Retinoid-Induced Adhesion in Cultured, Transformed Mouse Fibroblasts [1]

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ABSTRACT—Cultured, spontaneously transformed mouse fibroblasts (Balb/3T12-3 cells) were readily detached from the dish surface in an EDTA-mediated detachment assay. Retinoic acid-treated cells displayed increased adhesion to the culture dish surface. The effect of retinoic acid on the adhesion of Balb/3T12-3 cells was dose-dependent in the range of 0.05-5 μg/ml (0.17-17 μM) in an assay performed on cells cultured for 3 days in the presence of the retinoid. The earliest effect on adhesion was detected at 2 days of culture in the presence of 17 μM retinoic acid. The increase in adhesion displayed by retinoic acid-treated cells was rapidly lost upon removal of the retinoid from the culture medium. Synthetic retinoids were tested for their activity in inducing adhesion of cultured Balb/3T12-3 cells. Retinol, retinoic acid, and their 5,6-epoxy derivatives were the most active, showing activity at 1 μg/ml. 13-*cis*-Retinoic acid and the dimethylacetylcyclopentenyl and trimethylmethoxyphenyl derivatives were active at 10 μg/ml. However, active derivatives of retinoic acid invariably lost their activity upon chemical esterification or amide formation. Retinoids without biologic activity in other systems were also inactive in inducing adhesion. Among these were the synthetic derivatives of retinol, anhydroretinol and perhydromonoeneretinol, and the phenyl derivative of retinoic acid. β-Ionone, abscisic acid, and juvenile hormone were also inactive. Results showed that this adhesion assay may be used as an additional test for the biologic activity of retinoids containing a free carboxylic or carbinolic function. The phenomenon of induced adhesion may also aid in the study of the metabolic function of vitamin A.—JNCI 62: 1473-1478, 1979.

Vitamin A and its derivatives (retinoids) have been shown to exert a preventive activity in models of chemical carcinogenesis (*1-5*) and viral transformation (*6*). An antitumor activity has been demonstrated in some studies on transplanted tumors (*3, 7-9*). However, the mechanisms by which retinoids exert these distinct activities are unknown.

Previous studies from our laboratory have suggested a molecular involvement of phosphorylated vitamin A as a lipid intermediate in the biosynthesis of membrane glycoconjugates [for a review *see* (*10*)]. This mechanism of action of vitamin A may well explain the phenomenon of increased adhesion induced by retinoids in Balb/3T12-3 cells.

Here we report the findings that retinol, retinoic acid, and their biologically active derivatives increase the adhesion of spontaneously transformed mouse fibroblasts Balb/3T12-3 cells, a cell line characterized by a high saturation density, absence of contact inhibition of growth, and tumorigenicity in immunosuppressed hosts (*11*).

MATERIALS AND METHODS

Balb/3T12-3 mouse fibroblasts were obtained from the American Type Culture Collection (Rockville, Md.) and cultured in T25 flasks (Falcon Plastics, Oxnard, Calif.) in 3 ml medium. Dulbecco's modified Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.) was supplemented with 10% calf serum (Flow Laboratories, Inc., Rockville, Md.), 25 mM HEPES[6] (pH 7.3), and 50 μg gentamicin/ml (Microbiological Associates, Inc., Walkersville, Md.). The medium was changed daily. The initial inoculum, unless otherwise stated, was of 10,000 cells/cm^2 of the growth area. Trypsinizations (both for cell counting and passaging) were performed as follows: The trypsinization solution contained 2.5 g trypsin/liter (ICN Nutritional Biochemicals, Irvine, Calif.) and 0.2 g EDTA/liter in Hanks' balanced salt solution (Ca^{2+}-free and Mg^{2+}-free, pH 7.6). The solution was divided into 10-ml aliquots and frozen. After removal of the culture medium, the dishes were rinsed twice with 3 ml Dulbecco's PBS (Ca^{2+}-free and Mg^{2+}-free). The cells were rinsed quickly with 1.5 ml trypsin-EDTA solution. This solution was discarded, and the cells were incubated for 15 minutes at 37° C and then suspended by pipetting in a suitable volume of either complete medium (for passage) or 10% serum in PBS (for cell counting). Samples of the cell suspensions obtained by trypsinization were routinely screened microscopically: More than 95% of the cells were present as single cells. Moreover, the culture vessels were examined after trypsinization to verify that no significant amount of cells had been left attached after trypsinization. Cells were enumerated with the use of a model B Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Trypan blue dye exclusion test was performed by incubation of the cells with 1 volume of 0.4% trypan blue in PBS and 1 volume of complete medium. All-*trans*-β-retinoic acid (Eastman Kodak Co., Rochester, N.Y.) was dissolved in

