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Adsorption of Rous Sarcoma Virus to Genetically Susceptible and Resistant Chicken Cells Studied by Laser Flow Cytometry

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Quantitative binding of Rous sarcoma virus (RSV) of different antigenic subgroups to chicken cells was examined by using a laser flow cytometer/cell sorter. RSV of subgroups A, C, and E, labeled with the fluorescent membrane probe rhodamine-18, bound 2 to 10 times more to genetically susceptible chicken embryo fibroblasts than to resistant cells, as measured by flow cytometry on a single-cell basis. This suggested that susceptible cells possess both specific and nonspecific receptors for virus adsorption, whereas resistant cells bind virus only by means of nonspecific sites. Polybrene at low concentration increased eightfold the binding of virus. Higher levels of Polybrene inhibited adsorption. Cell binding sites were saturable, and attachment of labeled virus could be partially blocked by preexposure of cells to unlabeled RSV. Virus surface glycoproteins played an important role in adsorption, since their removal with bromelain decreased binding of virus to susceptible cells. Maximal binding of RSV to both susceptible and resistant cells occurred within 10 min, although the level of binding was up to 10-fold higher for susceptible cells. Binding to all cell types showed a broad distribution. This implies that there are considerable differences in the number of virions bound per cell.

Avian sarcoma viruses possess two types of envelope glycoproteins, gp85 and gp37, which interact with specific cell receptors to initiate infection and determine the virus antigenic subgroup and host range (6, 28). The presence of specific receptors which mediate cell susceptibility to infection by Rous sarcoma virus (RSV) is genetically controlled by dominant alleles of autosomal loci (3, 15–17, 29). Whereas lack of specific receptors is responsible for resistance to viruses of certain subgroups (6, 26), it is generally believed that virus adsorption to cells is nonspecific. In fact, genetically resistant cells (18) or cells rendered resistant by preinfection with other viruses (23) adsorb RSV, but virus penetration does not occur. However, studies of binding kinetics of RSV glycoproteins have shown some differences of adsorption between susceptible and resistant cells (13).

So far, early cell-virus interactions (adsorption and penetration) have been studied on large cell populations by using radioactive uptake, infectivity assays, or, less frequently, examination of a few cells by electron microscopy (5), but, to our knowledge, no quantitative data are available on the relative efficiency of adsorption leading to infection (specific adsorption) versus nonspecific adsorption of RSV.

In this study, we examined the adsorption of RSV to genetically susceptible and resistant chicken embryo fibroblasts (CEF) under different experimental conditions, using a laser flow cytometer. This instrument allows rapid, sensitive measurements on large numbers of single, live cells which can be sorted and recovered as subpopulations for additional analysis (8; J. F. Leary and M. F. D. Notter, Cell Biophys., in press). The results of our study indicate that more RSV particles adsorb to susceptible than to resistant cells and suggest that virus envelope glycoproteins are responsible for attachment to the specific receptors on susceptible cells. However, a certain amount of viral adsorption occurs also in the absence of functional envelope glycoproteins. This is probably nonspecific and was shown to be about 20% of the binding of RSV possessing envelope glycoproteins.

MATERIALS AND METHODS

Cells and viruses. Embryonated chicken eggs of genotype C/E were purchased from Spafas, Inc., Norwich, Conn. Eggs from line 15 of genotype C/C and eggs of genotype C/ABE from the F1 progeny of a cross between line 100B and line 7_2 were obtained from the Regional Poultry Laboratory, U.S. Department of Agriculture, East Lansing, Mich. CEF prepared from 10-day-old embryos were grown in Eagle minimal essential medium, 10% tryptose phosphate broth, 8% calf serum (CAS), 2% chicken serum (CHS), and 100 μl of streptomycin per ml. All cells were tested for chicken helper factor as well as for

susceptibility to viruses of subgroups A through E. Early-passage CEF (up to third passage) were grown in minimal essential medium with 2% CAS and 2% CHS. Japanese quail fibroblasts were kindly provided by C. Moscovici and maintained in Ham F-10 medium, 10% tryptose phosphate broth, 5% CAS, and 1% CHS. The RSV(−)-transformed quail cell line 16Q (14), which releases large quantities of noninfectious virus, was maintained on Dulbecco modified Eagle medium with 10% tryptose phosphate broth, 4% fetal calf serum, 1% CHS, and 1% dimethyl sulfoxide.

