INTERLEUKIN 6 DECREASES CELL-CELL ASSOCIATION AND INCREASES MOTILITY OF DUCTAL BREAST CARCINOMA CELLS

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Interleukin 6 is the major mediator of the early host response (the acute-phase response) to infection and injury (reviewed in reference 1). The defense mechanisms activated by IL-6 serve to contain tissue damage. The IL-6 gene can be expressed in a wide range of cell types, including fibroblasts, endothelial cells, endometrial stromal cells, keratinocytes, monocytes/macrophages, and also in a variety of tumor cells.

Whereas it is clear that IL-6 has highly significant effects on the expression of differentiated functions and on differentiation of cells of nonimmune and immune systems (2–13), its effects on cell proliferation mainly involve either stimulation or suppression of the proliferation of certain kinds of cells in which the genetic control of growth has been altered. IL-6 promotes the growth of certain murine hybridomas and plasmacytomas (14–16), and EBV-infected human B cells (17), but it inhibits the proliferation of a number of breast carcinoma cell lines and of myelomonocytic M1 cells (11, 18, 19).

Epithelial cells are connected to each other through a complex system of junctions (20–23). An important question concerns the mechanism whereby the junctions are modulated. Stoker and co-workers have reported that factors released from cells may either enhance or decrease junctional interactions between cells (24–26). A fibroblast-derived factor of M_r ~50,000 affects a variety of normal epithelial cells by increasing local motility, which results in scattering of contiguous cells (27, 28). This factor has little effect on cell proliferation and it does not affect carcinoma cells (27, 28). A 55,000 M_r factor produced by A2058 melanoma cells increases the motility of melanoma cells (29).

During the course of our studies of the inhibition of colony formation by recombinant and natural forms of human IL-6 in two lines of human breast carcinoma cells (ZR-75-1 and T-47D), we observed an IL-6–induced change in cell morphology from the typical polygonal-cuboidal shape seen in epithelial cells to a stellate or fusiform shape. This change was commonly associated with separation of cells from each other. We have defined the dynamics of these effects of IL-6, which persist for

This work was supported by National Institutes of Health research grants CA-18608 and CA-44365 and by a contract from The National Foundation for Cancer Research.

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at least 10 d in the continued presence of the cytokine, but are reversed by its removal. We show that IL-6 treatment causes increased local movement, as well as long-range locomotion of ductal carcinoma cells, which is associated with the dissolution of "adherens" (adhering) type cell junctions or structures.

Materials and Methods

Cell Lines and Culture Conditions. The T-47D and ZR-75-1 lines of human ductal breast carcinoma cells were obtained from the American Type Culture Collection, Rockville, MD. The T-47 line was established from the pleural effusion obtained from a female patient with an infiltrating ductal carcinoma of the breast (30). The differentiated epithelial subline T-47D (30) contains cytoplasmic junctions and receptors for 17β-estradiol and other steroids (31, 32). T-47D cells are responsive to but not dependent on estradiol for growth (33). The ZR-75-1 line was established from the ascitic effusion of a female patient with an infiltrating ductal carcinoma (34). The characteristic features of this cell line have remained constant irrespective of passage history and the cells closely resemble malignant cells in the original effusion (34). As described by Engel et al. (34), ZR-75-1 cells are often arranged in rosettes around duct-like lumens and are connected by occasional desmosomes. The ZR-75-1 cells possess receptors for estrogen and other steroid hormones and are dependent on estradiol for growth (33). Both the T-47D and ZR-75-1 lines were grown in RPMI 1640 medium containing 10% FCS. For T-47D cells the medium was supplemented with 0.2 U/ml insulin, and for ZR-75-1 cells, with 10^{-8} M 17β-estradiol. Stock cultures of T-47D and ZR-75-1 cells were split 1:5 once a week and refed once during the week.

IL-6 Preparations. The *Escherichia coli*-derived human IL-6 used represents a fusion protein that contains 181 amino acids of the IL-6 polypeptide fused to 34 amino acids from β-galactosidase (6). Pelleted material from *E. coli* was dissolved in buffered 8 M urea and partially purified using a MonoQ-FPLC column from which it was eluted with a stepwise NaCl gradient (6). Most of the IL-6 eluted between 100 and 150 mM NaCl. This preparation was ~50% pure ("*E. coli*-derived"). IL-6 was further purified on a preparative 4 M urea-acrylamide gel. IL-6 was eluted by crushing the appropriate gel band and leaving it overnight in 8 M urea. This preparation was >90% pure ("*E. coli*-derived, gel-purified"). Two batches of *E. coli*-derived IL-6 were used: batch 100688 was used in all experiments except that shown in Table I, in which batch 070288 was used.

The Chinese hamster ovary (CHO)[1] cell-derived preparation of recombinant human IL-6 (10, 35) was generously provided by Dr. Steven C. Clark (Genetics Institute, Andover, MA). The CHO-derived IL-6 was 90% pure and the specific activity, expressed in terms of stimulation of IgG secretion by CESS lymphoblastoid cells, was 2.5×10^6 U/mg.

Time-Lapse Cinemicrography. Cells were planted in 25-cm^2 flasks (no. 2510025; Corning Glass Works, Corning, NY) in 5.2 ml of growth medium at 1.3×10^3 cells/cm^2 (T-47D) or 5.2×10^3 cells/cm^2 (ZR-75-1). The following day the medium was replaced with growth medium containing an appropriate control solution or IL-6 in the respective solution, each diluted to the desired concentration with growth medium. The flasks were gassed with 5% CO_2 in air and capped. Time-lapse cinemicrography of control and treated cultures was carried out simultaneously using two Zeiss inverted microscopes equipped with phase contrast optics and Kodak cine cameras with EMDECO timing units. In most experiments a Ph 6.3× planar objective was used, the exposure time was 4 s, and the cultures were photographed every 6 min for 10 d. For film analysis an L-W International projector was used that permitted projection at speeds ranging from 1 to 24 frames per second as well as stop action viewing.

Serial Photomicrography. Grids of 1/4 × 1/4 inch squares were drawn on the bottoms of 25-cm^2 flasks (no. 3012; Falcon Labware, Oxnard, CA). Cells were planted in 2.6 ml of growth medium at 22 or 42 cells/cm^2 (T-47D) or 42 cells/cm^2 (ZR-75-1). The day after planting the medium was replaced with growth medium with or without IL-6 at the desired concentra-

[1] *Abbreviations used in this paper:* CHO, Chinese Hamster Ovary; IRS, immune rabbit serum; NRS, normal rabbit serum.

tion. 1 or 2 d later eight colonies in each flask were identified by their location within the grid. The number of cells per colony indicated that some had been initiated by small groups of cells not completely dispersed by trypsinization. Presence of substantial numbers of cells permitted more extensive evaluation of IL-6 effects on colony morphology. Daily serial photographs of the identified colonies were taken for up to 10 d using a M35 Zeiss inverted photomicroscope using a Ph-1 25× phase contrast objective and TRI-X 400 ASA or TMY 400 ASA film.