ABBREVIATIONS USED: DMSO = dimethyl sulfoxide; PBS = phosphate-buffered saline.

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[6] *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

DMSO (Pierce Chemical Co., Rockford, Ill.) at a concentration of 2 mg/ml or less, and the solution (freshly made every week) stored in the dark at room temperature. Retinoic acid in DMSO, DMSO alone, or nothing was added to the culture medium 24 hours after the cells were plated, unless stated otherwise. DMSO concentration in the culture medium was 0.5%.

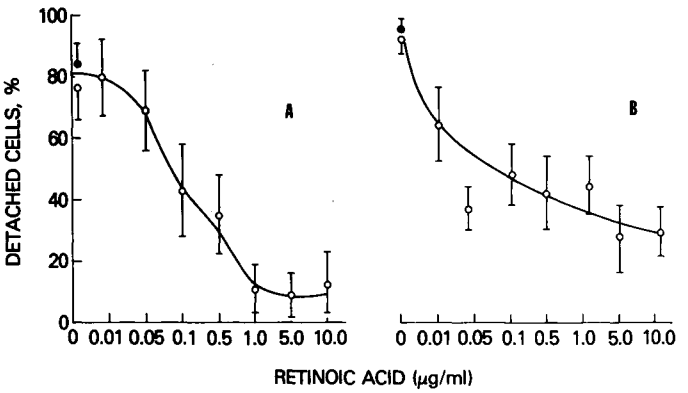
The adhesion assay is a modification of that described by Yamada et al. (*12*) and was performed as follows: Cells cultured in T25 flasks were rinsed with 3 ml PBS (Ca^{2+}-free and Mg^{2+}-free). After the addition of 1.5 ml EDTA (0.2 g/liter in PBS; Ca^{2+}-free and Mg^{2+}-free), the cells were shaken at 110 rpm on a Clay Adams variable speed rotator for 4 minutes at room temperature. At the end of the incubation, the detached cells were removed and these as well as the cells still adherent to the culture surface were trypsinized and dispersed as described to obtain a single-cell suspension suitable for automatic enumeration. When indicated, the adhesion test was preceded by a rinse of the cells with diluted trypsin (0.25 g/liter in PBS; Ca^{2+}-free and Mg^{2+}-free) for about 10 seconds at room temperature (pretrypsinization).

The following derivatives of retinol were prepared as follows: The hydrocarbon anhydroretinol was obtained as described by Dunagin and Olson (*13*); the hydrogenated derivative 3,4-monoeneperhydroretinol was prepared by catalytic hydrogenation of retinol (*14*); the 5,6-epoxyretinol was prepared from retinoic acid (*15*). Derivatives of retinoic acid were a gift from Dr. Beverly Pawson of the Hoffmann-La Roche Company, Nutley, New Jersey. Retinoids were solubilized in DMSO or ethanol before addition to the medium.

