Isolation and Characterization of Chinese Hamster Ovary Cell Variants Defective in Adhesion to Fibronectin-coated Collagen

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ABSTRACT Variant clones of Chinese hamster ovary (CHO) cells were selected for reduced adhesion to serum-coated tissue culture plates. These clones also displayed reduced adhesion to substrata composed of collagen layers coated with bovine serum or with fibronectin (cold-insoluble globulin). Wild-type (WT) and adhesion variant (AD^v) cells grew at comparable rates in suspension culture, but the adhesion variants could not be grown in monolayer culture because of their inability to attach to the substratum. The adhesion deficit in these cells was not corrected by raising the concentration of divalent cations or of serum to levels 10-fold greater than those normally utilized in cell culture. However, both WT and AD^v clones could adhere, spread, and attain a normal CHO morphology on substrata coated with concanavalin A or poly-L-lysine. In addition, the adhesion variants could attach to substrata coated with "footpad" material (substratum-attached material) derived from monolayers of human diploid fibroblasts or WT CHO cells. These observations suggest that the variant clones may have a cell surface defect that prevents them from utilizing exogeneous fibronectin as an adhesion-promoting ligand; however the variants seem to have normal cytoskeletal and metabolic capacities that allow them to attach and spread on substrata coated with alternative ligands. These variants should be extremely useful in studying the molecular basis of cell adhesion.

Cellular adhesiveness is a fundamental aspect of many normal and pathological processes. Wound healing, embryogenesis, and the invasive and metastatic behavior of cancer cells depend, to some degree, on the ability of the cell to make and break adhesive connections while migrating through the extracellular matrix (23, 36). The study of cell adhesion has been approached in several ways; cellular adhesiveness can be studied in terms of the rate at which cells attach to other individual cells, cell aggregates, cell monolayers, or to a variety of substrata (9, 33, 42). Alternatively, adhesion has been quantified in terms of the ease of cell detachment by trypsin, chelating agents, or shear (5, 9, 13). It seems likely, however, that these various assays may reflect different aspects of cellular adhesive behavior. While the process of substratum attachment is quite complex, it nonetheless is probably the simplest, most reliable measure of cell adhesion. In early studies, highly artificial substrata such as protein-coated glass or plastic were used (5, 9, 10, 15), but recently substrata with greater physiological relevance such as collagen (3, 5, 6, 11) and fibrin (26) have been employed. There is considerable evidence that the events that take place during cell-substratum attachment in vitro are biologically significant and bear some resemblance to the interaction between cells and the connective tissue matrix in vivo. Thus, adhesion in vitro is mediated by a layer of protein absorbed to the substratum (6, 9, 22), there is a divalent ion dependence (18), energy metabolism is involved (15, 24), and adhesion is inhibited if the cytoskeleton is perturbed (15, 16, 24).

Recently, a set of proteins thought to have an important role in cell adhesion has been identified and characterized. These components, termed fibronectins (39, 41), are a family of high molecular weight glycoproteins found in plasma, or associated with cell surfaces, especially at points of cell attachment (12, 20). There appear to be specific domains on the fibronectin molecule that have the capacity for interaction with the cell surface (34), with collagen (3), and with glycosaminoglycans

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(29, 35). In addition fibronectin, at low concentrations, promotes cell attachment and enhances the normal alignment and spreading of fibroblasts (38, 40). Fibronectin therefore, satisfies many of the criteria for an adhesive ligand, capable of bridging between the cells and the connective tissue matrix. There are a number of biochemical and functional differences between the circulating and cellular forms of fibronectin (27, 37), but the ability to promote adhesion is shared by both forms.

Although the discovery of fibronectin has provided many insights into the adhesive process, there still remain many unanswered questions as well. These include (*a*) the nature of the cellular binding site for fibronectin, (*b*) the possible role of other cell surface constituents in adhesion, and (*c*) the nature of the interplay between surface elements and components of the cytoskeleton. We have decided to attempt to answer some of these questions using an approach based on somatic cell genetics, namely the selection and characterization of variant cell clones with diminished adhesive capabilities. We believe that an understanding of the defects involved will yield further insights into the normal adhesive process.

In this paper, we describe a selection procedure for the isolation of CHO cell variants with an altered ability to adhere to serum-coated collagen or to other serum- or fibronectin-coated substrata. In addition we present evidence, based on an initial characterization, that the defect in these adhesion variants is likely to be at the cell surface.

MATERIALS AND METHODS

Cell Culture

Wild-type (WT) CHO cells and adhesion variants (AD^v) were routinely maintained in suspension culture in α minimal essential medium (α-MEM) plus 10% fetal calf serum at 37°C and 5% CO_2 (14).

Preparation of Fibronectin

The circulating form of fibronectin (cold-insoluble globulin [CIG]) was prepared from fresh bovine plasma by the gelatin affinity technique of Engvall and Ruoslahti (7). The CIG prepared in this manner was pure judged by SDS polyacrylamide gel electrophoresis criteria.

Selection of Adhesion Variants

Exponentially growing WT cells were pelleted by centrifugation and resuspended at 1×10^6 cells/cm^3 in fresh growth medium containing 50 $\mu g/cm^3$ ethanemethanesulphonate (EMS). After a 16-h exposure to the mutagen, the cells were washed free of EMS, resuspended in fresh growth medium, and allowed a 3- to 4-d recovery period. Exposure to EMS reduced the colony-forming ability of the treated cells to 71% of control cells. To enrich for less adhesive phenotype, the mutagenized cells were plated in serum containing medium at 5×10^5 cells/75-cm^2 tissue culture flask and allowed a 3-h period at 37°C to adhere; under these conditions better than 95% of WT CHO cells will adhere. The nonadhesive cells or "floaters" were recovered, returned to suspension culture for 3 h and then given another opportunity (2 h) to adhere to tissue culture flasks. The floaters, which initially constituted 5% of the original cell population, were replaced in suspension culture and grown back to the original cell density. The enrichment and regrowth procedure was repeated four times, recovering the floaters each time; at this point ~50% of the population were floaters and did not adhere to tissue culture flasks.

