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Virus-Receptor Interaction in the Adenovirus System: Characterization of the Positive Cooperative Binding of Virions on HeLa Cells

ROBERT PERSSON,* CLAES WOHLFART, ULLA SVENSSON, AND EINAR EVERITT

Department of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund, Sweden

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The established positive cooperativity of adenovirus 2 binding to HeLa cells revealed a strong temperature dependence. The degree of cooperativity, quantified by means of Hill coefficients, progressively increased from 10°C to reach a maximum level, which was maintained between 20 and 37°C. On the other hand, negative cooperativity of virion attachment was apparent at 3.0°C and on glutaraldehyde-stabilized cells. The corresponding monovalent ligand of the system, the fiber antigen, demonstrated only weak-positive cooperativity of the binding at 37.0°C, which was absent at 3.0°C. Dithiothreitol and dansylcadaverine, reagents inhibiting clustering of ligand-receptor complexes in the plasma membrane, markedly reduced the degree of positive cooperative binding at 37.0°C. Evidently, the positive cooperative binding of adenovirus to HeLa cells at 37.0°C is a consequence of both the multivalency of virus attachment proteins, i.e., fibers, on the virion and of the capacity of the receptor sites to migrate in the plane of the plasma membrane, forming local aggregates of virus-receptor site complexes.

Recently, it has been suggested that adenovirus attachment to cells leads to rearrangements in the plasma membrane (10, 11, 20, 21), resulting in cooperative binding and capping of the virus particles (21). On HeLa and KB cells there are 5,000 to 10,000 specific binding sites (13, 23), which are referred to as cellular receptor sites. The fiber antigen, localized in the vertex regions of the virus particle, has been identified as the virus attachment protein, since it specifically prevents virion attachment after cells have been pretreated with this protein (23). The number of receptor sites for the fiber has been estimated to be one to two orders of magnitude greater than that for virions on HeLa and KB cells (13, 23).

The importance of lateral migration and clustering of ligand-receptor complexes in the plasma membrane has been demonstrated as an integral part of the mechanism leading to cellular activation in response to peptide hormones (14, 27, 28). Furthermore, it has conclusively been shown by Prujansky et al. (25) that mitogenic stimulation of lymphocytes by lectins requires multivalent ligands. Positive cooperative binding is also apparent with such multivalent lectins, but not with the correspondingly monovalent ones. These authors put forward the idea that clustering of lectin-receptor complexes and conformational changes in membrane structure are prerequisites for mitogenic stimulation (25).

Positive cooperative binding of ligands could imply that binding of the first ligand to one receptor site promotes the binding of subsequent ligands to the other sites. On the other hand, negative cooperativity describes the situation when the binding of one ligand impairs the attachment of the subsequent ligands (for a review, see reference 17). The Hill coefficient (12) is a most convenient means to quantitatively express the degree of cooperativity, and this factor is determined from the slope of a corresponding Hill plot.

In this report attempts have been made to characterize the situation leading to positive cooperative binding of adenovirus 2 (Ad2) to HeLa cells. The attachment of virions to cells was compared with that of the fiber antigen with respect to obtained Hill coefficients and binding affinities at 3.0 and 37.0°C. The effect of physiologically interacting reagents and the temperature dependence of the cooperative binding between 3.0 and 37.0°C was also studied.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μg of gentamicin per ml. Ad2 was propagated, radioisotope ([^{3}H]thymidine) labeled, and purified as described previously (6).

Isolation and purification of the fiber antigen. Virus-infected cells were disrupted and processed for virion isolation as previously reported (6). The virus-free supernatants, containing soluble cell and viral antigens, were subjected to anion-exchange chromatography as described by Pettersson et al. (22), with the following modifications: the buffer system of choice was 50 mM Tris-hydrochloride (pH 7.7), and the sodium chloride gradient ranged from 0 to 0.4 M. Fiber antigen-containing fractions were collected after identification by double radial immunodiffusion (18) against monospecific antifiber antiserum. After extensive dialysis against 50 mM sodium phosphate buffer (pH 7.2) containing 0.2 M sodium chloride, the isolated fiber was applied on a column of wheat germ agglutinin-Sepharose 6MB (0.9 by 6.3 cm or 4 ml) equilibrated in the same buffer. After elution with 100 mg of *N*-acetyl-D-glucosamine per ml, the purified fiber was dialyzed extensively against double-distilled water and subsequently freeze dried.