The Schmidt-Ruppin strain of RSV of subgroup E (SR-E) was provided by C. Moscovici. SR virus of subgroup A (SR-A) and LA33C, a mutant of Prague strain RSV of subgroup C, temperature sensitive for transformation, were obtained from J. Wyke.

Virus stocks were produced on CEF or quail cells from freshly cloned virus by collecting fluids from infected plates every 4 h for 1 week. Before virus purification these fluids were kept at 4°C.

Virus purification. Virus was purified by a method of E. Humphries (personal communication) devised to preserve as much as possible the integrity of the virus envelope. Briefly, pooled media were clarified for 20 min at 15,000 rpm in a Beckman J21-B centrifuge. Supernatant fluids (30 ml) were then overlaid onto a short (7 ml) 15 to 55% discontinuous sucrose gradient in 0.05 M Tris–0.001 M EDTA, pH 8.0 (TE), and centrifuged for 1 h in an SW27 rotor at 25,000 rpm at 4°C. The supernatant was removed; additional fluid was added to the same gradient tubes and centrifuged as before. After all supernatant was centrifuged, the viral band at the interface was removed, pooled, diluted to 15% sucrose, and overlaid onto a continuous 20 to 55% sucrose gradient. This was centrifuged at 4°C for 1.5 h at 25,000 rpm. The viral band was removed through the side of the tube, or dropwise from the bottom, for density measurements.

Virus assays. The purified virus was assayed for infectivity by the focus-forming assay carried out according to standard procedures (27). The particle density was determined by optical measurements at 260 nm, as described by Smith and Bernstein (22). Optical density readings at 260 nm (OD_{260}) provide an approximate measure of the number of virus particles, both infectious and noninfectious, based on the following relationship: 1 OD_{260} = 158 μg of protein = 243 μg of virus = 3.1×10^{11} virus particles.

Reverse transcriptase (RTase) activity was measured on unconcentrated as well as purified viral preparations. Briefly, 50-μl samples were incubated for 2.5 h at 40°C with 50 μl of a standard reaction mixture consisting of 50 mM Tris-hydrochloride, pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 0.5% Triton X-100, 5 μg of poly(rA):poly$(dT)_{12-18}$, 20 μM [*methyl*-^{3}H]TTP (5 μCi/sample), and 10 mM dithiothreitol. After rapid cooling on ice, the mixture was transferred to Whatman 3MM filter paper. Filters were batch washed with 5% trichloroacetic acid (21), dried, and counted in toluene scintillation fluid with an Isocap 300 liquid scintillation spectrophotometer.

Fluorescent probe and virus labeling. Rhodamine-18 (R-18) is a fluorescent dye covalently linked to a saturated hydrocarbon 18 carbons long (octodecanol) provided by P. M. Keller. This probe partitions preferentially into bilayer lipid membranes (9).

The optimal concentration of fluorescent probe and the labeling technique which maintains infectivity of the virus as well as normal growth of CEF were established previously (Leary and Notter, in press).

Briefly, R-18 dissolved in dimethyl sulfoxide was added to a portion of virus, measured in OD, in 15% sucrose to a final concentration of 0.001 M for 18 h at 4°C. Unbound dye was removed by centrifugation on a discontinuous sucrose gradient, 20/40/60%, in TE in an SW50.1 rotor at 40,000 rpm for 1 h at 4°C. The fluorescent band at the 20/40% sucrose interface was removed through the side of the tube and tested for infectivity and RTase activity before storage at −80°C. Labeled concentrated virus could be stored for at least 2 months without loss of infectivity or fluorescence. The same virus preparation was used for several experiments.