The cells were cloned by dilution into microwells and only those wells containing a single cell by visual inspection were maintained. After the clones had reached 20–50 cells, they were transferred to bacteriological plastic petri dishes (D1906; Lab-Tek Products, Div. Miles Laboratories Inc. Naperville, Ill.). WT CHO cells do not adhere to these dishes but grow readily in suspension, therefore, we considered these dishes to be a nonselective substratum for the routine maintenance of both WT and AD^v CHO cells. The clones were grown to high density, numbered, and then stored by freezing at −70°C in growth medium plus 10% dimethyl sulfoxide. Individual clones were tested for their ability to adhere to serum-coated tissue culture dishes; adhesion behavior ranging from identity with WT to markedly impaired adhesion was found in different clones. In this report we concentrated on clones that are very different from WT, but a few examples of clones with intermediate behavior are included.

The AD^v cells of the E and F series are the results of two independent selections. The clones were numbered AD^v E11, E12, etc., or AD^v F11, F12, etc., corresponding to their initial positions on the microwell dishes. Because of the multiple enrichment steps employed, it is possible that the clones within each of the two series are sister clones (i.e., derive from a common progenitor). The parental WT cells were also re-cloned at this time; the WT subclones were all identical in terms of adhesion behavior. In these studies, we have interchangeably employed the parental WT cells and WT-P, one of the subclones. The variant phenotype is stable during at least 60 doublings whether grown under nonselective (in spinners or in bacteriological plastic dishes) or selective (Falcon tissue culture plastic; Falcon Labware, Div. Becton Dickinson & Co., Oxnard, Calif.) culture conditions. Thus both WT and AD^v cells can be maintained in continuous culture for at least 2 m without changing their respective phenotypes.

To test the dominant or recessive nature of the adhesion variants, we formed hybrids between AD^v F11 and EOTC5, a clone that displayed wild type adhesion behavior (80-90% adherence in 30 min) and which possessed genetic markers for sensitivity to HAT medium and for resistance to ouabain. Polykaryons were formed by fusion of AD^v F11 (HAT^R, $ouabain^S$, $adhesion^-$) and EOTC5 (HAT^S, $ouabain^R$, $adhesion^+$) in medium containing polyethylene glycol 6000, as described (8). As controls, AD^v F11 was fused with itself and EOTC5 and fused with WT cells. After fusion, 2% of cells from EOTC5 × AD^v F11, 3% of cells from EOTC5 × WT, and no cells from WT × WT or F11 × F11 survived selection in HAT-ouabain medium (α-MEM containing 10 μg/ml hypoxanthine, 10 μg/ml thymidine, 1 μM methotrexate, 3 mM ouabain, antibiotics, and 10% fetal calf serum [FCS]). The survivors were cloned in HAT-ouabain medium, numbered as Hyb^1, Hyb^2, etc., and frozen.

Preparation of Substrata

COLLAGEN SUBSTRATUM: Rat tail tendon collagen was prepared as described (4). A 0.25% wt/vol solution of this collagen in water was gelatinized by heating at 60°–70°C for 90 min. After slight cooling, 2 cm^3 was aliquoted onto plastic petri dishes (Falcon 1007) and allowed to dry overnight, thus forming a thin film on the surface of the dish. The gelatin film was then stabilized by fixation with 0.1% vol/vol gluteraldehyde in phosphate-buffered saline, pH 7.5 (PBS) for 10 min, followed by three washes with 1 M glycine and three washes with PBS. At this point the gelatin plates could be stored dry at room temperature indefinitely. The fixation procedure had no effect on the suitability of the gelatin plates as an adhesion substratum. This procedure is similar to those used by other investigators in the preparation of collagen substrata for adhesion assays (11, 17).

Before use as an adhesion substratum, 2-cm^3 aliquots of MEM-α-medium containing antibiotics (AB) (100 μ penicillin, 100 μ streptomycin, and 0.25 mg fungizone/cm^3) and sometimes FCS or purified serum proteins, such as bovine albumin (BSA) or fibronectin (CIG), were added and the dishes were incubated for 2 h at 37°C and 5% CO_2; the dishes were then washed thoroughly with PBS to remove any nonabsorbed protein. The resulting protein (FCS, CIG, or BSA) -coated gelatin substrata were used immediately in adhesion assays.

PLASTIC SUBSTRATUM: Tissue culture plastic petri dishes (Falcon 3030) were coated with MEM-α-medium plus AB plus FCS or BSA as described above. The washed plates were used immediately either in adhesion assays or further modified by lectin treatment as described below.

GLASS SUBSTRATUM: Serum-coated glass liquid scintillation vials were prepared as previously described (15).

LECTIN SUBSTRATUM: A 0.5 mg/ml solution of the lectin concanavalin A (Con A) in PBS was aliquoted onto plastic petri dishes previously coated with 10% FCS as described above. After incubation at room temperature for 30 min, the dishes were washed thoroughly with PBS to remove any unbound lectin and used immediately in adhesion assays.

POLY-L-LYSINE (PL) SUBSTRATUM: Plastic petri dishes (Falcon 3030) were treated with 2 cm^3 of a 1 mg/ml solution of PL in PBS for 30 min at room temperature. The plates were then washed thoroughly with PBS and used immediately in adhesion assays.

SUBSTRATUM-ATTACHED MATERIAL (SAM) SUBSTRATUM: Plastic substrata coated with "footpad" material or SAM were prepared essentially by the method of Rollins and Culp (32). Thus 80–90% confluent monolayers of human diploid fibroblasts (Hospital for Sick Children strain 160), or of WT CHO cells, were treated with 1 mM EGTA in PBS or 1 h at 37°. The cells were removed by washing the plates three times with PBS, leaving SAM attached to the substratum. The plates were used immediately in adhesion assays. It should be noted that SAM-coated plates probably also have serum components attached to the substratum as well.