Radioisotope labeling of the fiber antigen. The purified fiber, dissolved in 0.1 M sodium borate buffer (pH 8.1) containing 0.15 M sodium chloride, was ^{125}I-labeled with 1,3,4,6-tetrachloro-3a,6a-diphenyl-glucoluril (Iodogen) as described by Fraker and Speck (7). The specific radioactivity of the labeled fiber was 2.9×10^8 cpm/mg of protein.

Attachment studies. The general procedure of virion attachment was as outlined earlier (32). HeLa cells were

* Corresponding author.

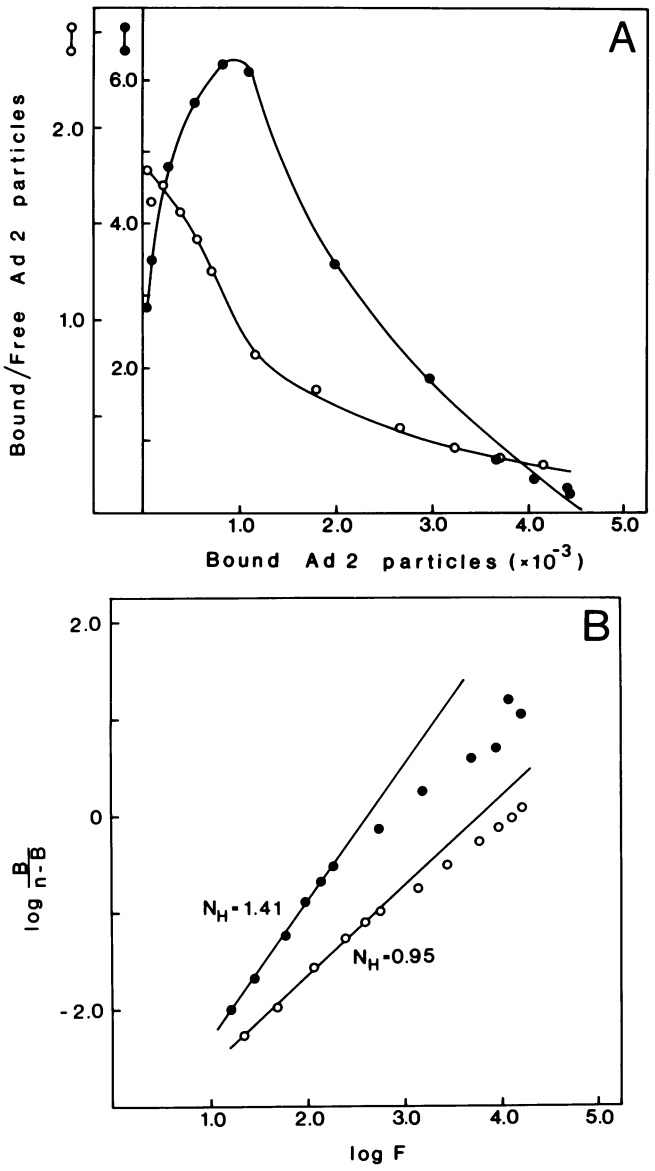

FIG. 1. Equilibrium binding of adenovirus to HeLa cells. Ad2 particles were added to cells at different MOIs, and the number of bound virions was estimated after 45 min at 37.0°C (solid circles) and 180 min at 3.0°C (open circles). Data from a typical experiment was converted into a Scatchard plot (A) and a Hill plot (B). N_H, Hill coefficient.

washed once in phosphate-buffered saline and suspended to 5×10^7 cells per ml, and in all experiments, cells were equilibrated at the appropriate temperature for 10 min before the addition of the ligand. [^{3}H]thymidine-labeled virions (1.0×10^7 to 1.5×10^7 cpm per 10^{12} virions) were allowed to attach to cells at multiplicities of infection (MOIs) between 50 and 20,000. Virus-cell mixtures were incubated for 45 min at 37.0°C or 180 min at 3.0°C. These periods were selected to ensure saturated binding at equilibrium (21). In temperature experiments, the virion attachment was assessed between 3.0 and 37.0°C, after selected incubation periods as described above.