Virus absorption assay. The binding of R-18-labeled virus to CEF was assayed as follows except where stated otherwise. Cell suspensions were obtained by trypsinization with 0.05% trypsin in Tris–0.02% EDTA for less than 3 min and by quenching the trypsin with 10% CAS. Volumes containing 0.01 to 0.03 OD unit of labeled virus (3×10^9 to 9×10^9 virions) in TE were added to 7.5×10^5 cells in 0.2 ml of minimal essential medium, 1% fetal calf serum, and 2 μg of Polybrene per ml on ice. This concentration of labeled virus allowed for reliable measurements of fluorescent signals. After 30 min, the cells were pelleted at 1,000 rpm for 5 min at 4°C and resuspended in 1 ml of fresh medium. For cytometer analysis, the samples were filtered through a 44-μm nylon mesh and kept on ice during sampling. Cold phosphate-buffered saline was used as a sheath medium for carrying the samples through the flow cytometer and to collect cells that had been sorted into separate sterile containers.

Virus-infected cells were examined by a multiparameter laser flow cytometer/cell sorter (EPICS IV; Coulter Electronics, Hialeah, Fla.). Cells were sorted by a sterile procedure according to user-specified windows on two parameters, e.g., fluorescence and light scatter. Data were stored on floppy disks of a PDP-11/03 minicomputer (Digital Equipment Corp., Maynard, Mass.) and displayed on a 4012 graphics terminal (Tektronix, Beaverton, Ore.). Software relevant to these experiments is an upgraded version of a published program (20) and has been described in detail (Leary and Notter, in press).

RESULTS

Cells bind labeled viruses. The amount of fluorescence bound to CEF upon mixing with R-18-labeled virus was recorded after excitation with 514-nm-wavelength light from the cell sorter's argon ion laser.

Simultaneous measurements of light scattering allowed identification of live versus dead cells, since dead cells scatter significantly less light than live cells of similar size (10). Virus alone scatters too little light to be detected by the instrument. Only fluorescence signals from live cells were analyzed, even if dead cells constituted less than 5% of the total cell population.

In several experiments, R-18-labeled RSV of

different subgroups was mixed with CEF of different genotypes at nonsaturation conditions (Table 1). When LA33C was mixed with C/E CEF in the presence of Polybrene (2 μg/ml), living cells showed a relative mean fluorescence of 54, or 18 times autofluorescence, i.e., fluorescence emitted by control C/E cells. Genetically resistant C/C cells mixed with the same concentration of virus showed a mean fluorescence of 22. This value is significantly lower than that obtained with susceptible cells. Repeated tests with each population showed a high reproducibility of results. The standard error of the mean in eight different samples (viral binding reproducibility) was less than 2%, and repeated sampling of the same cell population (cell sorter reproducibility) gave a standard error of the mean of less than 0.5% (Fig. 1). This high reproducibility was achieved with 0.02 OD units of virus, about fivefold less than saturation concentrations and allowed studies of virus binding to be carried out with small amounts of virus. The fluorescence values of 22 and 54 for resistant C/C and susceptible C/E binding are weighted arithmetic means of these distributions and would be equivalent to what one would obtain in a bulk measurement of a large number of cells by other methods. However, with the latter, the cell-to-cell variation in binding is unknown. One usually assumes a gaussian distribution expressing an error in measurement around a mean value. However, the distributions observed here were not errors in measurement around the mean of the distribution. Rather, cells with a fluorescence of 100 units really had 100 ± 2% relative units of virus, whereas cells with a fluorescence of 50 units really had 50 ± 2% relative units of virus. Thus, differences in fluorescence measurements per cell in the distribution reflect real differences in viral binding per cell. More important, the profile of fluorescence distribution with the two cell populations was altogether different. The distribution curve of fluorescence with susceptible cells showed a

