Adhesion of Cells to Polystyrene Surfaces

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ABSTRACT   The surface treatment of polystyrene, which is required to make polystyrene suitable for cell adhesion and spreading, was investigated. Examination of surfaces treated with sulfuric acid or various oxidizing agents using (a) x-ray photoelectron and attenuated total reflection spectroscopy and (b) measurement of surface carboxyl-, hydroxyl-, and sulfur-containing groups by various radiochemical methods showed that sulfuric acid produces an insignificant number of sulfonic acid groups on polystyrene. This technique together with various oxidation techniques that render surfaces suitable for cell culture generated high surface densities of hydroxyl groups. The importance of surface hydroxyl groups for the adhesion of baby hamster kidney cells or leukocytes was demonstrated by the inhibition of adhesion when these groups were blocked: blocking of carboxyl groups did not inhibit adhesion and may raise the adhesion of a surface. These results applied to cell adhesion in the presence and absence of serum. The relative unimportance of fibronectin for the adhesion and spreading of baby hamster kidney cells to hydroxyl-rich surfaces was concluded when cells spread on such surfaces after protein synthesis was inhibited with cycloheximide, fibronectin was removed by trypsinization, and trypsin activity was stopped with leupeptin.

Polystyrene dishes have been used for cell culture since about 1965. Many cell types adhere to and move on the surfaces of such materials and present a morphology that is very similar to that seen when the cells are grown on glass. However, it has long been known that the polystyrene must be subjected to a surface treatment to render the dishes suitable for cell attachment. Polystyrene surfaces, as pressed by the manufacturer, are unsuitable for cell attachment. This has been attributed to the surface chemistry of the polystyrene, and many different suggestions have been made as to the precise chemistry involved in the nonadhesive nature of polystyrene (1–3). Several processes appear to be in use commercially for making the dishes suitable for the attachment of eucaryote cells, (4, 5) though regrettably little information has been published by manufacturers about their own techniques. Martin and Rubin (6) reported that treatment of polystyrene with concentrated sulfuric acid followed by exposure to ultraviolet light (used by them for sterilization) converted the surfaces of the dishes into a state suitable for the adhesion of fibroblasts. It has been suggested that this treatment leads to the sulfonation of the polystyrene with a consequent increase in the number of charged groups per unit area (2). However it seems likely that the treatment used by commercial manufacturers is a corona discharge, which might induce mild oxidation of the surface of the plastic or destruction of any mold release agent used in manufacture. Klemperer and Knox (3) found that treatment

with chromic acid, which might produce hydroxyl, aldehyde, or carboxyl groups on the surface, led to increased cell adhesion. They suggested that cell adhesion required the presence of charged groups, which might be either carboxyl or sulfonate.

However there is much subjective or semiquantitative evidence (reviewed in 7) that cell attachment to surfaces decreases rather than increases as surface charge density is raised. Gingell and Todd (8) demonstrated in some elegant quantitative experiments that cell adhesion decreases as the charge density on a surface increases. This evidence appears to be in direct contradiction to the idea that charged groups on the cell surface are responsible for cell adhesion. Moreover the two studies on modification of polystyrene (2, 3) both used the crystal violet method for measuring the density of charged groups. This method is suspect because it requires the binding of several hundred dye molecules per $Å^2$ of the surface to detect one charged group. Furthermore measurements of the surface charges produced by sulfonation by Gingell and Vince (9), using electroendosmosis, gave a low value of only 1.3^3 ESU cm^{-2}. Thus there are two reasons for doubting whether the presence of charged groups on polystyrene is required for cell adhesion. It is perhaps appropriate to note at this point that in the foregoing section we have assumed that the charged groups are all anions: surfaces bearing cations are very adhesive for cells (7).

The first aim of this paper is to report on work directed to identifying the chemical groups generated on polystyrene by various types of oxidation or by sulfuric acid treatment. The second aim is to assess how the various types of group contribute to the adhesion of cells to these surfaces. It is usual to grow cells in the presence of serum. The consequence of this is that cells probably adhere to proteins bound to the substrate rather than to the surface itself (10–13). Different treatments of polystyrene may result in different degrees of adsorption of various proteins. We hope to give particular attention to the binding of serum proteins in a later paper.

MATERIALS AND METHODS

Cells: BHK21C13 cells were obtained from stocks routinely grown in this laboratory by trypsinization of confluent or near-confluent cultures, using the method of Edwards and Campbell (14). Polymorphonuclear leukocytes were obtained from fresh human blood by the technique described in reference 15. The baby hamster kidney (BHK[1]) cells were suspended in either Dulbecco's phosphate buffered saline or Ham's F10 medium plus 3% fetal calf serum plus the insulin-transferrin-selenite supplement (Collaborative Research, Inc., Waltham, MD) with insulin and transferrin each at 5 μg/ml, at a density of 2×10^5/ml. Leukocytes were suspended at a density of 1.5×10^6/ml in HEPES-buffered Hanks' balanced salts solution with or without 10% fetal calf serum. The fetal calf serum used in this investigation was purchased from Gibco-Biocult, Paisley, United Kingdom.

Culture Dishes: Polystyrene culture dishes, bacteriological and tissue culture grade, were purchased from the following makers: Sterilin, Teddington, Middx, United Kingdom, Nunc A/S, Roskilde, Denmark; and Corning, Ltd, Stone Staffs, England.

The bacteriological dishes were given the following treatments: (a) 98% sulfuric acid at 37°C for 10 min; (b) ozonolysis with 2% ozone for 25 min at 25°C; (c) exposure to ultraviolet light for 30 min ($\sim$5 mW/cm^2) at a peak of 254 nm; (d) chloric acid treatment by adding 3 ml of 70% perchloric acid and 2 ml saturated aqueous potassium chlorate to the dishes and allowing these to react for 10 min; and (e) hydrolysis with 5 M potassium hydroxide or with 10 M hydrochloric acid for 30 min. If no treatment was given the dishes were washed three times with ultradry petroleum ether to remove traces of mold release agents.