Adhesion Assay

The assay for adhesion to protein coated glass was essentially that of Juliano and Gagalang (15) but was modified as described below to measure adhesion to plastic or gelatin substrata.

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PREPARATION OF ^{3}H-LABELED CELLS: Exponentially growing suspension cells (2–4 × 10^5 cells/cm^3) were incubated overnight with 1 μCi/cm^3 of [^{3}H]-leucine. Immediately before use the cells were harvested by centrifugation and the incubation medium discarded. The resultant cell pellet was washed three times with MEM-α-medium plus AB plus 1 mg/ml BSA (adhesion buffer) and resuspended at ~5 × 10^6 cells/ml in adhesion buffer. It should be noted that it is important to have a certain amount of an inert protein such as BSA in the system so as to prevent nonspecific attachment of cells to plastic or glass surfaces.

PRE-EQUILIBRATION OF THE PLATES WITH MEDIUM: 2-cm^3 aliquots of adhesion buffer were dispersed into dishes containing the substrata which had been prepared as described above. The dishes were equilibrated at 37°C in 5% CO_2 for 30 min.

ADHESION ASSAY IN DISHES: At time zero known aliquots of the washed cells (~1 × 10^6 cells) were pipetted onto the pre-equilibrated dishes and the dishes placed at 37°C in 5% CO_2. In addition, known aliquots of the washed cell suspension were measured for total radioactivity. After the desired length of time (0–90 min) the dishes were taken from the incubator, the nonadherent cells were removed by aspiration and the plates were washed three times with PBS. The adhered cells were treated with 1% wt/vol SDS and the lysates transferred to liquid scintillation vials to determine the amount of radioactivity. With this procedure, there is a direct proportionality between radioactivity remaining on the plate and number of attached cells (15).

VISUAL DETERMINATION OF ATTACHMENT AND SPREADING: Nonradiolabeled suspension cells were plated on the appropriate substratum exactly as described above and incubated at 37°C and 5% CO_2 for 90 min. After removal of the nonadhered cells by washing with PBS, the remaining attached cells were fixed with 0.1% gluteraldehyde in PBS for 30 min and washed with PBS, the cells were examined under a phase-contrast microscope, and the presence of attached and spread cells noted.

Capping Assay

Cap formation induced by fluorescein isothiocyanate-labeled concanavalin A (FITC-Con A) was examined by fluorescence microscopy using techniques similar to those of Aubin et al. (2). Because AD^v cells do not readily attach, the capping assays for both WT and AD^v cells were done in suspension. The percentage of cells having caps was assessed by counting at least three samples of 200 cells and scoring the cells that formed tight central caps. Samples were scored "blind" by two independent observers. In preliminary experiments both WT and AD^v F11 cells were found to reach maximal levels of capping between 60 to 90 min using 50 μg/cm^3 FITC-Con A in isotonic phosphate buffer at 37°C.

RESULTS

Growth Characteristics

Fig. 1 shows the growth rate in suspension culture for WT and for several AD^v clones. Clearly the mutagenesis and selection procedures for the AD^v phenotype did not significantly alter the doubling times as compared to WT. The AD^v cells however, did show a greatly reduced ability to adhere to serum-coated tissue culture dishes; hence the AD^v clones could not be cultured in monolayer, but were routinely grown in suspension.

Adhesion Kinetics

The rate of attachment of AD^v cells to various substrata was radically different from that of WT cells. Thus when WT cells were allowed to attach to serum-coated gelatin (Fig. 2), 80% adhered within 15 min. The AD^v cells adhered at a much slower rate; thus 50% attachment for clone E15 was only achieved at 90 min, and no demonstrable attachment of clone F11 occurred. Extending the time-course up to 24 h did not result in increased levels of adhesion for F11. Under these assay conditions neither WT nor AD^v cells attached to substrata coated with BSA, a nonadhesive protein. The difference in adhesion capability between WT and AD^v was also manifest on other adhesive substrata such as FCS-coated glass or plastic.

A summary of the capacity of WT and AD^v clones to adhere to FCS-coated plastic culture dishes is given in Table I. Generally the AD^v clones adhered less well than WT, with the exception of E11 which adhered as well as WT. The F series

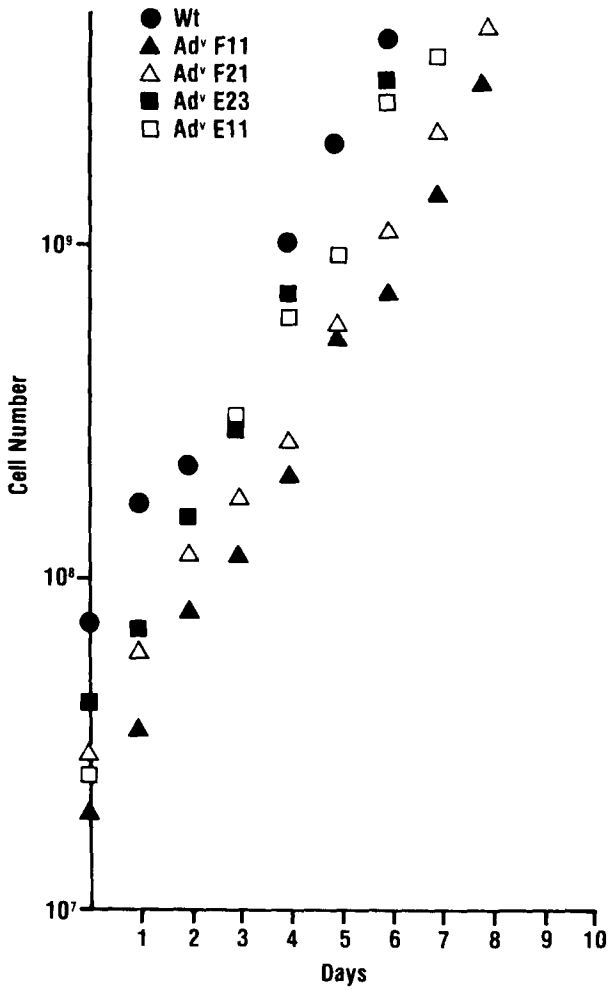

FIGURE 1 Growth of WT and AD^v cells in suspension culture. Suspension-adapted WT and AD^v cells were seeded at 1.5–4.0 × 10^5 cells/ml and maintained in exponential culture such that the cell density did not exceed 8 × 10^5 cells/ml. The cells were periodically counted by hemocytometry.