Attachment of virus particles at 37.0°C was also measured in the presence of the following reagents: sodium azide (50 mM), 2-deoxyglucose (50 mM), dithiothreitol (5 and 10 mM), EDTA (5 and 20 mM), ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA; 5 and 20 mM), cytochalasin B (60 μg/ml), colchicine (0.4 mM), trifluoperazine (50 μM), dansylcadaverine (0.5 and 1.0 mM), ammonium chloride (40 mM), chloroquine (0.4 mM), and methylamine (40 mM). The cells were preincubated with the appropriate reagent 30 min before addition of the virus.

Attachment of ^{125}I-labeled fiber antigen was assessed analogously to virion attachment as outlined above. Ratios of fiber molecules to cell numbers were between 2,000 and 400,000, and incubation periods for cells and added fiber antigen were 30 and 120 min at 37.0 and 3.0°C, respectively. For calculations, a 180,000-dalton molecular mass of the native fiber antigen was used (4, 30).

Scatchard and Hill plots. Graphical representations according to Scatchard (26) were made for binding data obtained for virions and fibers. For each graph a corresponding Hill plot (12) was constructed in which log ($B/n - B$) was plotted against log F; B and F represent bound and free ligands, respectively, and n is the actual number of receptor sites per cell for each series, derived from the intercept on the abscissa of the Scatchard plot.

Protein determination. Protein was determined by the method of Hartree (8) with bovine serum albumin as the standard.

Liquid scintillation spectrometry. Direct radioactivity measurements of water-containing samples were made in Ready-Solv HP/b. A Mark II scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) was used to determine radioactivity.

Chemicals and reagents. Eagle minimal essential medium, fetal calf serum, gentamicin, and L-glutamine were obtained from Flow Laboratories, Ltd., Irvine, Scotland. [^{3}H]thymidine (75 Ci/mmol) was purchased from New England Nuclear Chemicals Gmbh, Dreieich, Federal Republic of Germany, and the [^{125}I]iodine (13 to 17 mCi/μg) was from Amersham International plc, Buckinghamshire, England. Bovine serum albumin, chloroquine, colchicine, cytochalasin B, dithiothreitol, EGTA, monodansylcadaverine, sodium azide, and trifluoperazine were from Sigma Chemical Co., St. Louis, Mo. Ammonium chloride, 2-deoxy-glucose, EDTA, and methylamine were from E. Merck AG, Darmstadt, Federal Republic of Germany, and *N*-acetyl-D-glucosamine was from BDH Chemicals, Ltd., Poole, England. Wheat germ agglutinin-Sepharose 6MB was obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. Iodogen was from Pierce Chemicals Co., Rockford, Ill., and the Ready-Solv PH/b was purchased from Beckman Instruments AB, Bromma, Sweden.

RESULTS

Virion attachment. Ad2 was added to HeLa cells at different MOIs ranging from 50 to 20,000. To ensure proper equilibrium binding at 37.0 and 3.0°C, virus-cell mixtures were incubated for 45 and 180 min, respectively. Fifteen separate binding experiments with 12 measuring points for each series were performed at 37.0°C. A typical Scatchard plot for virion binding at this temperature revealed a maximum of the curve indicating positive cooperativity (Fig. 1A). The average number of CRS was estimated to be between 3,700 and 5,900, and a binding constant per site between 1.0×10^{10} and 2.2×10^{10} (M^{-1}) was obtained.

Graphical representation of virion binding data at 3.0°C according to the Scatchard plot revealed an upward curvature, indicative of negative cooperativity (Fig. 1A).