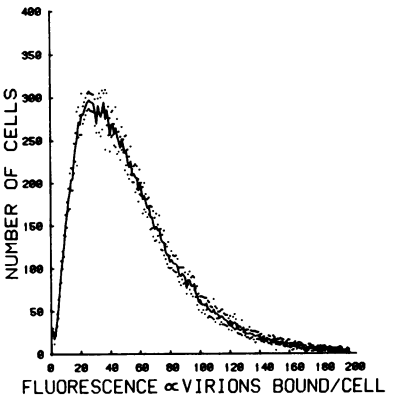

FIG. 1. Assay reproducibility. Distribution curve of susceptible C/E CEF with bound fluorescent LA33C. The curve is generated from eight different samples and shows a binding reproducibility of 54.15 ± 0.74 (mean fluorescence ± standard error of the mean). See text for discussion. Cell sorter reproducibility obtained by repeated determination of the same sample is 54.15 ± 0.23.

degree of skewness far greater than that of a resistant population, and the mean fluorescence values given in this study only partially describe the differences between the two populations (Fig. 2). C/C cells adsorbed 2.14 more SR-E virus than resistant C/E CEF at less than saturation concentrations. When SR-A was mixed with susceptible cells or with resistant C/ABE cells without polycation treatment, mean fluorescence binding was also higher for susceptible than for resistant cells, although the difference was not as great.

To evaluate the effect of Polybrene on virus adsorption, susceptible C/C cells were treated with two different concentrations of this polycation and mixed with labeled SR-E. Virus binding was compared with adsorption to untreated cells (Table 2). In the absence of Polybrene, SR-E bound to cells at a low level. In the presence of Polybrene (2 μg/ml), the mean fluorescence

TABLE 1. Relative mean fluorescence of CEF of different genotypes exposed to labeled virus of different subgroups[a]

Virus	Virus titer[b]	OD_{260}	Relative mean fluorescence[c]		
			C/E	C/C	C/ABE
SR-E	5×10^5	0.02	64 (R)	137 (S)	ND
LA33C	1×10^6	0.02	54 (S)	22 (R)	ND
SR-A	1.5×10^6	—[d]	60 (S)	ND	40
RSV(−)		0.09	18[e]	ND	ND
None			2.6	3.5	ND

[a] Data for different viruses are from different experiments.
[b] Focus-forming units of original harvest fluids per ml on susceptible cells.
[c] S, susceptible; R, resistant; ND, not done.
[d] OD was not measured; however, multiplicity of infection (0.3) is comparable to that of other viruses.
[e] RSV(−) is not infectious for any cell genotype.

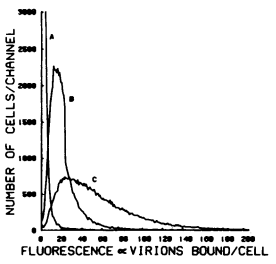

FIG. 2. Specific and nonspecific viral binding. Distribution curves of resistant and susceptible CEF with bound fluorescent LA33C. Curve A, Autofluorescence of control C/E cells (2.62 ± 0.02 = mean fluorescence ± standard error of the mean). Curve B, C/C cells (resistant), 22.80 ± 0.15. Curve C, C/E cells (susceptible), 54.15 ± 0.74. Curve B represents nonspecific viral binding; curve C represents both specific and nonspecific binding.

was sixfold higher. When 10 μg of Polybrene per ml was used, however, the mean fluorescence obtained was similar to that observed with untreated cells. Thus, high doses of Polybrene appeared to inhibit adsorption. Similar results were obtained also with resistant cells (data not shown).