Antibodies and Immunofluorescence Microscopy. Mouse mAbs to vinculin were obtained from Serotec (Oxford, England). AE1 and AE3 mouse mAbs to cytokeratins were obtained from Cappel Laboratories (San Diego, CA) and were used as a 1:1 mixture. Rabbit polyclonal antibodies to desmoplakins I and II were a generous gift from Dr. W. James Nelson (Institute for Cancer Research, Philadelphia, PA). FITC-conjugated phalloidin was obtained from Molecular Probes (Eugene, OR). FITC-conjugated $F(ab')_2$ fragments of goat antibodies to mouse IgG or to rabbit IgG were obtained from Tago Inc. (Burlingame, CA).

For immunofluorescence localization of specific proteins, colonies of cells were grown on sterile glass coverslips in the presence of IL-6–containing or control medium. Cells were fixed with 4% formalin in neutral PBS and subsequently treated with 1% Triton-X100 in PBS. Primary antibodies were reacted with fixed cells for 1–2 h and subsequently with FITC-conjugated second anibodies for 1 h. Fixed cells were directly reacted with FITC-conjugated phalloidin (100 ng/ml) for 1 h. Photomicrographs were taken on a Zeiss microscope equipped with epifluorescence optics. Original magnification of all photomicrographs is ×630.

Clonogenic Assay. 200 T-47D cells or 400 ZR-75-1 cells were seeded per well in six-well plates in growth medium. The following day the medium was replaced with growth medium containing an appropriate control solution or IL-6 in the respective solution, each diluted to the desired concentration with growth medium. The plates were usually incubated for 14 d and then stained with the Giemsa stain. Colonies consisting of 10 or more cells were counted in an inverted microscope. Colony morphology was evaluated as follows: a colony was designated as epithelioid if over half of the cells in the colony had a polygonal shape, and nonepithelioid if less than half were polygonal. The polygonal T-47D cells were flatter and had clearer borders than ZR-75-1 cells. The nonpolygonal cells were angular in shape and commonly possessed processes of variable length; such cells showed variable degrees of separation from each other.

DNA Synthesis. ZR-75-1 cells were seeded at a density of 5×10^4 cells/cm^2 in growth medium, 100 μl/well, in 96-well plates. 3 d later the medium was replaced with IL-6-containing or control medium. After 20–24-h incubation, 10 μl of [^{3}H]thymidine (6.7 Ci/mmol) was added to a final concentration of 10 μCi/ml for 2 h at 37°C. Each well then received 30 μl of 1 M citric acid for 10 min at room temperature. After two washes with cold PBS, 10% TCA was added for 15 min at 4°C, and the cultures were then washed twice with 5% TCA. Finally, the cells were lysed in 75 μl of 1% SDS, 1 mM EDTA, 0.1 N NaOH at 60°C for 1 h. The samples were then counted in a scintillation counter with Ready Safe liquid scintillation cocktail (Beckman Instruments, Fullerton, CA).

α_1-Antichymotrypsin Synthesis. Hep3B2 cells, obtained from the American Type Culture Collection, were used to assay the ability of IL-6 preparations to stimulate α_1-antichymotrypsin synthesis (6, 36). [^{35}S]Methionine labeling of α_1-antichymotrypsin was quantitated by immunoprecipitation, SDS-PAGE, autoradiography, and laser densitometry, and is expressed in arbitrary absorbance units.

Results

Cell Morphology. We have investigated the effects of IL-6 on two cell lines derived from metastic ductal breast carcinomas, T-47D, with a more differentiated morphology, and ZR-75-1, with a less differentiated morphology. The three left panels in Fig. 1 illustrate the morphology of colonies of the T-47D line of human breast carcinoma cells. The great majority of the T-47D cell colonies consist predominantly of contiguous flat polygonal cells with typical epithelial appearance, as illustrated by the top