To obtain quantitative, comparable, and more easily interpretable views of the Scatchard plots, the previous results and the binding data to follow were converted into Hill plots (Fig. 1B). Hill coefficients were calculated from the slopes of the plots, and coefficients ranging from 1.35 to 1.47 and 0.94 to 0.96 were obtained for virion binding at 37.0 and 3.0°C, respectively. It should be pointed out that all Hill coefficients in this report were based on the first six measuring points representing the low and medium multiplicity ranges, including the maximum regions of the corresponding Scatchard curves for all 37.0°C series. A value above 1.0 for the slope of a Hill plot indicates a positive cooperativity for the observed ligand-receptor interaction.

For the low-temperature series, the computer program "LIGAND" developed by Munson and Rodbard (16) was used to fit the experimental data. A two-receptor-site model with different affinities emerged as a result of the calculations. Figure 2 shows the Scatchard plot for the low-temperature series of Fig. 1A, with the addition of the computerized lines I and II depicting the suggested two component receptor system. Obtained intrinsic binding constants of 1.5×10^{10} and 7×10^{8} (M^{-1}) for the high (I) and low (II) affinity sites, respectively, are represented by the lines. Calculated receptor numbers for the high- and low-affinity sites were 1,200 and 5,500, respectively. The horizontal line III is the computer representation for nonspecific binding.

Attachment of the fiber antigen. Equilibrium binding studies of affinity-purified fiber antigen to cells were performed as described above. Scatchard plots of the fiber attachment yielded almost straight lines at 3.0°C (Fig. 3A). From corresponding Hill plots, Hill coefficients of 0.99 to 1.03 were obtained, thus strengthening the idea of noncooperative binding indicated in the Scatchard plot (Fig. 3B). The binding at 37.0°C showed slight downward curves in the Scatchard analyses and yielded Hill coefficients between 1.05 and 1.15, suggesting weak-positive cooperative binding

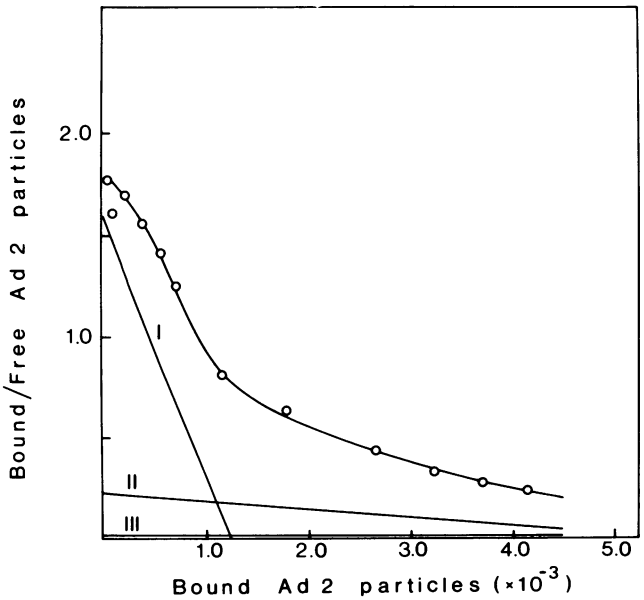

FIG. 2. Scatchard plot of adenovirus binding to HeLa cells at 3.0°C. Data shown in Fig. 1 was analyzed by the LIGAND computer program. Lines I and II represent the obtained virion binding to high- and low-affinity sites, respectively. Line III represents the nonspecific binding of the viruses.