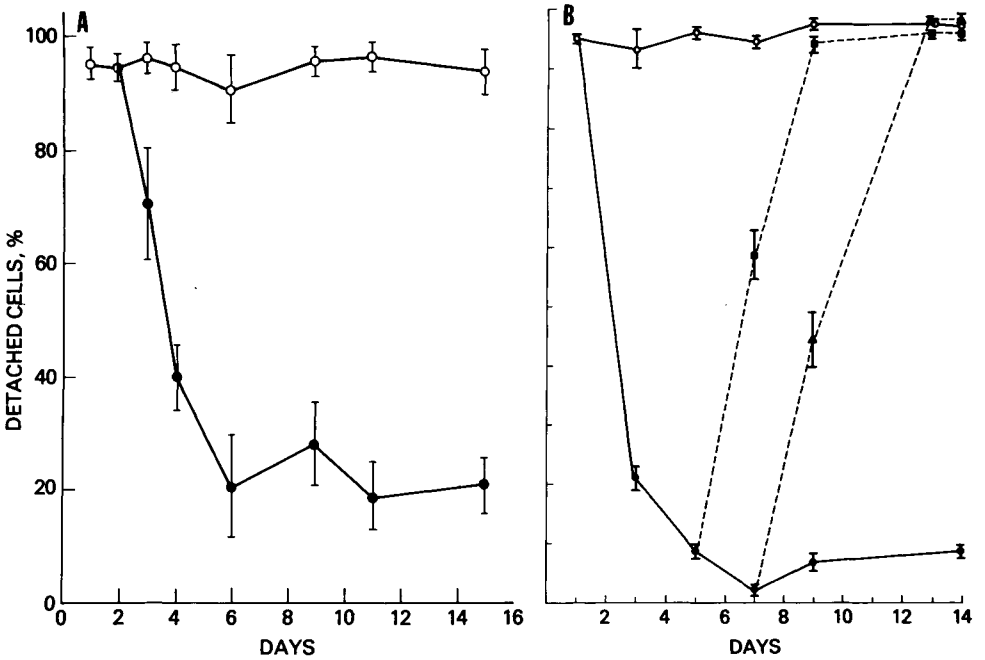
RESULTS

Balb/3T12-3 cells grew to reach a maximal density of about 450,000 cells/cm^2 by the end of the second week of culture. After this time, cell density did not increase, and cell death and detachment became evident. Nutritional deficiencies due to overcrowding might in part be responsible for this arrest of growth (*16*). These cells are easily detached from the dish surface in an EDTA-mediated detachment assay in the presence or absence of 0.5% DMSO.

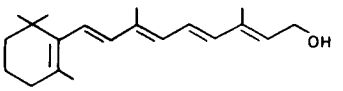
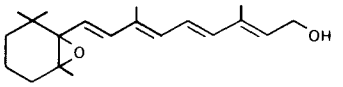
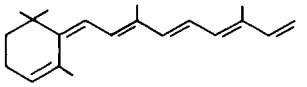
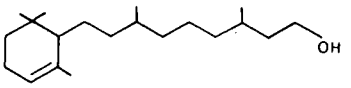
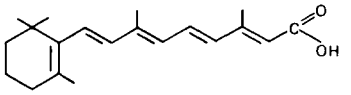
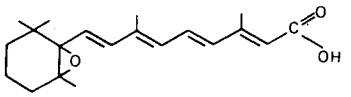
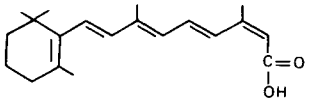
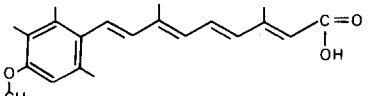
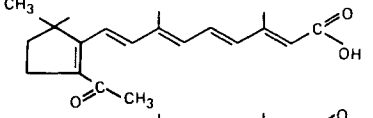
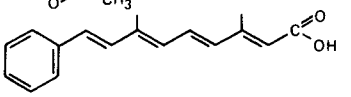
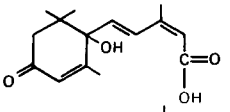
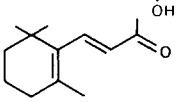
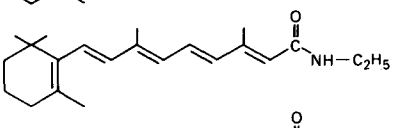
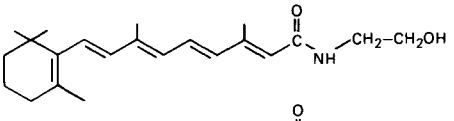
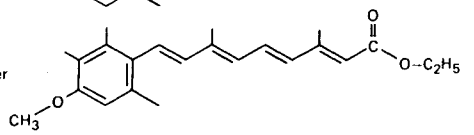
Treatment with 10 μg retinoic acid/ml (3.3×10^{-5} M)



TEXT-FIGURE 1.—Effect of retinoic acid on detachability of Balb/3T12-3 cells is shown in dose-response curves. Assay was performed as described under "Materials and Methods." A) Assay performed at day 4 of culture without pretrypsinization. B) Assay performed at day 4 of culture with pretrypsinization. ● refers to cells cultured without DMSO. *Each point* represents mean±SD.