To show whether a correlation exists between amount of cell fluorescence and virus adsorption leading to infection, labeled SR-A was mixed with C/E CEF. Cells with high levels of fluorescence were sorted, recovered in a sterile manner, and grown in culture dishes at 39°C. After 5 days the cultures were totally transformed. On the other hand, cells with low levels of fluorescence did not show appreciable transformation after the same period of time. The fact that the latter cells did not become transformed within 5 days, even if a certain amount of fluorescence was present, is probably a reflection of the high ratio of noninfectious to infectious particles. A similar experiment involving cells with high and low levels of fluorescence done with resistant C/ABE cells failed to show transformation in both cases, as expected. These experiments indicate that cell fluorescence is due to virus binding and confirm the known fact that virus adsorbed to genetically resistant cells does not successfully initiate infection.

Binding sites can be saturated. We estimated the number of specific and nonspecific virus receptor sites by investigating the relationship between fluorescence and concentration of labeled virus added to cell suspensions.

A constant number of cells was mixed with increasing concentrations of labeled virus. Saturation of viral receptors was reached with the addition of 0.12 OD unit of LA33C or about 5×10^4 virions/cell, as estimated by the OD–micrograms-of-protein relationship reported in Materials and Methods (Fig. 3).

TABLE 2. Effect of polybrene treatment on the binding of labeled SR-E virus[a] to susceptible C/C cells at 4°C

Polybrene (μg/ml)	Mean fluorescence
None	11
2	96
10	12

[a] 0.02 OD_{260} per 7.5×10^5 cells.

Further evidence that specific and nonspecific binding sites can be saturated derives from a competition experiment in which labeled SR-E was mixed with resistant C/E cells, susceptible C/C cells, and C/C cells which had been previously incubated for 40 min with 0.1 OD unit of unlabeled SR-E or Pr-A. This was done at less than saturation to eliminate any problems of steric hindrance. Pretreatment of C/C with unlabeled virus caused a decrease in mean fluorescence of about 30 to 50%, to a level close to that seen with the resistant C/E cells (Table 3).

These results can be best interpreted by assuming that the unlabeled virus adsorbs to specific or nonspecific sites (or both), making them unavailable for binding of the labeled virus.

Specific and nonspecific virus adsorption to cells is rapid. To investigate the rate of specific and nonspecific binding of labeled virus to resistant and susceptible cells, a newly developed time flow parameter was used as a measurement of rate adsorption (Leary and Notter, in press).

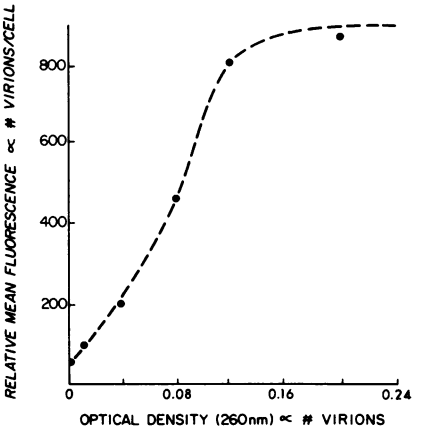

FIG. 3. Saturation of membrane receptors. Fluorescence measured by flow cytometry. Optical density of input virus inoculum measured by spectrophotometry. An approximately linear relationship exists between optical density (input number of virions) and bound fluorescence (number of virions bound/cell) until specific receptor sites are saturated at an OD of approximately 0.12 (equivalent to 5×10^4 virions/cell).

TABLE 3. Adsorption of labeled SR-E virus to susceptible (S) and resistant (R) cells as measured by the amount of fluorescence per cell at 4°C

Cell	Virus	Mean fluorescence
C/C (S)	SR-E*[a]	130
C/E (R)	SR-E*	67
C/C (S)	SR-E + SR-E*[b]	71
C/C (S)	Pr-A + SR-E*[c]	84

[a] SR-E*, Labeled virus, 0.01 OD_{260} unit per 7.5×10^5 cells.

[b] Cells were pretreated with 0.1 OD unit of unlabeled SR-E for 1 h before addition of 0.01 OD of labeled virus.

[c] Cells were pretreated with 0.12 OD unit of Pr-A for 1 h before addition of 0.01 OD unit of labeled virus.