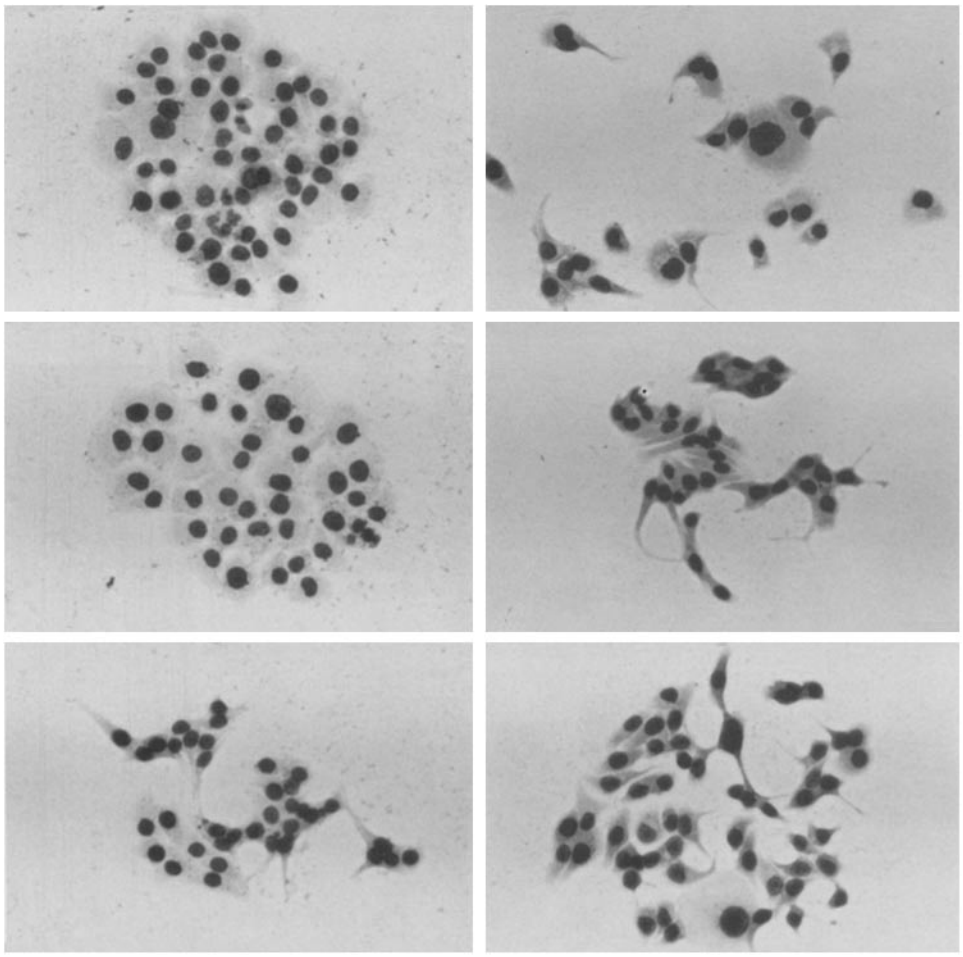

FIGURE 1. Altered shape of IL-6–treated T-47D ductal breast carcinoma cells. T-47D cells were planted at a density of 42 cells/cm^2 and incubated for 11 d in the absence (*left*) or presence (*right*) of *E. coli*–derived gel-purified rIL-6 (150 ng/ml). (*Left half*) The upper two colonies are representative of the majority of the control T-47D cell colonies composed of polygonal cells, and the third illustrates the occurrence of cells of angular shape. (*Right half*) Three colonies illustrate the spectrum of IL-6–induced shape change from polygonal to angular and the partial or complete separation of cells from each other. Giemsa-stained colonies were photographed on 35-mm film at a magnification of ×64 using a ×25 objective. Final magnification, ×111.

two colonies on the left. Some colonies are mixtures of polygonal cells and stellate or fusiform cells with elongated processes (lowermost colony on the left). Angular shape is associated with the separation of cells from neighbors. A small fraction of the T-47D cell colonies are characterized by very dense packing of cells (not shown).

IL-6 causes a change in cell shape from the polygonal to the stellate or fusiform with an associated development of spaces between cells. The three right-hand panels in Fig. 1 illustrate different stages in this process. The cells that are still connected to each other are seen as patches or as linear arrays of elongated cells. In addition,

some IL-6-treated colonies show increased variation in nuclear size and contour. The time and concentration dependence of the IL-6-induced changes in cell shape and colony organization are documented below.

Fig. 2 (three left panels) illustrates the morphology of the ZR-75-1 line of human breast carcinoma cells. The colonies of control ZR-75-1 cells are densely packed with polygonal or cuboidal cells that often form what appear to be multi-layered, elon-

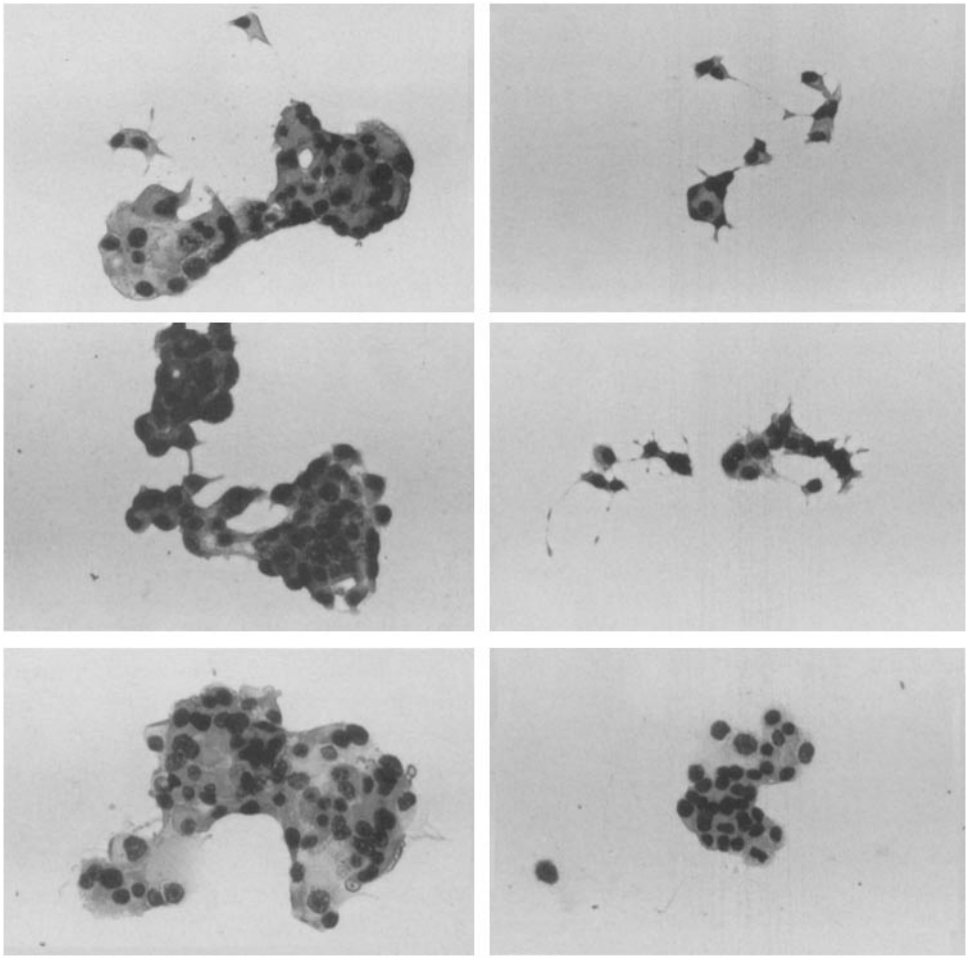

FIGURE 2. Altered shape of IL-6-treated ZR-75-1 ductal breast carcinoma cells. ZR-75-1 cells were planted at a density of 42 cells/cm^2 and incubated for 14 d in the absence (*left*) or presence (*right*) of *E. coli*-derived partially purified rIL-6 (150 ng/ml). (*Left half*) Three colonies demonstrate the typically very compact arrays of tightly packed control ZR-75-1 cells with only occasional cells or groups of cells located at a distance from the body of the colony, but often linked to the main part of the colony via intercellular bridges. (*Right half*) The upper two colonies are representative of the common form found in IL-6-treated cultures; the angular cells in these colonies often have little cytoplasm and are separated from each other except for the cytoplasmic processes which often connect the separated cells. The lowermost colony on the right is representative of a minority of colonies in the IL-6-treated cultures; their number is inversely related to the concentration of IL-6. Giemsa-stained colonies were photographed on 35-mm film at a magnification of ×64 using a ×25 objective. Final magnification, ×111.

gated, and convoluted aggregates. Parts of some colonies show evidence of separation from each other (center left panel), and in some cases single cells or small groups of cells, are present in the immediate vicinity of a colony (upper left panel).