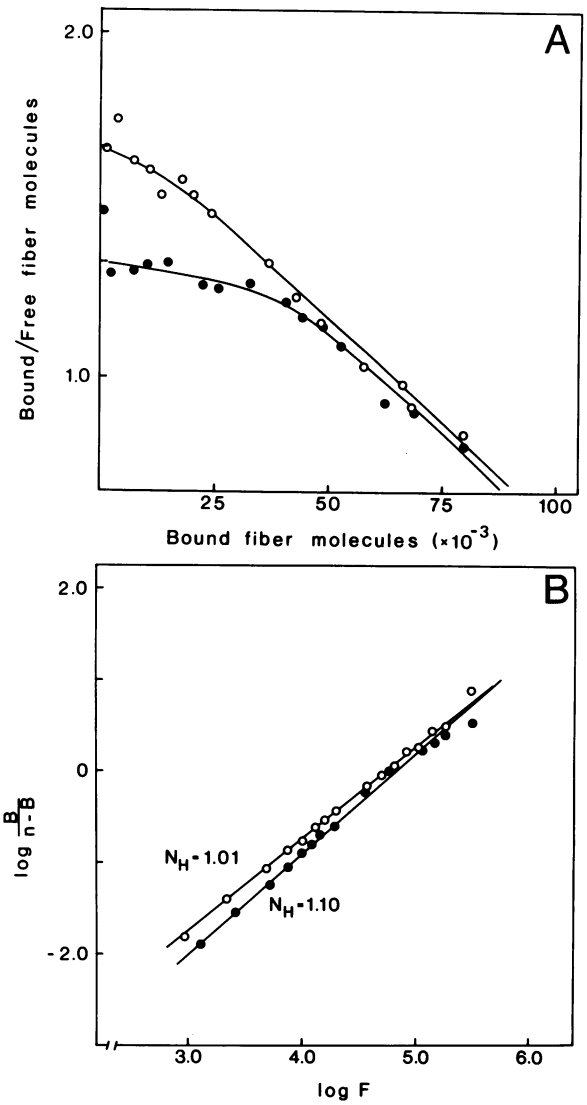

FIG. 3. Equilibrium binding of fiber antigen to HeLa cells. Fiber was added to cells at ratios of between 2,000 and 400,000 molecules per cell. The amount of bound fiber was measured after 30 min at 37.0°C (solid circles) and 120 min at 3.0°C (open circles). Data represent a typical experiment. (A) Scatchard plot; (B) Hill plot. N_H, Hill coefficient.

at this temperature. The estimated number of binding sites at both temperatures ranged between 85,000 and 95,000, and calculations of the association constants yielded values from 1.0×10^{8} to 2.7×10^{8} (M^{-1}). Attachment of fiber antigen, metabolically labeled with [^{35}S]methionine (5), displayed under identical conditions almost the same binding pattern as the ^{125}I-labeled fiber, with an estimated association constant of 2.9×10^{8} (M^{-1}) and ca. 110,000 binding sites (data not shown).

Cooperative binding of Ad2 at different temperatures. As graphically demonstrated by plotting calculated Hill coefficients against various attachment temperatures, the positive cooperative binding of virions exhibited strong temperature dependence, with a two-critical temperature appearance (Fig. 4). At temperatures below 6°C, the Hill coefficient displayed a lowest value of 0.95, whereas a shift occurred at 7 to 8°C, revealing a plateau with a coefficient of ca. 1.08. At

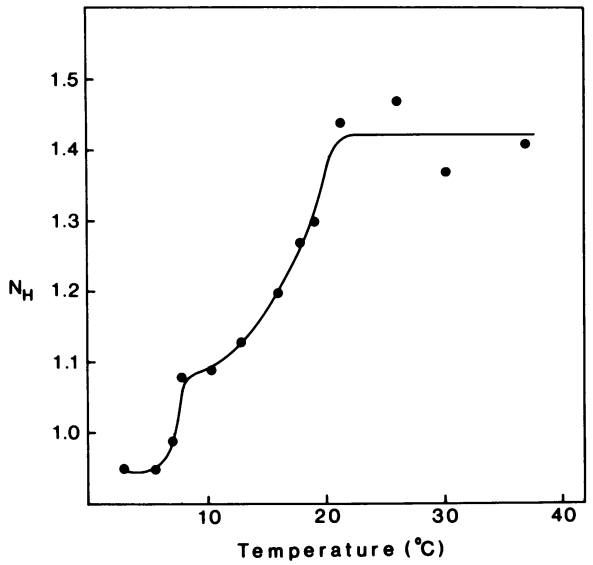

FIG. 4. Temperature dependence of the cooperative binding of adenovirions to HeLa cells. Different MOIs of Ad2 particles were added to cells equilibrated at different temperatures. The attachment was measured as equilibrium binding at appropriate temperatures, and for each temperature a separate Hill plot was constructed, from which Hill coefficients (N_H) were derived.

increasing temperatures up to 20°C, the Hill coefficient progressively increased to finally reach a maximum level of 1.42, which was maintained between 20 and 37°C.