Measurement of Cell Adhesion: 0.6×10^6 BHK cells (in a final volume of 3 ml) or 1×10^6 leukocytes (in a final volume of 4 ml) were placed in each culture dish which was then incubated at 37°C for 15 min (BHK cells) or 30 min (leukocytes). Cell spreading during these incubation periods is inappreciable on control surfaces. The cells that had not adhered in these culture periods were removed from the dishes by washing with HEPES-buffered Hanks' balanced salts solution three times. The number of adherent cells in each of 10 standard counting areas was then counted using phase contrast microscopy to detect the cells and a Quantimet 720 image analyzing computer (Cambridge Instruments, Cambridge, United Kingdom) to count the cells. The counting areas were 0.0026 cm^2. The results are expressed in terms of the number of cells adhering per cm^2.

High Purity Polystyrene Surfaces: Method 1. Highly purified polystyrene surfaces prepared by Mr. Ian Todd and Dr. D. Gingell (Middlesex Hospital Medical School, London, United Kingdom) were provided for us by the kindness of Dr. Gingell. These surfaces had been prepared by the same method as used by Gingell and Vince (9). Method 2: Commercial grade bacteriological polystyrene dishes were dissolved as a 2% wt/vol solution in benzene and the polystyrene was then precipitated by addition of anhydrous methanol. The precipitate was then redissolved in benzene and the precipitation repeated. A benzene solution of this purified polystyrene was used to pour thin layers on a glass substrate. These were air-dried and then annealed under high vacuum at 125°C, above the "glass" transition temperature.

X-ray Photoelectron Spectroscopy: This was carried out for us by the Department of Chemistry, University of Durham, United Kingdom. Treated and untreated polystyrene specimens were reacted with trifluroacetic anhydride to obtain specific detection of hydroxyl groups.

Attenuated Total Reflection (ATR) Spectroscopy and Contact Angle Measurements: Attenuated internal reflection spectra of samples cut from the bases of the dishes were recorded on a Perkin-Elmer

[1] *Abbreviations used in this paper:* ATR, attenuated total reflection; BHK, baby hamster kidney; XPS, x-ray photoelectron spectroscopy.

Model 580 infrared spectrophotometer using a TR-25 ATR unit. The analyzing crystal was of KRS-5 and the spectra were recorded at an angle of 45°. Contact angle measurements were made with a Wilhelmy surface balance (Bailey Engineering Co., Windsor, United Kingdom) using 3×1.5-cm plates of polystyrene.

Chemical Blocking of Groups on the Polystyrene: Hydroxyl groups were blocked by the following reactions: (a) silylation with N-trimethyl-silylimidazole, purchased from Pierce Chemical Co., (Rockford, IL). The reagent was dissolved at 0.5 % vol/vol in ultradry petroleum ether for 5 min; or (b) acetylation with 0.1% acetic anhydride dissolved in ultradry petroleum ether for 3 min. Possible carboxyl groupings were blocked by esterification with either N-butanol or ethanol under mildly acidic conditions. The efficacy of these treatments was checked by using radioactive reagents (see below). Silylation and acetylation were carried out in an atmosphere that had been dried over phosphorous pentoxide. At the completion of reaction ($\sim$3 min) the dishes were allowed to dry and the unreacted chemicals removed by washing with distilled water.

Measurement of Hydroxyl and Carboxyl Groups on Dishes: These were measured by esterifying with [^{14}C]acetic anhydride (specific activities quoted in subscripts to appropriate tables) in ultradry petroleum ether for 3 min, counting the activity bound by the dishes for hydroxyl groups, and esterifying with [^{14}C]ethanol (sp act 5.74 μCi/μmol) in mildly acidic conditions to detect carboxyl groups. Both radiochemicals were obtained from The Radiochemical Center, (Amersham, United Kingdom). The radioactivity bound by the dishes was measured on liquid scintillation counters (Models L200, Beckman Instruments, Fife, Scotland and Tricarb 300, United Technologies Caversham, England).

RESULTS

### Preliminary Experiments: Oxidation of Polystyrene and Cell Attachment

Bacteriological grade polystyrene dishes bound very few leukocytes or BHK cells from a serum-containing medium but if the dishes were exposed to concentrated sulfuric acid cell, attachment became appreciable (Table I). This result merely confirms the finding of other workers (2, 3, 6). If the sulfuric acid treatment was replaced with exposure to ultraviolet light or by chloric acid treatment, the polystyrene was similarly made adhesive for BHK cells or leukocytes in a serum-containing medium (Table I). Ozonolysis increased BHK and leukocyte adhesion over that seen on untreated surfaces but to a lesser extent than other oxidation methods. Cell attachment onto dishes that have been exposed to these oxidative conditions resembled that seen on glass or on "tissue culture" grade dishes (Figs. 1–4). Prolonged culture of cells on polystyrene surfaces that have been oxidized by these methods is as easy as prolonged culture of cells on commercial "tissue culture" grade surfaces. Figs. 5 and 6 show the lack of adhesion to bacteriological grade dishes.

