SOME EFFECTS OF POSITIVELY CHARGED SURFACE GROUPS ON CELL AGGREGATION

D. E. MASLOW AND L. WEISS
Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, N.Y. 14263, U.S.A.

SUMMARY
A study was made of the effects of 2,3-dimethylmaleic anhydride (DMA), a reagent removing positive charges, on the aggregation and surface charge of embryonic chick neural retina cells. Neural retina cells, recovered from the dissociation procedure, were cultured on a gyratory shaker and the aggregate diameters formed in the presence of DMA or DMA-serum dialysate, following DMA-pretreatment, or in appropriate control cultures measured. The electrophoretic mobilities of similarly treated cells were also determined. In addition, cellulose acetate electrophoresis was carried out on samples of serum containing DMA, and the incorporation of 14C-amino acids into DMA-treated cells studied.

Aggregates formed in the presence of DMA, or following DMA-pretreatment, were significantly smaller than aggregates from control cultures. The electrophoretic mobility of DMA-treated cells was significantly increased in serum-containing medium, but not serum-free Hanks’ solution. At 24 h after removal of DMA-containing medium, the mobilities of pre-treated cells were similar to those of controls.

The electrophoretic pattern of DMA-treated serum was changed only with concentrations of DMA many times that affecting cell aggregation or mobility. DMA-serum dialysate did not significantly reduce aggregate size. The incorporation of 14C-amino acids in DMA-treated cells and the structure of aggregates were unchanged from controls.

It is concluded that positively charged constituents of the cell periphery play a demonstrable, but not limiting, role in cell aggregation, while a minor role for positive charges on serum protein cannot be totally excluded.

INTRODUCTION
All populations of vertebrate cells studied so far have been shown to carry a net negative charge. However, Weiss (1974) has reported a small but significant increase in mobility to the anode after Ehrlich ascites tumour (EAT) cells were treated with agents which react with amino and other cationic groups, indicating the presence of positively charged groups, associated with a small decrease in the rate of adhesion of these cells to artificial surfaces.

Since contact between cells in close approximation may well involve Coulombic attractions between cationic and anionic sites in localized regions of the cell surface (Weiss & Harlos, 1972), the role of the positively charged surface groups may have more biological significance than their contributions to charge density might suggest. We report here the effect of 2,3-dimethylmaleic anhydride (DMA), a reagent removing positive charges (Mehrishi, 1970, 1972), on the aggregation and surface charge of embryonic chick neural retina cells.
MATERIALS AND METHODS

Cells

Neural retina (NR) cells were isolated from 10- or 11-day-chick embryos using Tryptar crystallized trypsin (Armour Pharmaceuticals, Kankakee, Ill.) according to the optimal procedure of Barnard, Weiss & Ratcliffe (1969), and cultured in Eagle's minimal essential medium with Hanks' salts (HMEM) supplemented with 10% horse serum (Grand Island Biological, N.Y.). Cells were initially cultured in a Vibromixer (Chemapac; Vineland, N.J.) (Ulrich & Moore, 1965), which permits recovery from the dissociation procedure without aggregation (Barnard et al. 1969; Maslow, 1970).

Aggregation was promoted by culture of 6 x 10^6 neural retina cells in 3 ml of medium in 25-ml flasks on a gyratory incubator shaker moving at 70 rev/min (New Brunswick Scientific) (Moscona, 1961). The diameters of 50 aggregates from each treatment group were measured using a Zeiss filar micrometer. Viabilities were consistently higher than 95%, as determined by trypan blue exclusion. Some cultures were fixed and stained for observations of the morphology of the aggregates formed.

DMA treatment

Unless otherwise specified, 10 μl of a 10^-4 M DMA (Sigma, St Louis, Mo.) in ethanol solution was added to 10 ml of cell suspension containing 2 x 10^6 cells/ml maintained at pH 6.8. Controls received equivalent doses of ethanol. The chemistry of the reactions between DMA and amino groups has been discussed by Mehrishi (1970, 1972) and Weiss (1974a).

Cell suspensions were removed from the Vibromixer, washed once in Hanks' balanced salt solution (HBSS), once in HBSS containing DMA, and once with medium containing DMA. In experiments in which cells were used in the presence of DMA, they were suspended directly in medium with DMA and either cultured to promote aggregation, or their mobilities measured. Cells which were used in the absence of DMA following DMA pretreatment were incubated for 1 h in medium with DMA and washed twice with medium before use.

Electrophoretic mobility

Measurements of electrophoretic mobilities were made on cells treated exactly as described for use in the aggregation experiments, in a modified cylindrical cell apparatus of the type described by Bangham, Flemans, Heard & Seaman (1958). All measurements were made at 37°C, with reversal of current, in a known voltage-gradient applied between grey, sintered platinum electrodes. Under these conditions, the mean electrophoretic mobility of human erythrocytes was -1.383 μm s^-1 V^-1 cm. Measurements were also made on cells treated initially with DMA and cultured in its absence for 24 h.