Effect of different reagents on the cooperative binding of Ad2 at 37°C. The most pronounced inhibitory effect on the positive cooperative Ad2 binding was obtained by dithiothreitol at a concentration of 10 mM, followed by the effect of 1.0 mM dansylcadaverine (Fig. 5). All the other reagents investigated were ineffective. Thus, the positive cooperative binding was not dependent on accumulated energy in the form of ATP, and the cytoskeleton system appeared not to be involved. EDTA, EGTA, trifluoperazine, and the lysosomotropic agents at the indicated concentrations did not affect the positive cooperativity of Ad2 binding to HeLa cells.

Attachment of virions at 37.0°C to HeLa cells stabilized with 0.015% glutaraldehyde (21) was comparable to the situation at 3.0°C, with an estimated Hill coefficient of 0.99.

DISCUSSION

The binding mechanisms of Ad2 virions and fiber antigen to HeLa cells were studied at equilibrium, i.e., after incubation periods long enough to achieve saturated binding (29, 33). In binding studies of ligands to cells it is of the greatest importance to be certain that the obtained results are reflections of true attachment, and do not include effects of, e.g., internalization of ligand-receptor complexes or modifications of the ligand as discussed by Beck and Goren (1). Ways to overcome this problem is to decrease the temperature and thereby prevent internalization or to add reagents that inhibit such side reactions without affecting the apparent attachment.

It was reproducibly shown that virion attachment at 37.0°C exhibited positive cooperativity, with an estimated mean Hill coefficient of 1.41. From the Scatchard plots the determined number of cellular receptor sites was between 3,700 and 5,900, and the calculated association constants ranged between 1.0×10^{10} and 2.2×10^{10} (M^{-1}), as previously proposed (21). The binding of the fiber antigen displayed noncooperativity at 3.0°C, whereas a tendency towards positive cooperative binding at 37.0°C was observed. Wadell and Norrby (34) have reported that a small fraction of the fiber antigen, from adenovirus belonging to subgroup C and isolated by ion-exchange chromatography, has the ability to dimerize. Such bivalent structures might bind to cells in a cooperative way, yielding the increased mean Hill coefficient of 1.10 at 37.0°C as compared with the value of 1.01 obtained at 3.0°C. The mean number of receptor sites for the fiber antigen on HeLa cells was estimated to be 90,000, with a mean association constant of 2.3×10^8 (M^{-1}), at both 37.0 and 3.0°C. This is in fairly good agreement with previously reported data on attachment of fibers to isolated KB cell plasma membranes (13) and also with the calculated number of fiber receptors on HeLa and KB cells (23). The possibility that the in vitro-labeled fiber antigen could yield erroneous binding data due to chemical modification(s) (2) was not considered in the presented study, since in vivo-labeled fiber exhibited attachment characteristics almost identical to those of the iodinated antigen. Attachment of virions at 3.0°C showed an upward curve in the Scatchard plot, and