In IL-6–treated ZR-75-1 cultures, the typical cells are highly angular and possess long processes that often connect noncontiguous cells or extend into areas without cells (Fig. 2, two upper right panels). Occasionally a compact colony is seen (Fig. 2, lowermost panel on right); the frequency of such colonies is inversely related to IL-6 concentration (see below).

Cell Motility by Time-Lapse Cinemicrography. Based on previous observations on colony-forming epithelial cells exfoliated in human milk (25), we will refer to cells that grow in contiguous cell sheets as junction forming and to those that grow into colonies of noncontiguous cells as deficient in junction formation.

Time-lapse cinemicrography of control T-47D cells (three experiments) shows that the majority of cells are junction forming, move little, and grow into colonies of flat polygonal cells as illustrated in Fig. 1 (two upper panels on the left). However, even within such colonies of contiguous cells forming epithelial sheets, individual cells may temporarily separate along their borders from neighbors in a series of undulating to- and fro- movements while remaining attached to the substrate and to other cells at the poles. Such spindle-shaped or angular cells rarely leave their place within the colony and soon become laterally reattached to neighbors and reassume flat epithelioid morphology.

A small fraction of cells in control cultures of T-47D cells consist of cells deficient in junction formation. Such cells move apart, and give rise to colonies of scattered stellate or fusiform cells among which some cells may be laterally attached to each other as illustrated in Fig. 1 (left lower panel). On rare occasions a junction deficient control cell moves a considerable distance in the vicinity of the parent colony, but long distance migration away from a colony was not seen.

IL-6 (*E. coli*–derived, 150 ng/ml; two time-lapse experiments) causes a marked increase in the proportion of T-47D cells deficient in junction formation, which scatter or may be seen in various states of partial separation from neighbors, as is illustrated in Fig. 1 on the right. After several days of treatment many cells are spatially separate. Some IL-6–treated T-47D cells engage in locomotion over considerable distances. At low magnification these cells appear round in shape.

Time-lapse cinemicrography of control ZR-75-1 cells (two experiments) revealed some local cell movement even within the very compact multilayered colonies. IL-6 treatment (*E. coli*–derived, 150 ng/ml; two experiments) led to the contraction and scattering of cells in most colonies.

To summarize, time lapse cinemicrography shows that IL-6 causes a marked increase in local movement of T-47D and ZR-75-1 cells with a small fraction of cells showing locomotion over distances many times greater than the cell diameter.

Cell Shape and Motility by Serial Photomicrography. Higher resolution serial phase contrast photomicrographs of individual colonies were taken to illustrate the changes seen by time-lapse cinemicrography. Fig. 3 shows photographs of two control colonies of ZR-75-1 cells (*A* and *B*), taken 3 and 5 d after medium change (i.e., 4 and 6 d after planting), which illustrate changes in the overall shape of the colonies and the partial separation of a few cells from the main body of the colony (*A*). The splitting of an entire colony into two parts was observed in another control colony (not

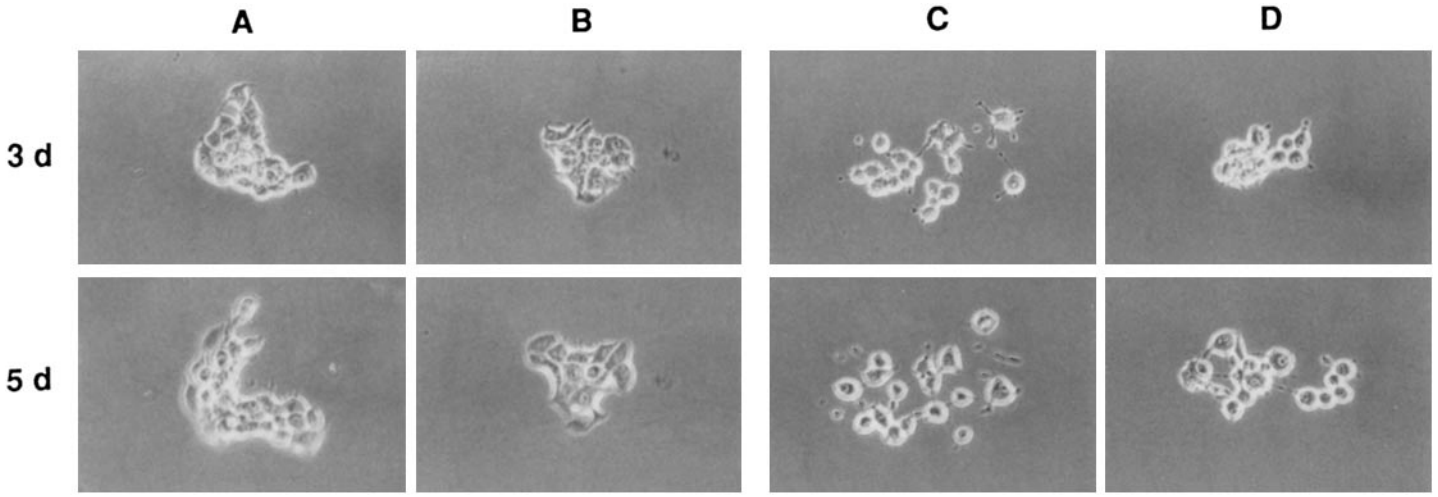

FIGURE 3. Time- and IL-6-dependent changes in ZR-75-1 cell colony organization. ZR-75-1 cells were planted at a density of 42 cells/cm^2 in 25-cm^2 flasks, and the medium was changed the following day. Photomicrographs taken 3 d and 5 d after medium change are shown. Final magnification, ×77. (*Left half*) Two control colonies (*A*, *B*); (*right half*) two IL-6-treated (CHO, 15 ng/ml) colonies (*C*, *D*).

shown). However, overall the compact epithelial character of control colonies was maintained over a prolonged period. The control cells showed few surface projections.

Treatment with IL-6 (CHO cell-derived, 15 ng/ml), begun 1 d after planting, caused a considerable change in cell shape and variable degrees of cell scattering by the third day (80 h) after the beginning of treatment (Fig. 3, colonies C and D). Photomicrographs taken on the fifth day (115 h) after addition of IL-6 show progressive separation of cells from each other. Many of the treated cells possess surface projections, at least some of which form connections between neighboring cells that had moved apart.

A second experiment showed that 2 d of treatment of ZR-75-1 cells with IL-6 (15 ng/ml) had not yet caused major changes in colony morphology, whereas by the third day most colonies displayed changes in cell shape and cell scattering.