TABLE I

The Effects of Sulfuric Acid Treatment and Other Agents on Cell Adhesion

Dish type	BHK cells	Leukocytes
Tissue culture grade	12,996 (23)	30,996 (22)
Bacteriological grade	234 (91)	617 (138)
H₂SO₄ treated	20,997 (10)	46,682 (16)
Ozonized	4,254 (7)	8,346 (48)
UV light	23,305 (13)	6,288 (49)
Chloric acid	32,485 (9)	35,335 (29)
KOH	760 (85)8	650 (91)
HCl	726 (54)	1,711 (79)

Values in parentheses are the standard deviations expressed as percentages of the mean. Each measurement is the mean of ten individual measurements made on each of at least four dishes. The maximum number of leukocytes available for adhesion was 52,910 cm^{-2} and the maximum number of BHK cells was 31,750 cm^{-2}. Cells adhering, in the presence of serum, is expressed as cells bound per cm^{-2}.
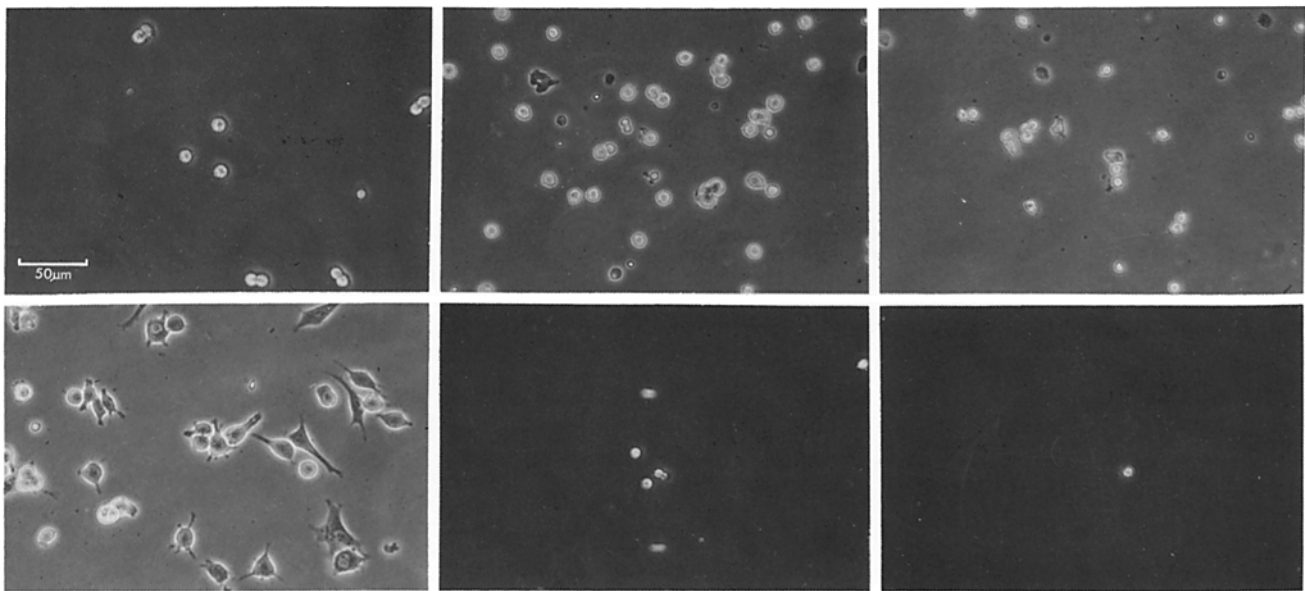
FIGURES 1-6   The appearance of cells adhering to polystyrene surfaces prepared by various surface treatments. Cells were grown in the presence of serum (3% plus insulin-transferrin-selenite supplement for BHK cells, 10% for leukocytes). Phase-contrast microscopy. Fig. 1 (*upper left*): Tissue culture grade; leukocytes. Fig. 2 (*upper middle*): Tissue culture grade; BHK cells. Fig. 3 (*upper right*): Chloric acid-treated; leukocytes. Fig. 4 (*lower left*): Chloric acid-treated; BHK cells. Fig. 5 (*lower middle*): Bacteriological grade; leukocytes. Fig. 6 (*lower right*): Bacteriological grade; BHK cells. BHK cells were allowed to settle for 15 min and leukocytes were allowed to settle for 30 min. Bar, 50 μm (applies to Figs. 1-6).

Identification of Groupings Produced on Polystyrene Surfaces by Various Oxidative Treatments

XPS and attenuated total reflection spectroscopy were used to detect the chemical changes induced by the various treatments of the dishes. The XPS technique revealed that there was an appreciable oxidation of the polystyrene by the chloric acid treatment. The carbon/oxygen ratio on the surface changed from 90:1 to 4:1 on treatment. About 30% of the surface oxygen was in the hydroxyl form; the remainder was ester-, ether-free carboxylate and perhaps some ketonic oxygen. ATR spectroscopy, which examines a surface and sub-surface layer, revealed that the chloric acid-treated material had relatively intense hydroxyl bands at 3,400 cm^{-1} whereas the sulfuric acid-treated material showed slight absorption at this wave number. Ozonolysis however markedly increased the intensity of both the hydroxyl and carbonyl band at 1,750 cm^{-1}. This result agrees with other findings (18) that have shown that ozonolysis produces extensive and nonspecific oxidation of polystyrene.

A very slight binding of ^{35}S to the dishes occurred when treated with [^{35}S]sulfuric acid under the same conditions as were used for sulfonation (see Table II). However there is reason to suspect that even this small binding could be attributed to adsorption of the acid because prolonged washing (16 h) was required to reduce the binding to this level. The resulting surface density of sulfonic groups was calculated as $\sim$5.7 groups/1,000 nm^2, which is very low.

Attempts to generate hydroxyl groups by strong acid or alkali hydrolysis were without effect either in the generation of hydroxyl groups (ATR data) or in rendering the surfaces adhesive for cells.