TEXT-FIGURE 2.—A) Time course of the effect of retinoic acid on detachability of Balb/3T12-3 cells. Retinoic acid (10 μg/ml or 3.3×10^{-5} M) in DMSO (●) or DMSO alone (○) was added 24 hr after seeding. The adhesion assay was performed at the times indicated as described in text-fig. 1 with pretrypsinization. B) Reversibility of the effect of retinoic acid on the adhesion of Balb/3T12-3 cells. Cells were cultured as described with DMSO or retinoic acid-supplemented medium. At days 5 (■) or 7 (▲), samples treated with retinoic acid were switched to DMSO medium. Adhesion assay was performed as described at the time indicated. *Each point* represents mean±SD.

Name	Structure	Activity At 0.1 μg/ml	1.0 μg/ml	10.0 μg/ml
Retinol		–	++	++
5, 6 epoxy Retinol		–	++	++
Anhydroretinol		–	–	–
Perhydromonoene Retinol		–	–	–
Retinoic Acid		–	+	++
5-6 epoxy Retinoic Acid		–	+	++
13-cis-Retinoic Acid		–	–	++
TMMP-Retinoic Acid		–	–	++
DACP-Retinoic Acid		–	–	++
Phenyl Analog of Retinoic Acid		–	–	–
Abscisic Acid		–	–	–
β-Ionone		–	–	–
Retinoic-acid ethylamide		–	–	–
Retinoic acid 2-hydroxyethylamide		–	–	–
TMMP-retinoid ethyl ester		–	–	–

– No Effect (70 to 100% of cells detached)
+ Moderate Effect (30 to 70% of cells detached)
++ Large Effect (0 to 30% of cells detached)

TEXT-FIGURE 3.—Structure-activity relationship of natural and synthetic retinoids.

had varying inhibitory effects on the final cell density and a profound effect on the morphology of Balb/3T12-3 cells. Cells treated for 6 days with the retinoid (7 days in culture) appeared flatter (fig. 1B) and displayed a more orderly alignment of cells than did their control counterparts (fig. 1A). Retinoid-treated and control cells had the same plating efficiencies in mass culture (≥90%) and in a clone-forming assay (20%) in which they were seeded at a density of 100 cells/ml. About 3% of control and retinoid-treated (3.3×10^{-5} M or 10 μg/ml) cells were stained with trypan blue.

During these experiments, it was observed that retinoic acid-treated cells were more adherent and more resistant to the procedures employed to obtain single-cell suspensions suitable for automatic enumeration. Thus strict control of the trypsinization conditions was necessary to avoid artifacts in cell counting.

Trypsinization was less effective in lifting retinoic acid-treated cells (fig. 1D) than in lifting control cells (fig. 1C) from the culture surface. A simple EDTA-mediated detachment assay was developed to measure the adhesion of the cells as described under "Materials and Methods."

The dose dependency of the effect of retinoic acid on the adhesion of Balb/3T12-3 cells at 4 days of culture is shown in text-figure 1A. Confluent monolayers of cells were not detached as single cells but as patches, probably containing cells with varying adhesive properties. If cells were treated with diluted trypsin (as described in "Materials and Methods") prior to the adhesion test, they could be detached mostly as single cells (text-fig. 1B); thus the reproducibility of the assay increased, though the relative effect appeared decreased.

The time course of the effect of retinoic acid on cell adhesion is shown in text-figure 2A. Adhesion increased significantly at the third day of culture (48 hr after the addition of retinoic acid), and the maximum effect was attained at the sixth day of culture.

We investigated whether the increase in adhesive properties of Balb/3T12-3 cells treated with retinoic acid was reversible after removal of the retinoid. Cells were treated with retinoic acid for 5 or 7 days and with control medium thereafter. The adhesion assay was performed at the times indicated in text-figure 2B. Within 48 hours of removal of retinoic acid, the induced adhesive properties of the cells were lost.