Fluorescence signals were recorded at minute intervals so that kinetics of adsorption of virus to CEF could be measured continuously. Figure 4 represents the kinetics of adsorption of 0.02 OD unit of labeled LA33C to C/C and C/E CEF which are resistant and susceptible, respectively, to this virus. Fluorescence measurements initiated after 2 min were carried out for 50 min. As seen in curve (a), about 75% of maximal binding to susceptible cells was observed within 10 min. With resistant cells (curve b), much less LA33C was bound, and the maximal level of fluorescence was reached after only 5 min.

Virus envelope glycoproteins are important but not necessary for binding. To determine whether glycoproteins are necessary for adsorption, binding of virus to cells was examined with and without bromelain treatment. Mild protease digestion of RSV (19) and influenza virus (1) with bromelain removes viral envelope glycoprotein with subsequent loss of infectivity. LA33C was treated for 1 h at 37°C with bromelain at a final concentration of 1.3 mg/ml. Treated virus and control virus kept at 37°C for the same amount of time were then labeled with R-18 dye, and 0.2 OD unit of virus, more than saturation concentrations, were mixed with susceptible CEF (Table 4).

Bromelain treatment decreased mean fluorescence even at these high virus concentrations to a value half of that obtained with control virus, whereas it reduced infectivity about 40-fold. However, RTase activity, a measure of internal viral protein function, was not decreased by the treatment. This suggests that bromelain does not affect the viral core and that decreased adsorption and infectivity might solely be due to the removal of envelope glycoproteins. As the effect of bromelain treatment on infectivity was greater than the effect on binding, it is tempting to speculate that a certain amount of nonspecific adsorption may occur even without intact envelope glycoproteins. To verify this assumption, the attachment of 0.2 OD unit of labeled RSV(−) which lacks surface glycoprotein gp85 was also investigated (Table 4). RSV(−) labeled by the standard procedure adsorbed to a limited but significant degree to C/E cells, up to 20% of the level seen with nondefective virus. This level, however, was four- to sixfold higher than the level of autofluorescence. These experiments at the same time confirmed the importance of envelope glycoproteins for virus adsorption and the fact that some nonspecific attachment can take place in the absence of functional glycoproteins.

DISCUSSION

By using a laser flow cytometer, we have observed that more virus adsorbs to susceptible cells than to resistant cells. This appears to be a general phenomenon, as it was observed with viruses of three different subgroups and cells of three different genotypes used in several combinations.

The amount of binding to susceptible and resistant cells, however, varies depending on the virus and the cells, and there is great heterogenicity in binding within each population. The difference in mean fluorescence ranges from less than 2-fold, as in the case of SR-A adsorbed to C/E or C/ABE, to about 10-fold, as in the case of the kinetic experiment with LA33C adsorbed to C/E or C/C cells. However, this latter experiment differed from the typical binding assays in that cells were not pelleted free from unbound virus or from virus that may have formed reversible attachments with the cell surface. Thus, this 10-fold difference may not be comparable to those observed in other experiments. Although we have reported here the weighted arithmetic means of these distributions, a very important aspect of our methodology is that it allows us to measure differences in viral binding in single cells. This difference between susceptible and

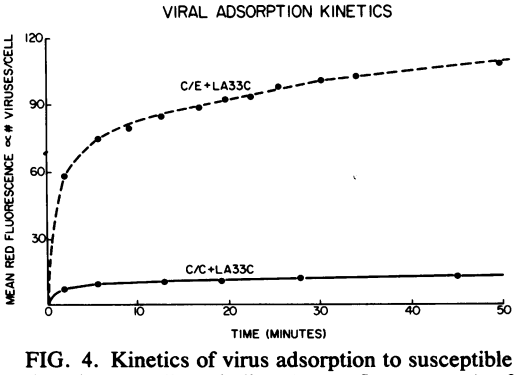

FIG. 4. Kinetics of virus adsorption to susceptible and resistant cells. Binding (mean fluorescence) of labeled LA33C to susceptible (C/E) or resistant (C/C) CEF as measured continuously from 2 to 50 min.