Chloric acid treatment was effective in generating hydroxyl groups on polystyrene surfaces as shown by the results of XPS

TABLE II
Sulfonation of Bacteriological Grade Dishes by 98% [^{35}S]Sulfuric Acid

Dish no.	Counts bound per cm^{2*}
1	224.9
2	150.0
3	303.9

[^{35}S]sulfuric acid, specific activity 106 μCi/μmol.
* After 20-min treatment and prolonged washing.

and ATR spectroscopy and by the increase in acetic anhydride binding (Table III). Chloric acid did not generate as large a density of carboxyl groups as the commercial treatment given for the preparation of "tissue culture" grade dishes (see Table III and V). The binding of [^{14}C]acetic anhydride to the chloric acid-treated dishes 17 disintegrations per minute (dpm)/cm^2 revealed 4,230 binding sites (hydroxyl?) per 1,000 nm^2, whilst the binding of the much higher activity [^{14}C]ethanol to ozonized dishes revealed only $\sim$15 binding sites (carboxyl?) per 1,000 nm^2. The bacteriological grade dishes showed hydroxyl densities of <10%, and the "tissue culture" grade dishes showed densities of 60% of that found on the chloric acid-treated dishes.

Chloric acid treatment was found to be the easiest and most reproducible method of rendering "bacteriological grade" material adhesive for cells in a serum-containing medium. This treatment also rendered the surfaces very wettable. Contact angle measurements by the Wilhelmy plate method showed that whereas the "bacteriological" grade polystyrene (stripped of the mold release agent) had a contact angle of 75° (receding) the chloric acid treated material had a contact angle of <8°. "Tissue culture" grade surfaces had a contact angle of 14°.

Table III

Hydroxyl and Carboxyl Groups on Polystyrene Dishes: Detection by Esterification

	Mean Count	SD	n
Tissue culture grade*	7.7	1.5	9
Bacteriological grade	1.5	0.6	8
Ozonized	11.0	3.2	7
Chloric acid-treated	17.1	1.2	7
H_2SO_4-treated	14.9	2.9	4
Bacteriological grade, H_2SO_4-treated[‡]	4.5	2.1	5
Tissue culture grade	3.5	0.8	4
Tissue culture grade[§]	4.9	2.9	3
Bacteriological grade	1.5	0.6	3
Ozonized	11.9	2.6	3
Chloric acid-treated	3.1	1.9	3

[^{14}C]acetic anhydride, specific activity 1.1×10^{-2} $\mu Ci/\mu mol$. [^{14}C]ethanol, specific activity 5.7 $\mu Ci/\mu mol$. Backgrounds were subtracted.
* Detection of hydroxyl groups by reaction with [^{14}C]acetic anhydride. Counts bound per cm^2.
[‡] Pre-esterified with cold acetic anhydride.
[§] Carboxyl groups detected by esterification with [^{14}C]ethanol. Counts bound per cm^2.

Are Hydroxyl Groups Involved in Adhesion of Cells to Polystyrene?

The evidence presented above is consistent with hydroxyl groups being required for adhesion of cells, but it affords no direct proof of this. Consequently we attempted to block the hydroxyl groups that were generated by chloric acid or other treatments, or present on the manufactured surface with acetylation or silylation. Carboxyl groups were present at relatively high density on "tissue culture" dishes (see Tables III and V, and the XPS data) and at rather lower density on chloric acid-treated dishes. We attempted to block carboxyl groups by esterification with alcohols (see Tables III and V for results). Esterification with alcohols (Table IV) had no effect on decreasing cell adhesion whereas acetylation or silylation were very effective in decreasing cell adhesion. The trimethylsilylimidazole reagent is believed to react only with hydroxyl groupings (16) and if this is correct it affords direct proof that much of the adhesiveness of these surfaces is due to the presence of hydroxyl groupings. Tissue culture grade and chloric acid-treated surfaces became unwettable after treatment with the acylating or silylating reagents, whereas esterification had no detectable effect on the contact angle with water. There was no evidence that blocking carboxyl groupings leads to any change in adhesion. It might of course be argued that the cells are capable of hydrolyzing such esters, but data in Table V shows that there was no apparent loss of labeled ethyl groups from such surfaces on incubation with cells. That adhesion of cells to sulfuric acid-treated dishes that were acetylated or silylated (Table IV) was diminished, argues strongly that adhesion of cells to these dishes requires the presence of hydroxyl groups. Sulfonic acid groupings would not be blocked by such reagents. It is of course possible that the reagents were used under conditions that did not effect complete reaction with the carboxyl or hydroxyl groups but the data in Table III show that reaction was taking place; the data in Tables IV and V show that hydroxyl blocking did affect adhesion whereas carboxyl blocking did not diminish adhesion.

Adhesion of Cells to Highly Purified Polystyrenes

Although the results described above are consistent with the oxidation or hydroxylation of polystyrene by chloric acid, perhaps at a tertiary carbon atom, the use of commercial grades of polystyrene may possibly reveal that the presence of additives or impurities in the plastic accounts for these results. To test whether some or all of the oxidative effects are upon polystyrene itself we repeated some of the experiments using ultrapure polystyrenes. The results are shown in Table VI. It is clear that chloric acid can render very pure polystyrenes adhesive.