Reversal of the Effects on Cell Shape, Motility, and Proliferation Upon Withdrawal of IL-6. To determine whether IL-6-induced changes were dependent on the continued exposure to IL-6, we performed the following experiment. T-47D cells were planted at low density and incubated in growth medium for 6 d to permit colonies to form. The cultures were then incubated for 7 d in fresh control medium or in the presence of IL-6 at 15 or 150 ng/ml, after which the medium in all cultures was replaced with fresh growth medium and incubation continued. Eight colonies in each of the three cultures were photographed serially, and the following representative samples are presented: −1 d: photographs taken one day before the final medium change, i.e., after 6 d of treatment; 3 d: taken 3 d after termination of treatment; 5 d: taken 5 d after termination of treatment.

In Fig. 4 control colony A is typical of most T-47D cell colonies (compare also with two top colonies on the left in Fig. 1). Control colony B illustrates cells that, based on time-lapse cinemicrographic observations, are undergoing local movements associated with separation and rejoining of neighboring cells. As a result, the colony has a looser structure.

Treatment of T-47D cell colonies for 6 d (cf., −1 d in Fig. 4) with IL-6 at 15 ng/ml (*C* and *D*) or 150 ng/ml (*E* and *F*) causes dose-dependent changes in cell shape and distribution. At 150 ng/ml, IL-6-induced separation of cells from each other became evident in most colonies over a period of 2–3 d, as revealed in photographs taken daily (not shown), and the cells assumed elongated and often curved shapes with polar processes. By the sixth day of exposure to IL-6 at 150 ng/ml, these changes were marked (cf., Fig. 4, *E* and *F*). Similar changes developed more slowly and were not as marked in cells exposed to IL-6 at 15 ng/ml (Fig. 4, *C* and *D*).

3 d after removal of IL-6, there is clear evidence of a reversal of the IL-6-induced changes as the cells are flatter and as neighboring cells have become associated into patches of cells (Fig. 4, 3 d, *C–F*). By the fifth day after removal of IL-6, reversal of the IL-6-induced changes has progressed further (Fig. 4, 5 d, *C–F*). It should be emphasized that in cultures of T-47D or ZR-75-1 cells continuously treated with IL-6 and observed by time-lapse cinemicrography for 10 d there was no evidence of reversion of IL-6-induced changes in cell shape or inter-cellular association.

The increase in cell number with time is readily apparent in T-47D cell colonies as the cells are flatter than and not as packed together as ZR-75-1 cells. Fig. 5 shows that T-47D cells proliferate with a doubling time of ~3 d and that IL-6 inhibits the proliferation of T-47D cells in colonies in a concentration-dependent manner. Within

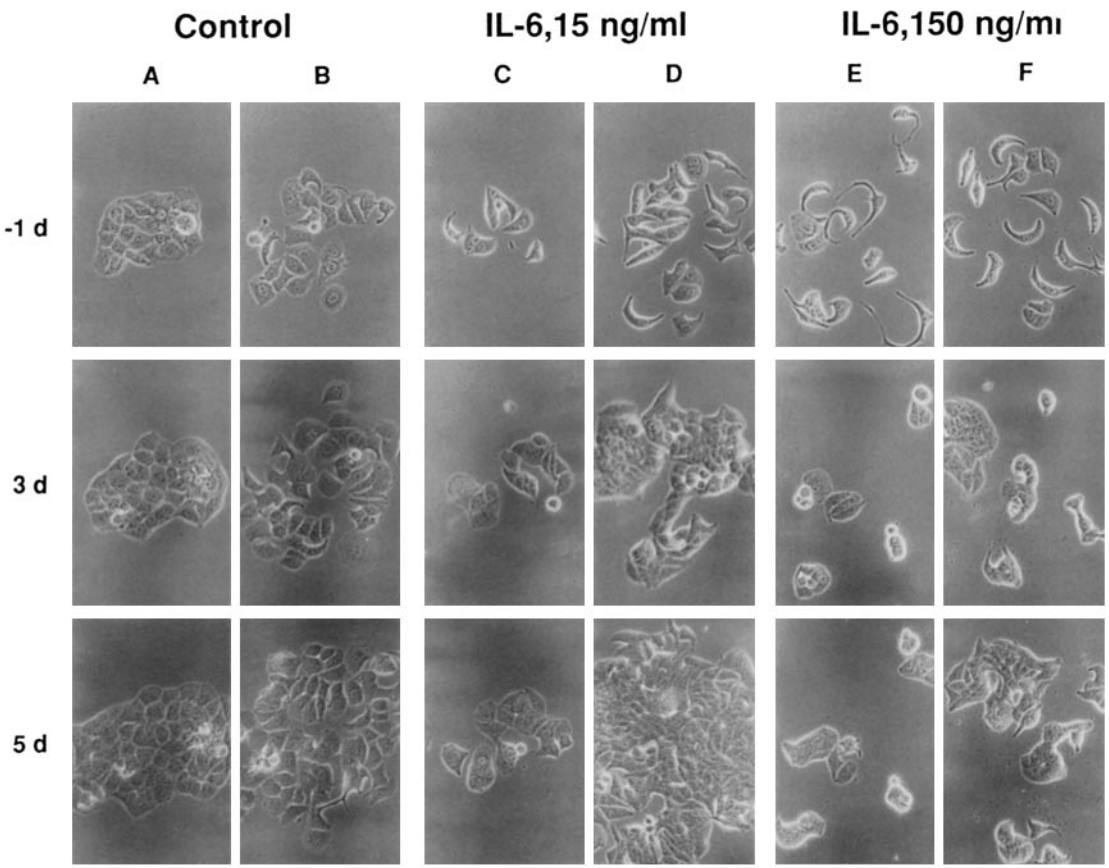

FIGURE 4. Reversal of the IL-6–induced morphological changes in T-47D cells upon withdrawal of IL-6. T-47D cells were planted at a density of 22 cells/cm^2 in 25-cm^2 flasks and the cultures incubated for 6 d. The medium was then replaced with control medium or with medium containing CHO cell–derived IL-6 and incubation continued for another 7 d at which time all media were replaced with control medium and incubation carried on further. As indicated in the left margin of the figure, photographs taken 1 d before and 3 and 5 d after the removal of IL-6 are shown. Final magnification, ×62. (*Left*) Two control colonies (*A*, *B*); (*center*) IL-6, 15 ng/ml (*C*, *D*); (*right*) IL-6, 150 ng/ml (*E*, *F*).