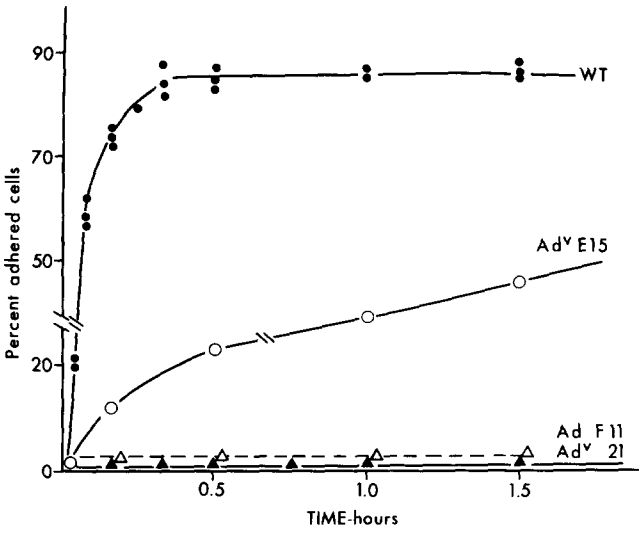

FIGURE 2 Adhesion of WT and AD^v clones to serum-coated collagen. The rate of cell attachment to collagen coated with 10% FCS was assayed as described in Materials and Methods. Each point represents the mean of three determinations.

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TABLE I

Adhesion of Various Clones to Serum-coated Culture Dishes

Clone	Adhesion	Clone	Adhesion
	%		%
WT	85	F11	1.8
E11	100	F21	2.7
E15	50	F31	2.4
E21	2.0	F32	2.9
E23	20.8	F24	14.5
E26	10.6		

Aliquots of ^{3}H-labeled cells were plated onto plastic substrata pre-equilibrated with serum and, after a 90-min incubation at 37°C, the number of attached cells was determined as described in Materials and Methods. Results represent the means of three determinations.

clones manifested only 2-20% attachment, while E series varied from 2 to 100%. Two independent WT clones were used in these studies, as both behaved in identical fashion.

Hybrids

The poorly adhesive characteristic displayed by AD^v cells seems to be fully recessive in nature. AD^v F11 was hybridized, as described in Materials and Methods, with EOTC5, a clone with wild-type adhesive characteristics. All of the hybrids isolated also displayed wild type adhesion behavior. For example, in one experiment, WT, AD^v F11 and hybrids (EOTC5 × F11)[1] and (EOTC5 × F11)[2] were tested for adhesion on serum-coated tissue culture plastic substrata; 76% of WT cells and 80% of both hybrids attached during 60 min, whereas <4% of AD^v F11 adhered. It was not possible to form hybrids between individual AD^v variants and to test these for adhesion because of the lack of accessory genetic markers in these cells. Heterokaryons of F11 × F11 were tested, and these proved to be nonadherent (<5%); however, at this time, we cannot rule out the possibility that gene dosage effects in the hybrids may also affect adhesive behavior.

Response to Serum and Divalent Ion Concentration

Other CHO variants, selected for nonadhesion to collagen have been described by Klebe et al. (19). The nonadhesive phenotype of these cells was corrected, however, if the serum concentration was elevated. In addition, increasing the divalent ion concentration to greater than normal levels also resulted in the adhesion of these variants (19). To test whether our AD^v clones resembled those of Klebe et al., we assayed for adhesion to substrata treated with concentrations of FCS of up to 100%; we also tested the effect of increasing the divalent cation concentration to supra normal levels.

Fig. 3 illustrates the serum dependence of CHO cell adhesion to collagen. At very low concentrations there was no significant attachment (<2%). Between 1 and 10% there was a dramatic increase (2–85%) in the adhesion of WT cells, while increasing the serum concentration above 10% did not result in any further increase in adhesion. By contrast AD^v F11 and F21 failed to adhere even at 100% serum concentration, whereas clone E15 did adhere somewhat with increasing serum concentration.

The adhesion of WT cells to serum-coated substrata was markedly divalent cation dependent; as seen in Fig. 4 and Table II, maximum adhesion after 90 min was observed at between 10^{-3} and 10^{-2} M calcium or magnesium. In contrast to the case for WT cells, clones F11 and F21 did not adhere significantly at any concentration of divalent ion, however clone E15 did adhere somewhat at higher divalent cation concentration.

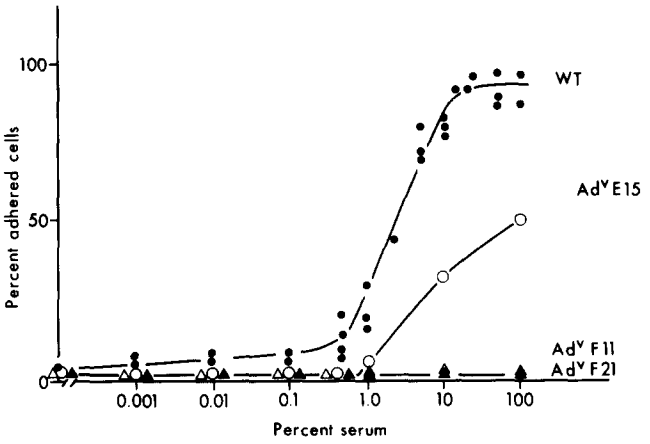

FIGURE 3 Serum dependence of adhesion. Adhesion to collagen substrata coated with from 0 to 100% FCS was measured after 90 min as described in Materials and Methods. For serum concentrations of 0–20% the total protein concentration was maintained at 10 mg/ml by the addition of BSA. Each point represents the mean of three determinations.