Adhesion and Spreading of Cells on Polystyrene Surfaces in the Absence of Protein in the Medium

Since there may be specific features of the adsorption of serum proteins to treated and untreated polystyrene surfaces, we also studied the adhesion of leukocytes and BHK cells to these surfaces in serum-free Hams F10 medium. The results

Table IV

Effects on Cell Adhesion of Blocking Surface Groupings

Dish treatment	BHK cells		Leukocytes	
TC control*	13,926	(25)	19,193	(28)
B control	1,080	(24)	886	(93)
TC acetic anhydride	71	(250)	696	(98)
TC, TIMS	131	(300)	564	(87)
B, acetic anhydride	5,358	(96)	3,045	(47)
B, TIMS	683	(91)	223	(183)
B, chloric, acetylated	2,492	(52)	5,293	(85)
B, chloric, silylated	2,275	(69)	1,168	(66)
B, H_2SO_4 acetylated	589	(118)	1,694	(80)
B, H_2SO_4, silylated	1,306	(76)	0	(0)
B, chloric acid-treated	13,598	(13)	49,276	(20)
B, chloric, butanol-treated[‡]	11,809	(32)	51,386	(8)

The data are the means and standard deviations (expressed as percentages) of at least ten individual measurements made on four dishes. Maximal number of leukocytes available for adhesion was 52,910 cm^{-2}; maximal number of BHK cells was 31,750 cm^{-2}. Cells adhering in the presence of serum is expressed as cells bound cm^{-2}. TC, tissue culture grade; B, bacteriological grade.
* Acetic anhydride or trimethylsilylimidazole was used to block hydroxyl groups.
[‡] Carboxyl groups were blocked by esterification with N-butanol.

Table V

Does Esterification of Surface Carboxyl Groups Block Cell Adhesion?

		[^{14}C]ethanol bound	
Treatment	Cell adhesion	Before cells added	After cells added
	cells/cm²	*dpm/cm²*	
Leukocytes			
Bacteriological dishes	8,844 (17)	—	—
Chloric acid treated	56,192 (10)	—	—
Chloric + Esterif.	56,809 (28)	1.7	1.6
BHK cells			
Bacteriological dishes	840 (43)	—	—
Chloric acid treated	14,932 (15)	—	—
Chloric + Esterif.	15,719 (23)	0.9	0.8

Cell adhesion conditions were as described in Tables I and IV. Ethanol specific activity as in Table III. Values in parentheses are the standard deviations expressed as percentages of the mean.

TABLE VI

Adhesion of Cells to Specially Purified Polystyrenes before and after Chloric Acid Treatment

Method of preparation	Cell adhesion	
	BHK cells	Leukocytes
Method 1	4,086 (77)	15,456 (52)
Method 1, chloric acid-treated	29,390 (16)	34,753 (18)
Method 2	287 (123)	2,970 (128)
Method 2, chloric acid-treated	29,747 (26)	41,229 (15)

Measurements were made in the presence of serum. Conditions as for experiments reported in Tables I to V. Adhesion is expressed as cells binding cm^{-2}.
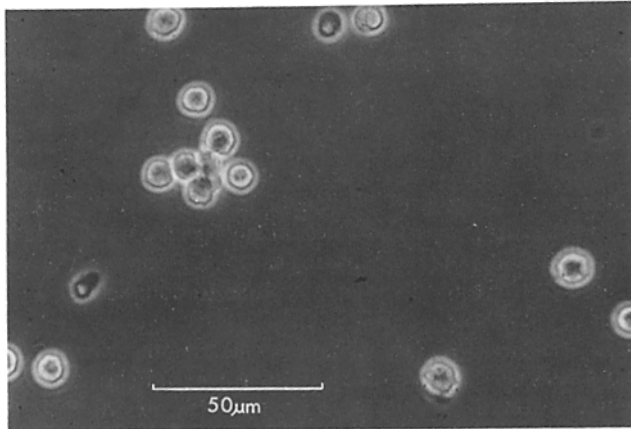
FIGURE 7 The adhesion but lack of spreading of BHK cells on bacteriological grade polystyrene in the absence of serum after 100 min. Scale bar in this figure refers to this and all subsequent photographs (except Fig. 8).

(Table VII, Figs. 7–11) showed that cell adhesion to untreated or to oxidized polystyrene is appreciable, unlike the situation with adhesion of cells to hydrophobic surfaces in the presence of serum. The leukocytes in these experiments (Fig. 8) appeared to be unusually flattened on chloric acid-treated surfaces yet trypan blue was excluded by the cells. BHK cells attached relatively well to both hydrophobic or hydrophilic (chloric acid-treated surfaces; Figs. 7 and 9) in the absence of serum or any other protein addition to the medium. They did not flatten well on the hydrophobic or slightly hydrophobic surfaces such as those produced by 60 min of exposure to ultraviolet light or on tissue culture grade polystyrene. They flattened well on the hydrophilic surfaces (Fig. 9). When the medium was replaced with the insulin-transferrin-selenite serum-supplemented Hams medium, the cells grew well. Thus it could be suggested that adhesion occurred in the absence of serum, whether the surfaces are hydrophobic or hydrophilic, and that in the presence of soluble proteins hydrophobic surfaces adsorbed them differentially by comparison with hydrophilic surfaces. Thus untreated polystyrene might adsorb proteins that were not suitable for cell adhesion whilst hydroxyl group-rich polystyrene might fail to adsorb such proteins or even adsorb proteins that promote adhesion more effectively. We hope to report further on this in a later paper.

The finding that cells spread well on the chloric acid-treated polystyrene in the absence of serum proteins raises the possibility that there is no requirement for such proteins in adhesion. It also raises the possibility that cellularly derived proteins removed in this disaggregation of cells are not required

for adhesion on such substrates. If this interpretation is correct cells that have not been exposed to serum after trypsinization and whose protein synthetic abilities have been poisoned with an agent such as cycloheximide should be able to adhere and perhaps spread even in the absence of protein added to the medium.