Various retinoids were tested in the adhesion assay at concentrations of 0.1, 1, and 10 μg/ml and are rank ordered in text-figure 3. Retinol, retinoic acid, and their 5,6-epoxy derivatives were the most active compounds, showing activity at 1 μg/ml. 13-*cis*-Retinoic acid, trimethylmethoxyphenyl-retinoic acid, and dimethylacetylcyclopentenyl-retinoic acid were active at 10 μg/ml (text-fig. 3). Derivatives of retinol without in vivo growth-promoting activity, such as anhydroretinol and perhydromonoeneretinol, were inactive in inducing adhesion. These compounds and the 5,6-epoxyretinol were prepared as described in "Materials and Methods." Derivatives of retinoic acid without vitamin A activity, such as the phenyl analog of retinoic acid (text-fig. 3), were inactive in inducing adhesion. Abscisic acid, juvenile hormones I, II, and III (not shown in text-fig. 3), and β-ionone were also inactive.

Ester and amide derivatives of retinoic acid and its various derivatives were inactive in increasing adhesion (text-fig. 3).

DISCUSSION

The results show a profound effect of retinoic acid on cellular adhesion of spontaneously transformed mouse fibroblasts (Balb/3T12-3 cells). The effect is dose-dependent within the range of 0.05–5 μg retinoic acid/ml (0.17–17 μM) and can be detected as early as 2 days after culturing in the presence of 17 μM retinoic acid. Moreover, this increase in adhesion is lost within 48 hours after the cells are refed with control medium.

Retinoic acid also caused a decrease in the maximal density of Balb/3T12-3 cells. An inhibitory effect of vitamin A on cell proliferation and saturation density has been reported for L-929 cells (*17*), and an inhibitory effect on cell growth of cultured murine melanoma cells even in sparse culture has also been reported (*18*). In our studies on Balb/3T12-3 cells, plating efficiencies under mass and sparse culture conditions and trypan blue staining of retinoid-treated and control cells were the same.

The activity of retinoids in inducing adhesion of Balb/3T12-3 cells correlated very well with their in vivo growth-promoting activity and/or their activity in the in vitro differentiation assay of Sporn et al. (*5*). However, esters or amides of retinoic acid and its derivatives (text-fig. 3), such as the retinoic acid ethylamide or the ethyl ester of TMMP retinoic acid, were inactive in the adhesion assay, though they showed activity in the differentiation assay (*5*). This apparent discrepancy may be due to the lack in the fibroblastic system of secreted hydrolytic enzymes that may be necessary for the release of the active retinoid from its ester or amide derivative.

The mechanism by which retinoids increase the adhesive properties of Balb/3T12-3 cells is not known. A cell-surface glycoprotein has been suggested to be involved in cellular adhesion. This glycoprotein restores normal adhesive properties, normal morphology, and contact inhibition of movement to transformed cells (*12, 19–22*). Moreover a glycoprotein that restores contact inhibition of growth to cultured malignant melanocytes has been partially characterized (*23*).

Patt et al. (*24*) have reported recently that contact orientation and cell-density-dependent inhibition of cell growth are induced by vitamin A in cultured mouse and hamster fibroblasts. Changes in cell-surface glycoproteins and glycolipids were also observed by these authors in retinoid-treated cultures.

Inasmuch as vitamin A affects the biosynthesis of glycoproteins in a variety of tissues [*see* (*10*) for a review], further investigation is warranted to verify whether the effect of retinoids on adhesion of Balb/3T12-3 cells may result from their involvement in the biosynthesis of specific glycoconjugates of the cell surface.