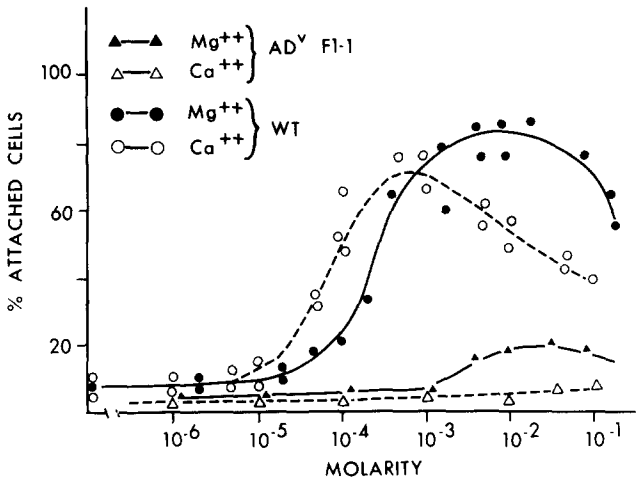

FIGURE 4 Divalent ion dependence of adhesion. The adhesion to serum-coated glass scintillation vials was determined after 90 min by the method of Juliano and Gagalang (15). The adhesion buffer in this assay consisted of PBS plus 0.1% dextrose and from 0 to 100 mM calcium or magnesium chloride. Each point represents the mean of three determinations.

TABLE II

Divalent Cation Dependence of Adhesion to Serum-coated Collagen

	Calcium			Magnesium		
Clone	0	10 mM	100 mM	0	10 mM	100 mM
WT	4.5	79	56	6	89	45
F11	3.4	1.4	1.4	2.5	1.1	5.8
E15	3.8	22.5	37	3.8	49	22.8
F21	4.0	1.5	4.7	3.3	4.7	16

The adhesion during 90 min to serum-coated collagen in the presence of 0, 10, or 100 mM calcium or magnesium was assayed as described in Materials and Methods. The adhesion buffer used in this assay consisted of PBS, AB, BSA, and 1 mM EGTA; the latter was added to chelate the divalent ions associated with the BSA. Results present the means of three determinations.

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These data indicate that some of our clones such as E15 may resemble the clones described by Klebe, as both increased serum concentration and increased divalent ion concentration resulted in increased adhesion of these cells. The AD^v F11 and F21 clones however, did not adhere in response to changes in serum or divalent ion concentration and seem to be quite different from the CHO adhesion variants previously described by others (19).

Response to Fibronectin

As seen in Fig. 5 purified bovine plasma fibronectin effectively promoted attachment of WT cells and thus can fully substitute for serum in this regard. However, clone F11 did not adhere to a substratum coated with as much as 5 mg/ml fibronectin, a concentration 50-fold in excess of that required for complete attachment of WT cells; several other F series clones behaved in a manner similar to F11.

Response to Con A and PL

The results described above suggest that the AD^v phenotype might be caused by a cell surface defect leading to impaired utilization of fibronectin as an adhesive ligand but did not rule out the alternate possibility of a defect in the cytoskeleton leading to a loss of adhesive ability. We attempted to distinguish between these two possibilities, namely, (*a*) a cell surface defect for recognition of serum components such as fibronectin, and (*b*) a cytoskeletal defect, as follows. We reasoned that if the AD^v cells possessed a normal cytoskeletal apparatus, and if they could be attached to the substratum by ligands other than serum components, then the cells should spread and attain normal morphology. If, on the other hand, the AD^v cells attached but did not attain a normal morphology, we might suspect a defect in the cytoskeleton. The ligands chosen for these studies were the multivalent lectin Con A, which can bridge between the cell surface and a serum-coated substratum, and PL which can promote adhesion by a charge interaction (21).

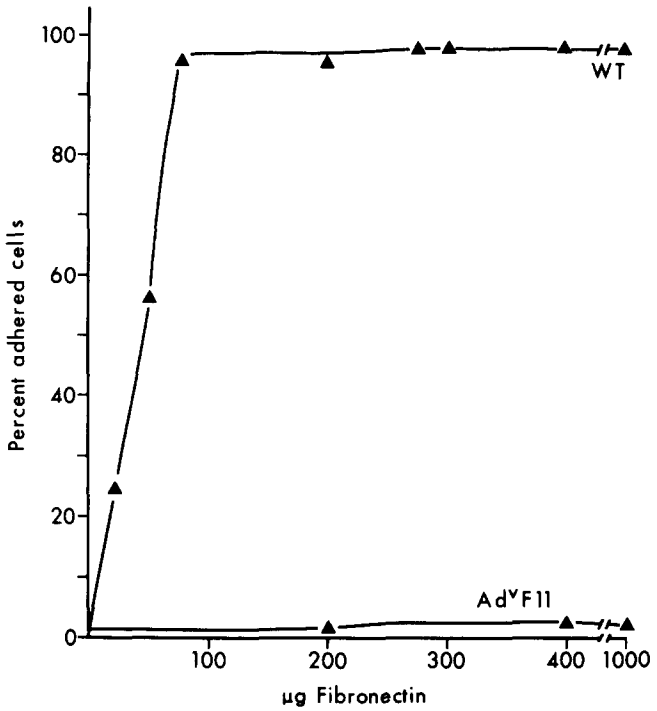

FIGURE 5 Adhesion to fibronectin-coated collagen. The adhesion of WT and AD^v F11 cells to collagen coated with various amounts of bovine serum fibronectin (CIG) was determined as indicated in Materials and Methods. The protein content of the solution used to treat the collagen plates was maintained at 1,000 μg/ml by admixture of the appropriate amount of BSA. The duration of the experiment was 90 min and the temperature was 37°C. Each point represents the means of three determinations.