To test this hypothesis BHK cell suspensions were prepared by trypsinization from cells that had been preincubated for 3 h with 25 μg/ml cycloheximide to ensure that the cells would be unable to synthesize fibronectin or other surface proteins. The cells were then trypsinized and the trypsin activity was stopped with 25 μg/ml leupeptin. They were then incubated with cycloheximide at a concentration of 25 μg/ml for 60

TABLE VII

Binding of Cells to Polystyrene Dishes in Serum-free Medium

Dish type	BHK cells	Leukocytes
Bacteriological grade	7,880 (24)	51,725 (20)
Tissue culture grade	22,159 (19)	49,323 (17)
Chloric acid treated	25,399 (17)	45,675 (32)

Maximal possible adhesion; leukocytes, 52,910 cm^{-2}; BHK cells, 31,750 cm^{-2}. The measurements are mean and standard deviations (%) of at least 28 sets of measurements, except data for the bacteriological grade which is based on 13 measurements. Adhesion is expressed as cells bound cm^{-2}.
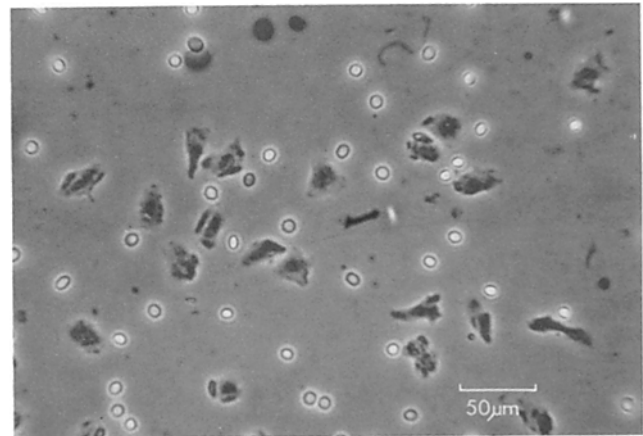

FIGURE 8 Spreading of leukocytes on chloric acid-treated polystyrene in serum-free medium after 15 min.
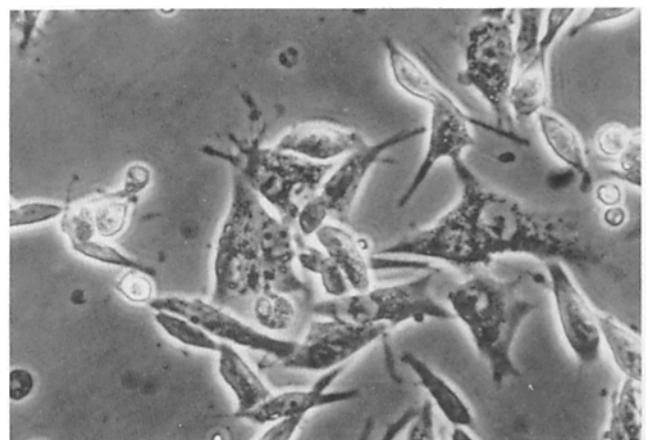

FIGURE 9 Spreading of BHK cells on chloric acid-treated polystyrene in serum-free medium after 50 min.

min at 37°C before being plated out on plain "bacteriological" or on chloric acid-treated polystyrene. Control cultures were prepared from cells whose trypsinization was stopped with antitrypsin or leupeptin but that had been grown in the absence of cycloheximide Figs. 9–11 show the appearance of the cells. Clearly attachment and spreading of the BHK cells is perfectly adequate in the absence of added fibronectin (or other proteins) and under conditions in which synthesis of fibronectin is impossible. It is interesting to note that adhesion of cells inhibited in the same way to "bacteriological" or "tissue culture" grade dishes hardly took place and that no spreading of the few attached cells occurred on such surfaces.

DISCUSSION

It is appropriate to start the discussion by reminding readers that reactions at surfaces are often substantially different from the reactions of the same compounds in solution. Thus the use of various reactions to block possible groupings (e.g., hydroxyl in this work) might behave in an unexpected manner on surfaces. However we have endeavored to meet this problem by including the results of both XPS and ATR spectroscopy to identify surface groupings, as well as using radiochemical methods. The results of the three methods are substan-
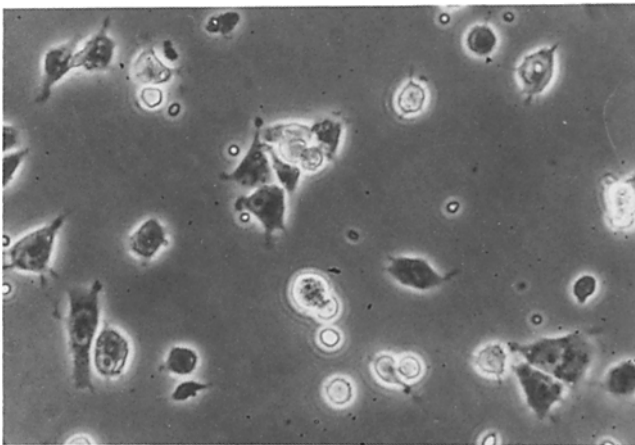

FIGURE 10 Spreading of BHK cells on chloric acid-treated polystyrene after cycloheximide treatment for 3 h prior to trypsinization and leupeptin inhibition in serum-free medium after 60 min.
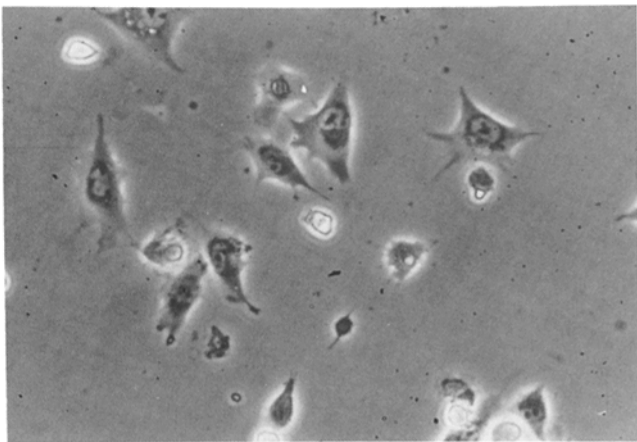

FIGURE 11 Spreading of BHK cells on chloric acid-treated polystyrene with cycloheximide treatment before and after trypsinization and leupeptin inhibition of trypsin in serum-free medium after 320 min.

tially consistent. The cells themselves, of course, should provide a very sensitive method of investigating surface chemistry but understanding the mechanism of their attachment is the aim of this work.