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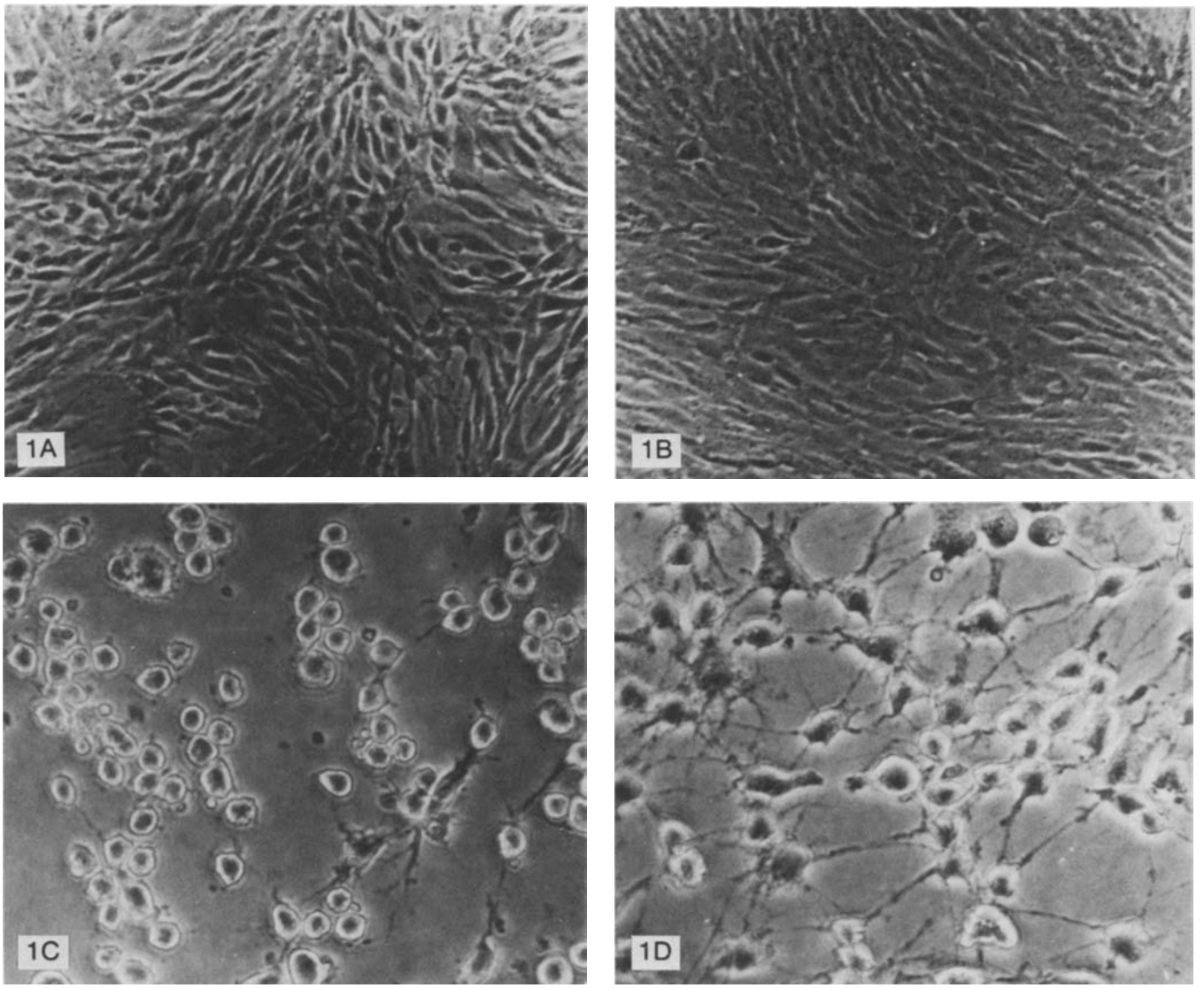

FIGURE 1.—Effect of retinoic acid on morphology of Balb/3T12-3 cells. A) Cells were seeded at 10,000/cm² and grown for 7 days as usual in the presence of 0.5% DMSO. Cells were photographed in phase contrast. × 130. B) Cultures of cells were prepared as above and cultured in the presence of 10 μg retinoic acid/ml (3.3×10^{-5} M) for the last 6 days of culture. × 130. C) Control cells (DMSO only) treated as described below. D) Cells treated with 10 μg retinoic acid/ml (3.3×10^{-5} M) 24 hr after plating and cultured for 4 days were incubated with trypsin-EDTA for 2 min at room temperature. Trypsinization was stopped by addition of calf serum to a final concentration of 10%, and the cells were photographed in phase contrast. × 130