Dialysis

In some experiments, the serum in the medium was reacted separately with DMA. In these cases, a solution of 8 mg/ml of DMA in serum was dialysed against HBSS for 24 h at 10°C. The dialysate was then added to cultures of neural retina cells at the same volume of DMA solution to that in the undialysed medium, indicating a potential maximum concentration of 6 times that in the cultures of undialysed DMA solution. The same amount of dialysed serum was added to control cultures.

14C-amino acid incorporation

The effect of DMA treatment on cellular protein synthesis was studied by measuring the incorporation of 14C-amino acid mixture (New England Nuclear) by DMA-treated cells and appropriate controls. Ten microcuries of the 14C-amino acid mixture were added to 10 ml of treated or untreated cell suspensions containing 2 x 10^6 cells. After 1 h of incubation at 37°C, the cells were washed with cold medium and the trichloroacetic acid (TCA)-precipitable material counted using a Packard Tri-carb liquid scintillation counter.
**Electrophoresis**

Cellulose acetate electrophoresis was carried out on samples of serum containing DMA at concentrations of 8–0.005 mg/ml and the pattern of proteins was analysed by a Beckman Microzone electrophoresis system.

**RESULTS**

Table 1, which indicates the combined data from 6 experiments using different cell preparations, shows that the presence of DMA in suspensions of NR cells resulted in the formation of aggregates that were significantly ($P < 0.001$) smaller after 3, 6 and 24 h of culture. The combined results in Table 2 from 2 separate experiments indicate that pretreatment of cells with DMA produces an inhibition in aggregate size compared with controls, which is not significantly different ($0.4 > P > 0.3$) from incubation in medium containing DMA. Variation between experiments is responsible for the difference in aggregate sizes between the 2 sets of data shown in Tables 1 and 2.

The effect of DMA treatment on the mean electrophoretic mobilities of neural retina cells is shown in Table 3. The 8% increase in mobility occurring after the addition of DMA to HBSS was not statistically significant ($0.1 > P > 0.05$), but cells in medium containing DMA showed a highly significant ($P < 0.001$) 20% increase in net cell surface negativity.
The recovery of cells from DMA treatment was studied by remeasuring their electrophoretic mobilities 24 h after initial treatment. The results in Table 4 confirm that an initial treatment with DMA results in a significant ($P > 0.001$) increase in electrophoretic mobility measured immediately after washing. At 24 h after replacement of the DMA-containing medium, the mobilities of the pretreated and untreated cells were similar ($P < 0.9$), indicating that the effect of the DMA pretreatment was not permanent.

Table 3. Electrophoretic mobility of cells treated with DMA in medium and Hanks' solution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mobility, $\mu m s^{-1} V^{-1} cm \pm S.E.$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks' (control)</td>
<td>$1.58 \pm 0.04$ (100)</td>
</tr>
<tr>
<td>Hanks' + DMA</td>
<td>$1.71 \pm 0.05$ (98)</td>
</tr>
<tr>
<td>Medium (control)</td>
<td>$1.32 \pm 0.04$ (107)</td>
</tr>
<tr>
<td>Medium + DMA</td>
<td>$1.59 \pm 0.04$ (122)</td>
</tr>
</tbody>
</table>

Table 4. Electrophoretic mobility of cells after DMA treatment followed by 24 h culture without DMA

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Treatment</th>
<th>Mobility, $\mu m s^{-1} V^{-1} cm \pm S.E.$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>$1.24 \pm 0.03$ (166)</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>$1.37 \pm 0.03$ (122)</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>$1.31 \pm 0.02$ (218)</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>$1.33 \pm 0.02$ (220)</td>
</tr>
</tbody>
</table>

Since the effect of DMA on electrophoretic mobility was significant only in serum-containing medium, cellulose acetate electrophoresis was done on samples of serum containing a wide variety of DMA concentrations up to 8 mg/ml. At DMA concentrations less than 4 mg/ml, no effect on the electrophoretic distribution of the serum components was observed. At 4 mg/ml (which is $4 \times 10^9$ the concentration used in culture and cell electrophoretic mobility experiments) some variation in the mobility of the albumin and $\alpha_1$ components was found, while with 8 mg/ml the $\alpha_2$ and $\beta$ components were also affected. The $\gamma$ components remained unchanged at all concentrations tested. Thus it seems unlikely that the effect of DMA at the concentration used on cells is due solely or directly to its interaction with serum coating them.