Earlier work by others (2, 3) had led to the suggestions that sulfonate or carboxyl groups are involved in cell adhesion as binding groups. Our experiments show that this interpretation is almost certainly inaccurate. First, the extent of sulfonation by 98% sulfuric acid was very slight as judged by ATR spectroscopy or by the appearance of ^{35}S-activity on the dishes after exposure to radioactive sulfuric acid. This treatment in fact induced oxidations that appeared to produce carboxyl and hydroxyl groups detectable by both ATR spectroscopy or by the binding of [^{14}C]ethanol or acetic anhydride, respectively. Chloric acid-treatment produced hydroxyl groupings detectable by the same methods and by X-ray photoelectron spectroscopy, whose detection was confined to a more superficial layer than ATR techniques. The density of hydroxyl groups produced by these methods was at least 1,000-fold greater than the highest possible density of sulfonate groups. Adhesion to surfaces is blocked by treatments that block hydroxyl groups, in particular the highly specific trimethylsilylimidazole reagent (16). Blocking carboxyl groups was without effect. These results refer of course to adhesion in the presence of serum.

The results of the XPS technique and the others we have used for surface characterisation are, as we said above, generally consistent, but XPS revealed more carboxyl than hydroxyl groups on both the chloric acid and the "tissue culture" grade surfaces, whereas the direct chemical estimation and the ATR data show the reverse. However all three techniques showed that chloric acid-treatment yields surfaces richer in hydroxyl groups than does the commercial treatment used to make "tissue culture" grade surfaces, which produces surfaces with a dense covering of carboxyl groups and some hydroxyl groups (17). The biological data on adhesion to acetylated surfaces which is much reduced, as opposed to adhesion to alcohol-esterified surfaces, which is little affected, argues that carboxyl groups play a limited role in adhesion while hydroxyl groups may be very important.

All methods of oxidation produced some carboxyl groups (see Tables III and V). Tissue culture dishes have a higher density of carboxyl groups than bacteriological ones but are also characterized by a much higher density of hydroxyl groups. Blocking the hydroxyl groups diminished adhesion (Tables III–V), but blocking the carboxyl groups by esterification did not reduce adhesion (Tables IV and V). Ozonization generated a very high density of carboxyl groups and produced a surface of poor adhesiveness (see Table I).

It appears that one conventional explanation of adhesion is brought into question by our results. Many papers (for instance 18, 19) have suggested that those surfaces that are adhesive for cells are wettable ones, whilst the nonadhesive surfaces will be unwettable. It is of course correct on the basis of this theory that surfaces rich in hydroxyl groups are very adhesive. Nevertheless, surfaces that are unwettable by serum- or protein-free media still support appreciable cell adhesion (Table VII). However it is possible that metabolically active cells produce their own wetting agents (i.g., secreted proteins as they settle), which may make the question of the relevance of wettability unanswerable at present. However the finding that cells in which protein synthesis has been inhibited for 3 h prior to and during the experiment still spread on chloric

acid-treated surfaces argues against the release of wetting agents.

Rubin et al. (20) reported that hepatocytes spread well in the absence of fibronectin. Our finding that cells on whose surfaces endogenous or exogenous fibronectins are lacking argues again that fibronectin is not an essential component for cell attachment or spreading for BHK cells. This conclusion would seem to be at variance with other reports (10, 21) and in particular those of Grinnell (1, 22) who found that fibronectin or serum was an essential component for the spreading of fibroblasts (of a suspension growth adapted clone of BHK) on "tissue culture" grade dishes. We, of course, also find that the cycloheximide-inhibited fibroblasts, subjected to trypsinization and subsequent inhibition of the trypsin with leupeptin, will not spread appreciably on such surfaces, though they appeared to be ready for spreading. The chloric acid-treated surfaces were appreciably richer in hydroxyl groups than the "tissue culture" grade surfaces (see Table III) while their surface density of carboxyl groups was lower. Perhaps the greater density of hydroxyl groups was sufficient to make the surfaces adhesive enough for spreading. In any event the results strongly suggest that spreading can be a passive event that does not require fibronectin nor the resynthesis of any other cell surface material depleted by trypsinization.

Another way of looking at our findings is to point out that the role of fibronectin in culture situations is parallel to that provided by a high density of hydroxyl groups; this view should be compared with that in reference 23.

We suggest that hydroxylation of a polystyrene surface makes it very adhesive for cells. This would appear to be the main feature provided by the commercial treatment which renders polystyrene suitable for tissue culture. The concomitant generation of a rather high density of carboxyl groups on such surfaces may in fact reduce the adhesiveness which could be attributed to hydroxylation. Carboxyl groups would of course be expected to reduce adhesion for reasons of elementary physical chemistry (7, 9).

Our aim in using both leukocytes and BHK cells in this work was to check whether there might be individual reactions peculiar to particular cell types. However, we generally found that these two dis-similar cell types behaved similarly to a wide variety of surfaces.