As expected, WT cells were capable of attachment and subsequent spreading on substrata coated with FCS, fibronectin, Con A, or PL (Table III). The AD^v F11 clone attached very poorly on substrata coated either with FCS or fibronectin; however, AD^v F11 attached readily to both Con A and PL substrata and subsequently attained a well-spread morphology.[1] In all instances the spreading was blocked by low concentrations of cytochalasin B, suggesting cytoskeletal involvement in the process. These results indicate that the AD^v clones possess a relatively normal cytoskeletal system, because when cells were provided with a suitable (although nonphysiological) adhesive substratum, a normal morphology was attained. A comparison under the scanning electron microscope (Fig. 6) also confirmed that there were no gross morphological differences between AD^v F11 and WT cells when they were attached and spread on Con A, a substratum to which both cell types readily adhere, or between AD^v F11 cells on Con A and WT cells on FCS.

Thus AD^v cells failed to attach or spread on FCS or fibronectin (CIG) coated substrata, but could attach, spread, and attain a seemingly normal CHO cell morphology on alternative substrata such as Con A or PL. These results are consistent with the notion that the AD^v phenotype is caused by a cell membrane defect resulting in failure to utilize exogenous fibronectin, the usual mediator of CHO cell adhesion, rather than to an altered cytoskeleton.

Adhesion to SAM

While the artificial ligands, Con A and PL promoted attachment of the AD^v clones, we wondered if the nonadhesive phenotype could be corrected by more physiologically relevant substrata. We chose to test for adhesion to SAM (footpad material), which is known to be enriched in fibronectin, glycosaminoglycans, and other cell surface components, and which has been reported to promote the attachment of virally transformed cells (5, 6). We prepared plates coated with SAM from confluent human diploid fibroblasts or WT CHO cells, and used these in our adhesion assay. As expected, after 90 min WT cells adhered readily to SAM-coated plates (Table IV), but so did the majority of the adhesion variants, although some variability between clones was observed; further experiments indicated that maximal adhesion to SAM was attained by 30 min for both WT and AD^v F11 cells. Neither the AD^v nor WT cells spread well on the SAM substrata, however this might result from a patchy distribution of SAM on the plate yielding insufficient attachment points for fully developed cell spreading. This data would seem to indicate that SAM may contain a factor or factors which can correct the phenotype of the AD^v cells and promote attachment. This evidence further supports the possibility of a cell surface defect underlying the AD^v phenotype, as provision of exogenous macromolecular material can "correct" the adhesion deficit.

[1] The $t_{1/2}$ for attachment of AD^v F11 to Con A and PL was 5 and 2 min, respectively, indistinguishable from WT cells.

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TABLE III

Adhesion to Substrata Coated with Various Adhesive Ligands

	BSA		FCS		FN		PL		Con A	
Clone	att	spr	att	spr	att	spr	att	spr	att	spr
WT	−	−	+	+	+	+	+	+	+	+
WT + 1 μg/ml cyto B	−	−	+	−	+	−	+	−	+	−
F11	−	−	−	−	−	−	+	+	+	+
F11 + 1 μg/ml cyto B	−	−	−	−	ND	ND	+	−	+	−

Both WT and F11 were plated on substrata coated with 1 mg/ml BSA, 10% FCS, 100 μg/ml fibronectin, 1 mg/ml PL, or 0.5 mg/ml Con A. In some cases 1 $\mu g/cm^3$ cytochalasin B was added. Cell attachment was assessed at 90 min both visually and by determining the percent radioactivity remaining on the plate. In all instances where adhesion was observed, at least 70% of the cells were attached. Spreading was determined by observing whether the cells became fibroblastic in appearance and a positive score for both WT and F11 was given when at least 40% of the attached cells were spread after 90 min.
FN, fibronectin; att, attached; spr, spread.

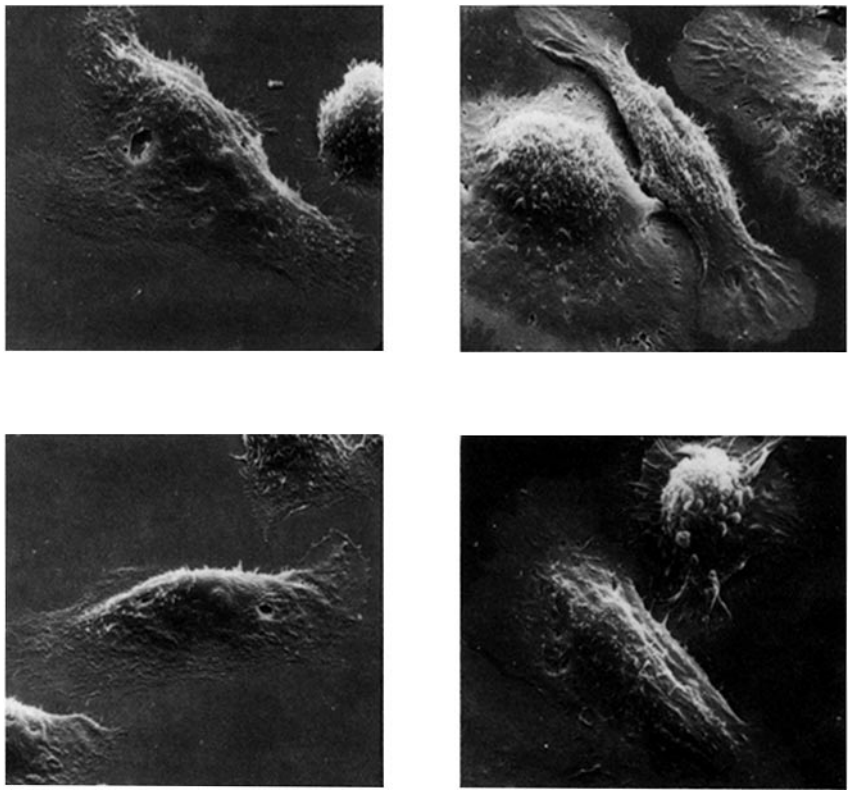

FIGURE 6 Scanning electron micrographs of WT and AD^v cells on Con A substrata. WT and AD^v F11 cells were allowed to attach to Con A-coated substratum, as described in Materials and Methods, the samples were then fixed, prepared for scanning electron microscopy, and examined by this procedure. The two right-hand panels are views of WT cells and the left-hand panels are views of AD^v F11.