TABLE 4. Effect of bromelain treatment on the adsorption of labeled LA33C to susceptible C/E cells

Virus[a]	FFU/ml[b]	RTase[c]	Density (g/ml)	Mean fluorescence
LA33C control	2×10^5	176,000	1.15	72
LA33C enzyme treated	5×10^3	213,000	1.14	37
BH RSV(−)	—[d]	100,000	1.14	18

[a] 0.2 OD_{260} unit per 7.5×10^5 cells.
[b] FFU, Focus-forming units.
[c] Expressed as cpm of [^{3}H]TTP incorporated in 2.5 h at 40°C.
[d] This virus does not possess the major glycoprotein gp85 and therefore is not infectious.

resistant cells does not depend on cell size, as the data were normalized to a standard surface unit, nor statistical error, but rather suggests that susceptible CEF possess on their surface both specific and nonspecific sites for RSV adsorption, whereas resistant cells possess only nonspecific sites. This hypothesis is at variance with earlier beliefs that adsorption of RSV to either susceptible or resistant CEF is the same. Also, the fact that viruses of different subgroups show consistent differences in binding to susceptible cells suggests that different specific receptors may exist for different types of glycoproteins. This is in agreement with data of Moldow et al. (12, 13), who have shown the presence of different binding sites for viruses of subgroups A and B on the surface of susceptible CEF.

The higher level of binding of RSV to susceptible cells appears to be due to a specific virus-cell interaction mediated by virus glycoproteins. The removal of glycoproteins by a treatment with bromelain decreased the attachment of virus to a level comparable to that observed with similar amounts of RSV(−), a virus which lacks envelope glycoprotein gp85 (6). The residual fluorescence observed in these experiments suggests that nonspecific binding does not require functional virus glycoproteins.

Treatment of cells with low concentrations of Polybrene increases virus adsorption in a way that can be detected by this technique. Of interest is also the fact that high levels of Polybrene inhibit adsorption. This observation suggests that the inhibition of infectivity observed by others with high levels of polycations may not be entirely due to toxic effect of these compounds on the cells (25).

Pretreatment of susceptible cells with unlabled virus decreases binding of labeled virus to the level observed with resistant cells. It is not possible to conclude from these experiments which type of sites, specific, nonspecific, or both, are blocked; however, in other experiments, cell receptors for RSV adsorption could be saturated by exposing the cells to increasing amounts of virus. The saturation of receptor sites may be governed by spatial limitations, since a saturation density of up to 5×10^4 input virions per cell is required for saturation. This is approximately the number of virus particles that can cover the surface of a chicken fibroblast, assuming that RSV has a diameter of 80 to 90 nm and that the mean diameter of CEF is 9 μm. However, the conclusions on these experiments are based on input virus, and actual numbers of attached virions are likely to be lower, either because of lack of initial attachment or subsequent loss during washing.

Whether or not spatial limitations for RSV exist on susceptible cells, specific virus binding still most likely depends on the presence of "recognition areas" as judged by the consistent differences found between susceptible and resistant cells. The best evidence on this point comes from the kinetic study in which virus binding to both types of cells was monitored over time under conditions of less than saturation concentrations of virus, where spatial problems most likely did not exist.

These preliminary studies do not tell us if more than one virus particle is bound to a recognition area, but simply argue for a larger number of sites on susceptible cells. Also, we cannot determine if the virus is "fused" to the cell membrane, although by carrying out all experiments at 4°C over a short period of time we have probably minimized viral penetration.

In summary, this study demonstrates the feasibility of distinguishing different amounts of viral receptor sites on the surface of susceptible and resistant cells by a method which is more sensitive and adaptable than others previously applied. Recently McGrath et al. (11) have applied methods similar to ours to the investigation of the receptors for murine leukemia virus on thymic lymphoma cells. It is reasonable to assume that laser flow cytometry might be useful for investigating other parameters of virus-cell interactions.

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