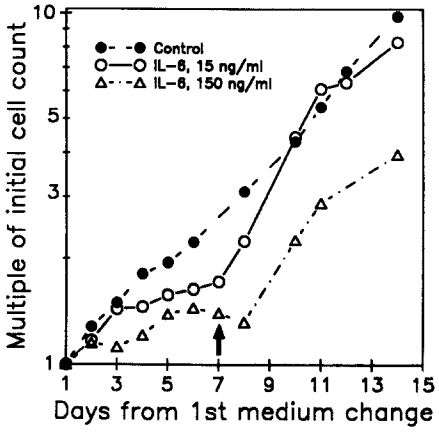

FIGURE 5. Inhibition of T-47D cell proliferation by IL-6 and reversal of the inhibition upon withdrawal of IL-6. Serial photographs taken in the experiment described in Fig. 4 were analyzed for increases in cell number in five colonies per variable, chosen because accurate cell counts could be performed. 1 d after the first medium change, at which time the first set of photographs was taken, the mean numbers of cells per colony were as follows: control: 13; IL-6, 15 ng/ml: 11; IL-6, 150 ng/ml: 12. The cell numbers on subsequent days were expressed as multiples of the initial cell counts and the geometric means of such multiples were calculated for each group of five colonies and plotted. In the instances where a colony no longer fitted within the field of the ×25 objective, it was rephotographed using a ×16 or ×10 objective for cell counting purposes. The arrow indicates time of second medium change, i.e., when IL-6 was removed from the treated cultures.

24 h from the removal of IL-6, 15 ng/ml, the rate of proliferation increased markedly and for a period exceeded that of control cells. After removal of IL-6, 150 ng/ml, there is a 24-h delay, after which proliferation proceeds at a rate comparable to that observed in control cells.

The compactness of control ZR-75-1 cell colonies precludes accurate counting of cells within the colonies; however, as reported below, IL-6 inhibits [^{3}H]thymidine incorporation in subconfluent cultures of ZR-75-1 cells. In a time-lapse cinemicrographic experiment, in which ZR-75-1 cells were incubated with CHO cell-derived IL-6 (15 ng/ml) for 10 d and then for 5 d in the absence of the cytokine, the following was observed: after removal of IL-6 a number of previously motile single cells became stationary, flattened out, and proceeded to divide; with time, large colonies of adhering cells formed.

Effects of IL-6 on Focal Adhesions and Desmosomes. Cellular adhesion to culture surfaces is mediated through focal adhesions (adhesion plaques) and close contacts in many cell types. In epithelial cell types, focal adhesions and adherens-type intercellular junctions are membrane insertion points for actin-containing filaments. Antivinculin mAbs were used to visualize focal adhesions in T-47D cells grown in control medium (Fig. 6, *A* and *B*). Prominent focal adhesions are visualized throughout the central cell surface as well as at the peripheral edge of boundary cells in a colony (Fig. 6 *B*). In contrast, T-47D cells grown in IL-6 (*E. coli*-derived, gel-purified, 150 ng/ml) for 8 d show either absence or marked reduction in the number of vinculin-containing focal adhesions (Fig. 6, *C* and *D*). Whereas colonies of T-47D cells grown in control medium show prominent F-actin stress fibers, such microfilament bundles are greatly diminished in T-47D cells grown in the presence of IL-6 (data not shown).

Because fewer intercellular bridges were seen in many IL-6-treated cells, the effects of IL-6 on intercellular desmosome formation were investigated by indirect immunofluorescence microscopy with antibodies directed against desmoplakins I/II. T-47D cells grown in control medium showed frequent intercellular desmosomal attachments as indicated by the punctate intercellular fluorescence in Fig. 7, *A* and *B*. T-47D cells grown in the presence of IL-6 (*E. coli*-derived, gel-purified, 150 ng/ml)

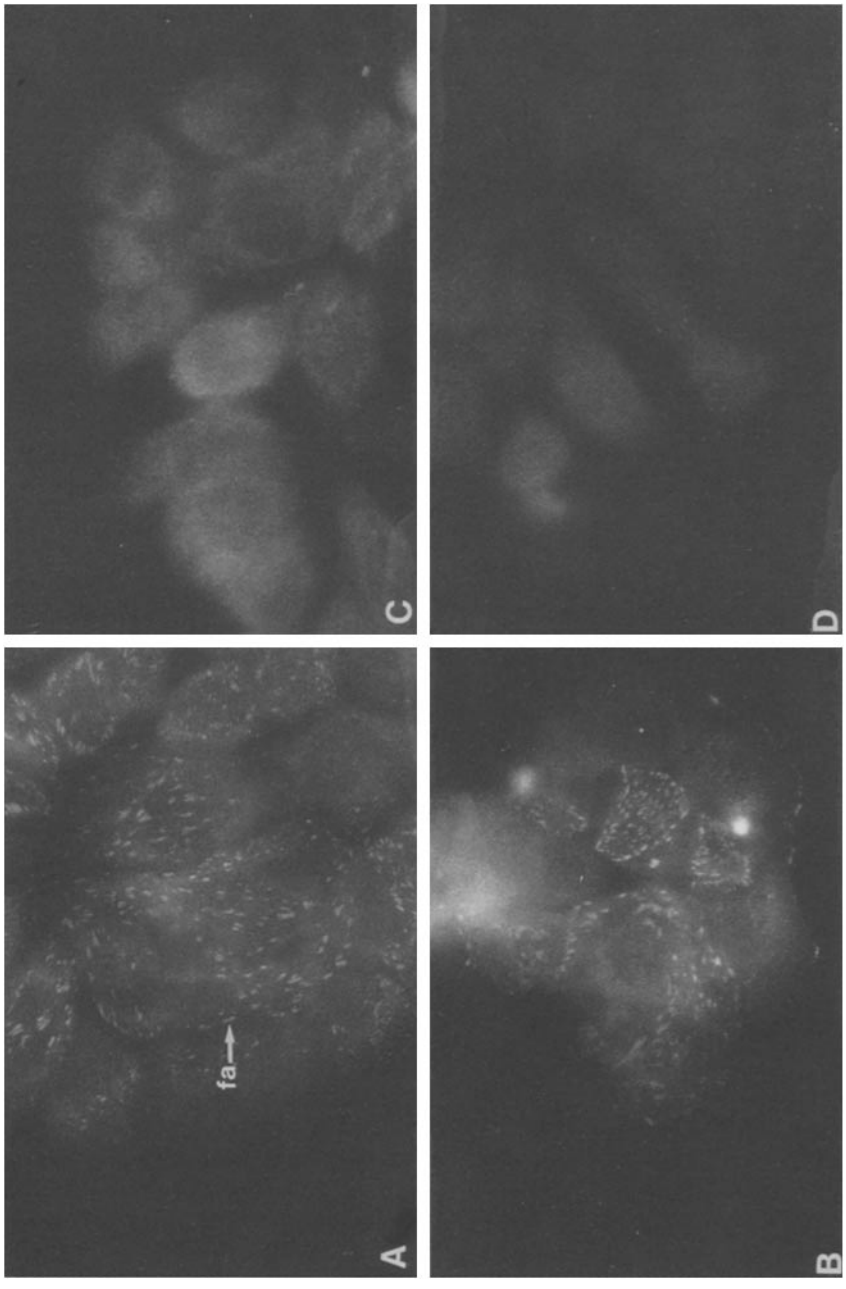

FIGURE 6. Focal adhesions in T-47D cells visualized with antivinculin mAbs in control cultures (*A*, *B*) or cells cultured in IL-6–containing medium (*C*, *D*). "fa" in A points to focal adhesion in the ventral cell surface. Final magnification, ×1466.