Test of Cytoskeletal Activity: FITC-Con A Capping

Unfortunately, there is no single assay which might allow one to test the functional status of all components of the cytoskeleton. However, lectin-induced capping is a complex cytoskeletal activity that requires coordination of both microfilaments and microtubules (25). Thus the ability of a cell to cap would seem a good indication of the functionality of some of its most important cytoskeletal components. When the FITC-Con A capping behavior of WT and AD^v F11 cells was examined, it was found that both types of cells could form the tight centrally located caps (Fig. 7) typical of CHO cells (2).

TABLE IV

Adhesion to SAM-coated Substrata

Clone	Serum	SAM
WT	72	83
F11	1	44
F21	1.8	42
F26	9.5	39.6
E14	8.2	37.5

Several AD^v clones and WT were plated on substrata coated either with serum or with SAM prepared from human diploid fibroblasts. After 90 min the percentage of adhered cells was assayed as described in Materials and Methods. Results represent the means of three determinations. Adhesion of AD^v cells to SAM from WT cells was more variable (data not shown).

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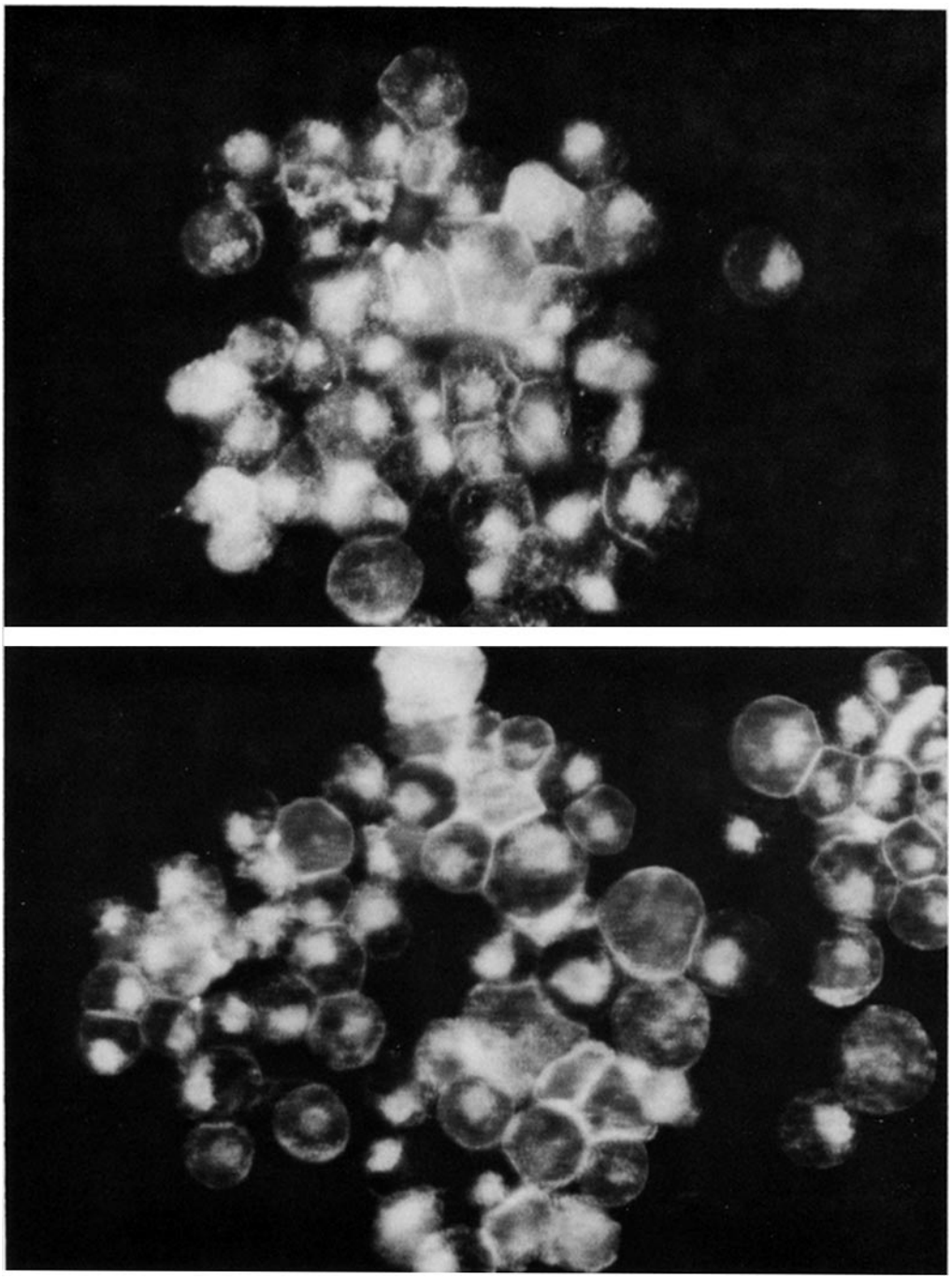

FIGURE 7 FITC-Con A-induced capping of WT and AD^v cells. WT and AD^v F11 cells (1×10^7) were treated with 50 μg of FITC-Con A in PBS (1 cm^3) for 15 min at 4°C, afterwhich the lectin-treated samples were transferred to 37°C for 90 min. Thereafter the cells were fixed in paraformaldehyde and observed and photographed in a Leitz fluorescence photomicroscope. Top: WT cells. Bottom: Ad^v F11 cells. × 630.

The extent of capping was quite similar in WT and AD^v cells with a level of 50-60% capping attained during 90 min at 37°C. The percentage capping of both cell types is well within the range of variability (20-80%) of capping observed in a series of CHO clones (2). Capping in both WT and AD^v F11 was inhibited by colchicine, cytochalsain B, or lidocaine, and thus presumably is a reflection of cytoskeletal activity (25).

