase pretreatment of the intact cell. RSV (RAV-2) binding is sensitive to both trypsin and pronase degradation. Membranes obtained from cells treated with these proteolytic enzymes in the presence of 10% calf serum at 4°C showed only minimal reduction in binding activity, confirming that enzymatic digestion of the cell

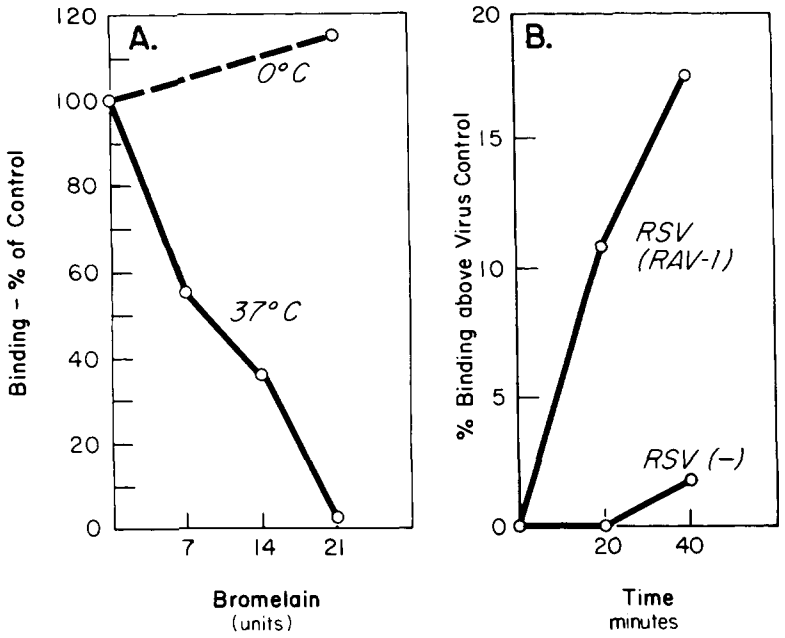

Fig. 3. Removal of ATV glycoprotein: effect upon CEF plasma membrane binding. (A) 5 × 10^4 FFU RSV (RAV-2) incubated with increasing concentrations of bromelain for 2 hr at 37°C or 0°C (control). Binding assay was performed at 0°C in the presence of 10% calf serum to protect the membranes from bromelain degradation during the binding assay. (B) RSV (–) and RSV (RAV-1) binding to CEF plasma membranes. Equivalent amounts of virus as acid precipitable radioactivity were incubated with 200 μg of membrane for times shown prior to measurement of binding.

TABLE III. Virus Binding. Comparison of Avian and Mammalian Cell Membranes

Source of plasma membranes	RSV (RAV-1) % binding	RSV (RAV-2) % binding
C/O CEF	100	100
C/A CEF	100	100
C/B CEF	100	100
Human erythrocyte	9	7
Human urothelium	22	36
Human embryonic kidney	18	43

The mean of five separate studies is shown in each case except experiments with C/A cells (resistant to infection with RSV [RAV-1]) and C/B cells (resistant to RSV [RAV-2]). These values are the mean of duplicate experiments.

surface is responsible for this effect. Pronase-treated cells were also found to be transiently resistant to infection with RSV (RAV-1) or RSV (RAV-2) as determined by reduced focus formation (29), whereas cells incubated with heat-inactivated pronase do not show this effect. RSV (RAV-2) shows increased binding to membranes obtained from cells from which at least 60% of membrane neuraminic acid has been removed by exposure to neuraminidase. This is not an unexpected result, since this virus shows increased absorption and infectivity in the presence of polycations (11). Exposure of CEF to neuraminidase in

TABLE IV. Virus Binding. Effect of the Enzymatic Perturbation of the Cell Surface

Cell treatment	RSV (RAV-1) % binding	RSV (RAV-2) % binding
None	100	100
Neuraminidase	100 (86–110) N = 5	150 (120–180) N = 10
Trypsin	100 (95–110) N = 5	33 (20–46) N = 5
Pronase	11 (0–18) N = 5	12 (7–16) N = 5

Confluent CEF were harvested and the washed cells were suspended in the appropriate enzyme solution.
Neuraminidase: 5×10^7 cells were with 1.5 U/ml at pH 6.0.
Trypsin: 1.5×10^8 CEF were incubated in 0.25% trypsin at pH 7.0.
Pronase: 1.5×10^8 cells were mixed with 300 μg/ml at pH 7.0.
Following exposure for 1 hr at 37°C the cells were washed and membranes prepared as in Methods. Cells viability was 95% as determined by trypan blue exclusion and plating efficiency of enzyme-treated cells was 60% of control. Occasional samples became badly clumped and were discarded. The mean and range of all experiments is shown.
N = number of studies.

the presence of bovine submaxillary mucin (a neuraminidase substrate) blocked any effect of neuraminidase.

DISCUSSION

These data indicate that certain structures on the CEF plasma membrane bind ATV. These sites are presumably on the cell surface, since exposure of intact cells to proteolytic enzymes results in altered binding activity when plasma membranes are isolated from these cells. It seems unlikely that these enzymes would exert their effect intracellularly and we have shown that treatment of cells with immobilized proteolytic agents release material which will antagonize ATV binding by CEF membranes (29). Binding activity for ATV is heat stable, protease labile, and apparently not directly mediated by surface neuraminic acid residues. The binding sites for the two viral subgroups used in this study seem separate on the basis of the disparate binding activity of the plasma membranes obtained after trypsin and neuraminidase treatment. Subgroup B virus (RSV [RAV-2]) binding activity is sensitive to both trypsin and pronase digestion while RSV (RAV-1) an A subgroup virus, is sensitive only to pronase digestion. Membranes obtained from neuraminidase-treated cells bind RSV (RAV-2) with increased avidity. This is not unexpected since the infectivity of subgroup B viruses is increased by polycation treatment of CEF and this correlates with increased virus absorption (11). Polycation treatment and neuraminic acid removal both would be expected to reduce the net negative charge at the cell surface, although other explanations such as receptor clustering on the surface following enzymatic exposure are certainly possible (30, 31).

The significance of binding activity seen with the various mammalian cell membranes tested is difficult to ascertain at present. While this may represent nonspecific binding, alternative explanations such as quantitative reduction in the number or availability of binding sites in these "nonpermissive" cells are possible. These questions may be quantitatively answered when antireceptor antibody and purified receptor are available.

While the kinetics of virus binding to membranes closely follow those reported for virus absorption to intact CEF, the assay requires at least 75 μg of plasma membrane in each tube for reproducible recovery in the gradient. This represents the surface from approximately 5×10^6 CEF; measurement of saturating levels of ATV binding therefore will require large quantities of purified virus of high specific activity. Since we have no direct way of equating acid-precipitable radioactivity or FFU with total viral particles capable of binding, saturation studies will only crudely estimate the actual number of receptor sites.

Our assay permits the study of the initial binding site for Rous sarcoma virus pseudotypes, and may be of some utility in the investigation of other virus-cell membrane interactions. Purification of this receptor will allow study of the relationship of the virus binding site to the cell membrane structure and provide insight into the later stages of viral penetration which appear to be important in the mechanism of genetic resistance in these cells.

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