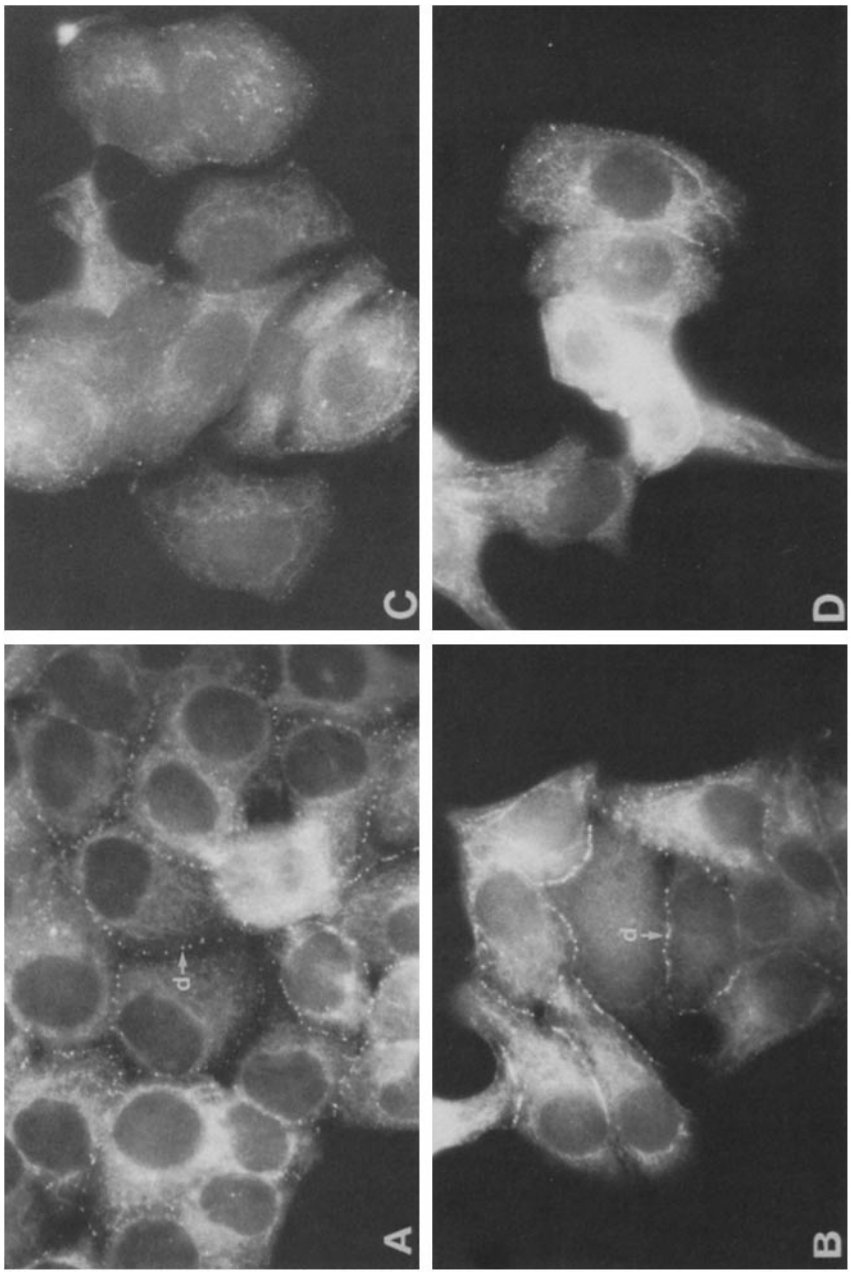

FIGURE 7. **Intercellular desmosomes in T-47D cells stained with polyclonal anti-desmoplakin I/II antibodies. Control cultures (*A*, *B*) or cells cultured in IL-6-containing medium (*C*, *D*). (*A* and *B*) "d" indicates punctate intercellular desmosomal attachment. Final magnification, ×1466.**

show a marked reduction in the number of desmosomal attachments (Figure 7, *C* and *D*). Colonies of IL-6-treated T-47D cells also show perinuclear retraction of cytokeratin filaments and greatly diminished intercellular keratin filament connections, corresponding to the decrease in desmosomal attachments (data not shown).

DNA Synthesis. We have determined the dose-response relationship for the inhibitory effect of CHO cell-derived IL-6 on DNA synthesis in ZR-75-1 cells and related it to that for its stimulatory effect on the synthesis of an acute-phase protein, α_1-antichymotrypsin, in the hepatoma line Hep3B2. Fig. 8 shows that inhibition of DNA synthesis by IL-6 is detectable at 0.15 ng/ml and is maximal at 15 ng/ml. Anti-IL-6 immune rabbit serum (1%) completely neutralized the inhibition of DNA synthesis when IL-6 was used at 15 ng/ml; when the concentration was increased to 150 ng/ml, the neutralization was partial (data not shown). Fig. 8 also shows that the major IL-6-induced increase in α_1-antichymotrypsin synthesis occurs when the concentration is raised from 1.5 to 15 ng/ml. In terms of the stimulatory activity of CHO cell-derived IL-6 on IgG secretion by CESS lymphoblastoid cells, the range from 0.15 to 1.5 ng/ml corresponds to 0.36-36 U/ml (see Materials and Methods). Thus, CHO IL-6 shows three widely different biological activities within a comparable concentration range in three different systems.

In two of the DNA synthesis experiments shown in Fig. 8 (● and ▼) we also examined the relationship between concentration of CHO cell-derived IL-6 and decreases in the number of ZR-75-1 cell colonies and in the fraction of predominantly epithelioid colonies. Decreases in both parameters were detected at 0.5 ng/ml and they were maximal at 15 ng/ml (data not shown).

Table I illustrates the inhibitory effect of *E. coli*-derived IL-6 on DNA synthesis in T-47D cells and the complete blocking of the inhibition by anti-IL-6 immune serum.