Our results provide perhaps the first evidence that hydroxyl groups play a major role in cell adhesion to solid surfaces. It also suggests that hydrogen bonding may play a major role in cell adhesion but it should not be assumed that this bonding is necessarily between surfaces. Hydrogen bonding with the water in the system may also effect forces of attraction. It is also of interest that our results may be connected to those many papers that have reported on the importance of glycoproteins in cell adhesion. The glycosidic moieties of these molecules will have a high density of hydroxyl groups and it is pertinent to enquire whether the effects attributed to specific binding of glycosidic groups to cell surfaces are at least in part explained as reactions to the presence of a high density of hydroxyl groups on the surface to which a cell is adhering.

Weigel et al. (24) reported that there is a threshold level of sugars bound to polyacrylamide gels for cell adhesion. The same effect seems to appear in our work in that adhesion did not become appreciable until the hydroxyl group density rose to ~1,000 per 1,000 nm^2. Unfortunately it is impossible to make an exact comparison with their work because they express densities of sugars per unit volume whereas we express them per unit area.

REFERENCES

1. Grinnell, F. 1978. Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* 53:65–144.
2. Maroudas, N. G. 1977. Sulphonated polystyrene as an optimal substratum for the adhesion and spreading of mesenchymal cells in monovalent and divalent saline solutions. *J. Cell. Physiol.* 90:511–520.
3. Klemperer, H. G., and P. Knox. 1977. Attachment and growth of BHK and liver cells on polystyrene: effect of surface groups introduced by treatment with chromic acid. *Lab. Pract.* 26:179–180.
4. Amstein, C. F., and P. A. Hartman. 1975. Adaptation of plastic surface for tissue culture by glow discharge. *J. Clin. Microbiol.* 2:46–54.
5. Ambroski, L. E. 1966. U.S. patent 3,274,090.
6. Martin, G. R., and H. Rubin. 1974. Effects of cell adhesion to the substratum on the growth of chick embryo fibroblasts. *Exp. Cell Res.* 85:319–333.
7. Curtis, A. S. G. 1973. Cell adhesion. *Prog. Biophys. Mol. Biol.* 27:317–375.
8. Gingell, D., and I. Todd. 1980. Red blood cell adhesion. II. Interferometric examination of the interaction with hydrocarbon oil and glass. *J. Cell Sci.* 41:135–149.
9. Gingell, D., and S. Vince. 1982. Substratum wettability and charge influence: the spreading of *Dictyostelium discoideum* amoebae and the formation of ultrathin cytoplasmic lamellae. *J. Cell Sci.* 54:255–285.
10. Olden, K. H., L.-H. E. Hahn, and K. M. Yamada. 1980. Fibronectin: properties and role in cellular morphology and adhesion. *In* Cell Adhesion and Motility. A. S. G. Curtis and J. Pitts, editors. Cambridge University Press, Cambridge, England. 357–387.
11. Brown, A. F., and J. M. Lackie. 1981. Fibronectin and collagen inhibit cell-substratum adhesion of neutrophil granulocytes. *Exp. Cell Res.* 136:225–231.
12. Cassiman, J.-J., P. Marynen, M. Brugmans, F. van Leuven, and H. van den Berghe. 1981. Role of the alpha-2-M-receptor in attachment and spreading of human fibroblasts. *Cell Biol. Int. Rep.* 5:901–911.
13. Unhjem, O., and H. Prydz. 1973. Interference of serum and serum components with attachment of HeLa 71 cells to the surface of plastic culture vessels. *Exp. Cell Res.* 83:418–21.
14. Edwards, J. G., and J. A. Campbell. 1971. The aggregation of trypsinized BHK21 cells. *J. Cell Sci.* 8:53–72.
15. Forrester, J. V., and P. C. Wilkinson. 1981. Inhibition of leukocyte locomotion by hyaluronic acid. *J. Cell Sci.* 48:315–331.
16. Horning, M. G., A. M. Moss, and E. C. Horning. 1967. A new method for the separation of catecholamines by gas-liquid chromatography. *Biochem. Biophys. Acta.* 148:597–600.
17. Andrade, J. D., G. K. Iwamoto, and B. McNeill. 1977. XPS studies of polymer surfaces for biomedical applications. *In* Characterization of Metal and Polymer Surfaces, Vol. 2. Academic Press, Inc., New York. 133–141.
18. van Oss, C. J., C. F. Gillman, and A. W. Neumann. 1975. Phagocytic Engulfment and Cell Adhesiveness as Cellular Surface Phenomena. Dekker, New York.
19. Edebo, L., E. Kihlstrom, K.-E. Magnusson, and O. Stendahl. 1980. The hydrophobic effect and charge effects in the adhesion of enterobacteria to animal cell surfaces and the influences of antibodies of different immunoglobulin classes. *In* Cell Adhesion and Motility. A. S. G. Curtis and J. Pitts, editors. Cambridge University Press, Cambridge, England. 65–101.
20. Rubin, K., S. Johansson, M. Hook, and B. Obrink. 1981. Substrate adhesion of rat hepatocytes. On the role of fibronectin in cell spreading. *Exp. Cell Res.* 135:127–135.
21. Yamada, K., and K. Olden. 1978. Fibronectins-adhesive glycoproteins of cell surface and blood. *Nature (Lond.)* 275:179–84.
22. Grinnell, F., and M. Feld. 1979. Initial adhesion of human fibroblasts in serum-free medium: possible role of secreted fibronectin. *Cell.* 17:117–129.
23. Virtanen, L., T. Vartio, R. A. Badley, and V.-P. Lehto. 1982. Fibronectin in adhesion, spreading and cytoskeletal organization of cultured fibroblasts. *Nature (Lond.)* 298:660–663.
24. Weigel, P. H., R. L. Schnaar, M. S. Kuhlenschmidt, E. Schmell, R. T. Lee, Y. C. Lee, and S. Roseman. 1979. Adhesion of hepatocytes to immobilized sugars. A threshold phenomenon. *J. Biol. Chem.* 254:10830–10838.