To establish further the possible role of DMA-serum interactions in the changes in aggregation of neural retina cells, the sizes of aggregates were determined following treatment with serum to which DMA had been added, and then dialysed. This would result in the cells being exposed only to DMA bound to serum, and not to free DMA. Table 5 indicates that while the aggregates formed in cultures to which DMA was added directly were significantly smaller, there was no significant difference ($0.2 > P > 0.1$) in the size of aggregates to which dialysed serum was added compared
Cell aggregation and positive charge

223
to those to which dialysed serum-DMA was added in the 2 experiments made. Thus the addition of DMA bound to serum does not significantly reduce the size of aggregates, while the presence of initially unbound DMA does. The presence of small quantities of dialysed serum also reduced the size of the aggregates formed.

Some aggregates formed in the presence of DMA were examined following histological preparation. They appeared similar in their internal structure and the formation of characteristic rosettes (Moscona, 1961) to untreated aggregates.

Table 5. Effect of DMA-serum dialysate on the size of NR aggregates formed in gyratory shaker cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aggregate diameter, ( \mu m \pm S.E. ) (no. of observations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>233.45 ± 10.15 (100)</td>
</tr>
<tr>
<td>DMA</td>
<td>200.45 ± 8.01 (100)</td>
</tr>
<tr>
<td>Serum dialysate control</td>
<td>185.60 ± 6.14 (100)</td>
</tr>
<tr>
<td>DMA-serum dialysate</td>
<td>170.55 ± 7.13 (100)</td>
</tr>
</tbody>
</table>

A comparison was also made of the \(^{14}\)C-amino acids incorporation of DMA-treated cells as further indication of their viability. Cells treated with DMA at levels equivalent to those used in aggregation experiments showed no decrease in levels of protein synthesis (35,377 total cpm for the control as compared with 37,624 cpm for DMA) whereas at doses 50 times the normal, the level of incorporation was 8\% that of the control (26,86 cpm). Thus, it can be assumed that the cells remained viable when treated with the normal DMA dose.

DISCUSSION

The experiments of Weiss (1974a) indicated that cationic groups at the cell surface which are susceptible to DMA treatment on the evidence of cell electrophoresis, influence the rates of cell adhesion to protein-coated plastic surfaces, but only to a minor extent. Our results (Tables 1 and 2) indicate that the treatment of cells with DMA caused a reduction in the size of aggregates formed after 24 h of culture in a gyratory shaker. The size of aggregates formed in gyratory shaker cultures from suspensions of one cell type under varying conditions is thought to reflect their 'collision efficiency', which is a composite parameter of the 2 variables of adhesiveness and resistance to separation by the shearing forces of the medium (Weiss, 1974b). Thus, the reduced aggregate size obtained following DMA treatment indicates reduced cell collision efficiency.

The electrophoretic mobility of DMA-treated cells was increased only on cells measured in the presence of serum. It was therefore necessary to determine if the reduced aggregation of the cells following DMA treatment resulted from its effect on the cell surface or on the protein coating which probably surrounds cells in serum-containing medium (Baier & Weiss, 1975; Revel & Wolken, 1973). The results of
cellulose acetate electrophoresis indicate that at high doses of DMA a change occurs in the mobility of some of the serum components. However, this technique is insufficiently sensitive to detect minor changes in the serum proteins which may occur in the presence of lower DMA dosages.

The data on aggregation in the presence of DMA-serum dialysates (Table 5) do not show significant reductions in aggregate diameter, but the reduction, although not significant, occurred consistently in the 3 experiments made. This suggests a possible small effect of DMA on serum proteins which may be reflected in the cellular aggregation behaviour, although at a level just at the threshold of sensitivity of this aggregation system. It is therefore impossible to rule out a partial role for DMA-serum protein interactions in the reduced aggregation obtained following DMA treatment. It is unfortunate that experiments on cell aggregation cannot be made in the absence of serum without causing artifactual trauma, since this would have provided a control for the contribution of serum to the DMA-inhibitory effect.

The effect of the DMA treatment on net surface negativity was reversible within 24 h in the absence of DMA. However, the size of aggregates formed by DMA-pretreated cells in the absence of DMA was the same as that found in its continued presence. This emphasizes the importance of the initial period of aggregation in determining final aggregate size.

The overall conclusion which we draw from these experiments is that positively charged constituents of the cell periphery play a demonstrable but not limiting role in cell aggregation. The positive charges on serum proteins present in the medium, play at most a minor role in cell aggregation.

We are grateful to Dr T. M. Chu for doing the cellulose acetate electrophoresis and densitometry. We also thank Mr G. Johnson and Mr D. Graham for their technical assistance. This work was partially supported by grants CA 14370 from NIH and BC-87G from the American Cancer Society.

REFERENCES


Cell aggregation and positive charge


(Received 17 November 1975)