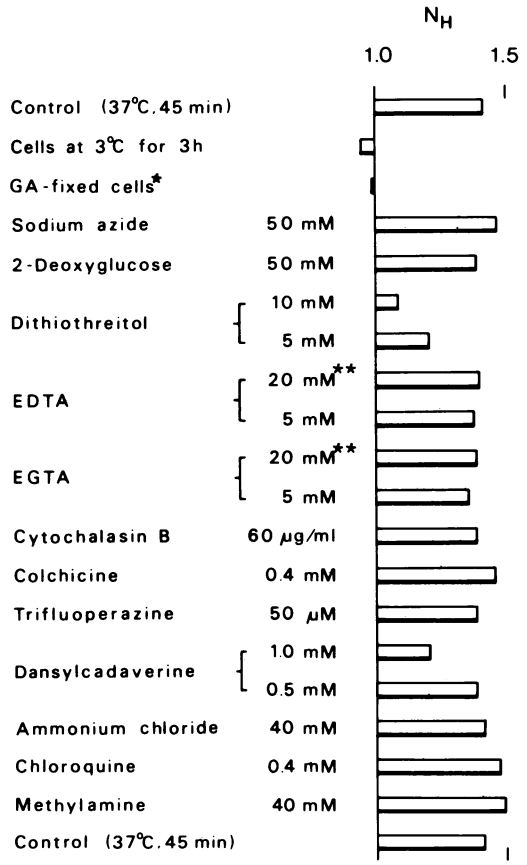

FIG. 5. Influence of different reagents on the positive cooperative binding of adenovirus. Cells were preincubated at 37.0°C with the appropriate reagent for 30 min before the addition of virus. After 45 min of virus-cell interaction, the number of bound virions was estimated, and Hill coefficients (N_H) were calculated. *, Cells were fixed with 0.015% glutaraldehyde (21); **, cells treated with 20 mM of either EDTA or EGTA had a tendency to aggregate.

a Hill coefficient slightly below 1 (i.e., 0.95) was obtained, indicating negative cooperative binding. The engagement of high- and low-affinity sites was also revealed after computerized model fitting of the experimental data. Even though the low number of measuring points, especially at high MOIs, made the calculations not statistically significant, it is reasonable to believe that attachment of virions at 3.0°C and low MOIs yields an association with cells with the same binding affinity as that at 37.0°C. On the other hand, at high MOIs, the competition for receptor sites will cause virions to bind monovalently with respect to the fiber antigen and with an affinity in the range of that of isolated fiber.

The positive cooperativity was shown to be highly temperature dependent, with two shifts in the temperature regions at 7 to 8°C and 19 to 20°C. We interpret the shifts in the temperature-dependent positive cooperativity in terms of local phase transitional-separational effects on the receptor-ligand domains of the plasma membrane. A similar change in the physical status of the lipid matrix has been suggested for the temperature-dependent uncoating of adenovirions associated with the plasma membrane surface (31). Correspondingly local thermal transitions have been proposed as an explanation for the temperature-dependent intercellular adhesiveness of HeLa cells (3), in spite of failure to establishing temperature-dependent shifts in membrane microviscosity. These studies of changes in microviscosity were performed by fluorescence polarization of incorporated 1,6-diphenyl-1,3,5-hexatriene in the bulk of the cell surface lipids. If one wishes to study the interactions of receptor-ligand complexes within the lipid maxtrix, the annular lipids constituting the immediate environment are those of interest. Therefore, the most relevant means of studying the migrational behavior of viruses attached to cellular receptor sites in the plasma membrane might be the employment of rhodamine-labeled virions (21) in connection with the FRAP (fluorescence recovery after photobleaching) technique, as introduced by Poo and Cone (24) and Liebman and Entine (15).

Different reagents previously employed to manipulate the steps connected with internalization of Ad2 virions (31) were used to further characterize the positive cooperative binding of Ad2 at 37.0°C. The presence of dithiothreitol and dansylcadaverine markedly reduced the degree of positive cooperativity of the binding. These reagents have been shown to efficiently block the clustering of receptor-ligand complexes in the membrane (9, 19). The above results, together with the facts that attachment of virions at 37.0°C to glutaraldehyde-fixed cells exhibited no positive cooperativity (Fig. 5) and the negative cooperative binding at 3.0°C (Fig. 1 and 5), all strongly suggest that aggregation in the plasma membrane of virion-receptor site complexes is a prerequisite for the achievement of positive cooperative binding of Ad2 virions. The addition of sodium azide, a potent inhibitor of Ad2 internalization (31), or the reagents interfering with the cytoskeletal elements had no effect on the positive cooperativity. This implies that the aggregation in the membrane is a relatively local phenomenon and distinct from the previously reported capping of bound virions (21).

From the presented results on the Ad2 virion and fiber attachment to HeLa cells, we propose that the unequivocal positive cooperativity of virion attachment at 37.0°C is a function of *both* the multivalency of virion attachment proteins on the actual virus particles and the ability of the receptor sites to move laterally in the plasma membrane, forming local aggregates of virus-receptor site complexes.

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