DISCUSSION

The data presented above describe both a selection procedure and an initial characterization of CHO variants (AD^v) which differ from WT cells in their ability to adhere to serum or fibronectin-coated collagen substrata. While the degree of non-adhesiveness differed among clones, the phenotype was stable for any particular variant clone. The AD^v characteristic seems to be recessive, because hybrids formed between AD^v F11 cells and cells with wild-type adhesion behavior also displayed wild-type adhesion behavior. The variant cells grew as well in suspension culture as did WT cells, but obviously could not be grown in conventional monolayer culture because of their inability to attach. Unlike other CHO adhesion mutants that have been described (19), increasing concentrations of FCS or divalent cations did not increase the adhesion of most of the

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AD^v clones. Even fibronectin, a ligand that can promote attachment of WT cells at rather low concentrations, was without effect on AD^v cells. However, AD^v cells were capable of attaching, spreading, and attaining a normal CHO morphology on substrata coated with ligands such as Con A and PL. In addition AD^v cells, as well as WT cells, could attach and partially spread on substrata coated with SAM derived from diploid fibroblasts. Thus, in contrast to the WT cells, AD^v cells cannot attach to fibronectin-coated substrata, however both types of cells can attach to substrata coated with other ligands or with the complex macromolecular mixture represented by SAM.

The results presented are suggestive of a surface defect in the AD^v cells but do not unequivocably rule out the possibility of an abnormality in the cytoskeletal apparatus. During cell adhesion, the initial attachment process is followed by a distinctive change in morphology resulting in a flattened and spread appearance. The cytoskeleton seems to play an integral role in this change in morphology as well as in the process of adhesion, because the addition of cytochalasin B, an agent that interacts with microfilaments, slows cell adhesion and blocks cell spreading (15, 24). The fact that the AD^v cells are capable of achieving a normal CHO cell morphology when attached to the substratum by nonphysiological ligands such as Con A strongly argues against the involvement of the cytoskeleton in the nonadhesive phenotype. It is possible, however, that the morphological changes observed when plating cells on Con A or polylysine substrata are controlled by mechanisms other than those responsible for the adhesion to serum-coated substrata, but this is unlikely, as the spreading of AD^v cells on Con A or polylysine-coated substrata is blocked by cytochalasin B, as is spreading of WT cells on serum-coated substrata. In addition, FITC-Con A induced capping is similar in WT and AD^v F11 cells, suggesting that the adhesion variant cells have relatively normal microfilament and microtubule systems, capable of mediating complex cytoskeletal activities.

Although fibronectin is clearly implicated in the cell adhesion process, it probably is not the only factor involved. There is a growing body of evidence suggesting that cell surface components other than fibronectin are involved either directly or indirectly in cell adhesion. Thus Juliano and Gagalang (15) showed that the adhesion of WT CHO cells to a serum layer was inhibited by trypsinization of the cells, but only at enzyme concentrations far in excess of those needed to remove cell surface fibronectin, thus implying the involvement of relatively trypsin-insensitive surface components. In another study, ricin-resistant baby hamster kidney (BHK) cell variants have been described (28) which appear to synthesize normal fibronectin but fail to retain it on the cell surface, indicating some alteration of fibronectin binding leading to a defect in cell adhesion. The work of Wylie et al. (43) using an antibody which is directed against BHK cell surfaces and which blocks adhesion, suggests that two glycoproteins of 100,000-140,000 mol wt are involved in the adhesion process in these cells. The above-mentioned observations, coupled with those presented in this communication, suggest the existence of a binding site for fibronectin at the cell surface. They also suggest that AD^v cells manifest a defect in this site which prevents the utilization of exogenous fibronectin in the adhesion process. CHO cells, in contrast to some other cell types (9, 11), cannot attach to denatured collagen substrata in the absence of exogenous fibronectin; the small amount of fibronectin-like protein on the CHO cell surface (14, 15) seems to be inadequate in this regard.

A substance enriched for many of the components involved in adhesion is SAM (6), which is prepared by detaching cells from the substratum with chelating agents. In the process, the adhesive bonds between the cell and the substratum are not broken, but rather the cell rounds up, remaining attached via retraction fibrils which are then sheared, leaving cellular material attached to the substratum. This material consists mainly of fibronectin, collagen, a complex array of glycoaminoglycans and cytoskeletal components. It was interesting to note that fibronectin alone, even at very high concentrations, was ineffective in promoting adhesion of the AD^v F11 cells, whereas SAM was able to do so. Thus some component(s) of SAM, such as the glycosaminoglycans, may be supplying a factor to the AD^v cells which either promotes the ability of fibronectin to act as an adhesive ligand, or which acts in an independent manner to promote adhesion. In any case the fact that provision of exogenous macromolecules "corrects" the nonadhesive phenotype of AD^v cells, supports the concept that the defect in these variants occurs at the cell surface.

A number of other workers have previously described mammalian cell variants with altered adhesive ability. Thus cells selected for lectin resistance (28), and for sensitivity or resistance to trypsinization (1, 30) have also displayed alterations in their ability to adhere to each other or to the substratum. In all of these cases altered adhesion ability was reflected in alteration of the cell surface biochemistry including reduced fibronectin levels (28), altered surface glycoproteins (31), and altered glycosaminoglycan patterns (1). A problem with these studies is that cell adhesion is a complex phenomenon likely to be affected directly or indirectly by a variety of cellular subsystems. Thus it would seem desirable to utilize variants which have been directly selected for alterations in adhesive ability. In addition to the present communication, there has been one other report of variants selected directly for altered substratum attachment (19). These variants could, however, be "corrected" by raising the concentration of serum or of divalent cations; thus the adhesion defect in these cells was not complete. By contrast, several of the variant clones described here display a radical impairment in their ability to adhere to a "normal" substratum (i.e., serum- or fibronectin-coated collagen). Hopefully an examination of the membrane biochemistry of these cells should lead to new insights into the identity and organization of the cellular macromolecules involved in adhesion.

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