Comparison of IL-6 Effects in T-47D and ZR-75-1 Cells with Respect to Colony Number and Morphology. *E. coli*-derived IL-6 was used to quantitate the effects of IL-6 on T-47D and ZR-75-1 cell colony number and morphology in the same experiment.

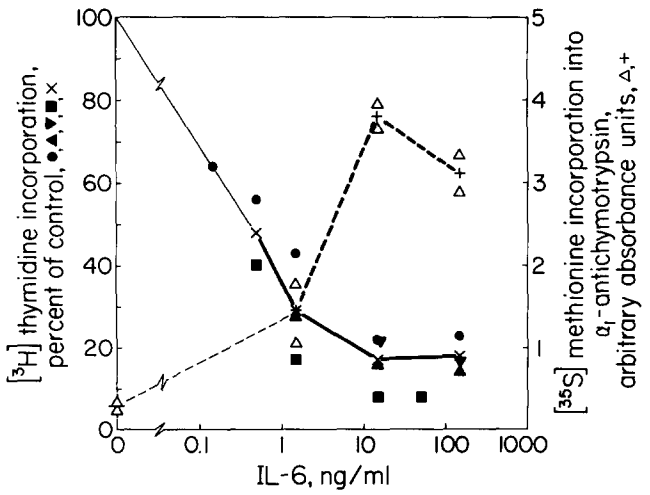

FIGURE 8. Relationship between the concentration of IL-6 and inhibition of DNA synthesis in ZR-75-1 cells and stimulation of α_1-antichymotrypsin synthesis in Hep3B2 cells. CHO cell-derived IL-6 was used and the assays were performed as described in Materials and Methods. Results of four separate experiments (●, ▲, ▼, ■) on DNA synthesis and one experiment (Δ) on α_1-antichymotrypsin synthesis, assayed twice, are shown. One of the DNA synthesis experiments (▲) and the α_1-antichymotrypsin synthesis experiment (Δ) were done at the same time. The control cpm values for [^{3}H]thymidine incorporation were as follows: (●) 11,539, (▲) 9,270, (▼) 13,281, and (■) 8,730. (X) Means of [^{3}H]thymidine incorporation in different experiments; (+) means of duplicate assays of [^{35}S]methionine incorporation.

TABLE I

Inhibition of DNA Synthesis by E. coli-derived IL-6 in T-47 D Cells and Neutralization of the Effect by Anti-IL-6 Immune Serum

	[^{3}H]Thymidine incorporation with the following sera:		
IL-6	None	Normal	Anti-IL-6*
ng/ml		*cpm*	
None	31,932	36,704	32,950
		Percent of control	
1.5	72	69	103
15	54	57	105
150	36	37	106

* Immune rabbit serum was prepared using *E. coli*-derived human recombinant IL-6 (6). It was used at a dilution of 1:100.

The latter was evaluated on the basis of the fraction of the colonies consisting of predominantly epithelioid cells. Table II shows that ZR-75-1 cells are more sensitive to the effects of IL-6 than the better differentiated T-47D cells both in terms of the inhibition of colony formation and the decrease in the fraction of predominantly epithelioid colonies. Both effects of *E. coli*-derived IL-6 were completely blocked by anti-IL-6 antiserum in both cell lines. Table II also shows that the CHO cell recombinant IL-6 preparation had a higher specific activity than the *E. coli* recombinant IL-6. This difference was also found in [^{3}H]thymidine incorporation experiments:

TABLE II

Effects of E. coli- and CHO Cell-derived IL-6 on Colony Formation by T-47D and ZR-75 Cells

		Number of colonies (percent of control)		Fraction of epithelioid colonies (percent of control)	
Cell line	Reagents	15 ng/ml	150 ng/ml	15 ng/ml	150 ng/ml
T-47D*	*E. coli* IL-6	90	53	82	72
ZR-75-1‡	*E. coli* IL-6	65	30	89	31
T-47D	*E. coli* IL-6 + IRS, 1:100§	112	101	98	101
ZR-75-1	*E. coli* IL-6 + IRS, 1:100	116	102	98	98
T-47D	CHO cell IL-6	-	30	-	58

* Controls for *E. coli*-derived rIL-6 were incubated in the presence of 0.8 or 8 mM urea in growth medium, resulting in 68 and 76 colonies of T-47D cells per well, respectively, with a mean of 72 and a plating efficiency of 36%. Of the control colonies 98 and 97%, respectively, were epithelioid.

Controls for CHO cell-derived recombinant IL-6 were incubated in the presence of 0.2 mM Tris/0.2 mM glycine in growth medium, which resulted in 56 colonies of T-47D cells per well and a plating efficiency of 28%. Of the control colonies 100% were epithelioid.

‡ Controls for *E. coli*-derived rIL-6 were incubated in the presence of 0.8 or 8 mM urea in growth medium, resulting in 48 and 50 colonies of ZR-75-1 per well, respectively, with a mean of 49 and a plating efficiency of 12%. Of the control colonies 98 and 99%, respectively, were epithelioid.

§ Immune rabbit serum was prepared using *E. coli*-derived rIL-6 (6).

as shown in Fig. 8 (▲), in ZR-75-1 cells the percent of control values for CHO cell-derived IL-6 at 1.5, 15, and 150 ng/ml were 28, 16, and 14%, respectively; in the same experiment the corresponding values for *E. coli* IL-6 were 50, 56, and 10%, and for *E. coli* gel-purified IL-6 they were 67, 49, and 19%. *E. coli* IL-6 is prepared and stored in 8 M urea, and thus may not always fully renature when diluted into tissue culture medium.

Discussion

We have demonstrated that ductal breast carcinoma cells treated with IL-6 undergo striking morphological changes, separate from each other, and migrate apart. The effects of IL-6 on cell shape and cell-cell association are reversible upon withdrawal of the cytokine from the medium. Although the morphological characteristics of untreated colonies of ZR-75-1 and T-47D cells are strikingly different, with the flatter T-47D cells often forming round colonies and the ZR-75-1 cells forming tightly packed colonies suggestive of duct walls, IL-6 causes similar effects in the two lines. In IL-6-treated T-47D cells, vinculin-containing focal adhesions as well as intercellular desmosomal attachments are decreased.

IL-6 not only causes a marked change in colony morphology, which in T-47D colonies has been independently observed in another laboratory (37), but it decreases both colony formation and cell proliferation. All of these effects can be blocked with anti-IL-6 antiserum. ZR-75-1 cells are more sensitive to IL-6 effects than T-47D cells.

There is a sharp distinction between the effects of IL-6 and those of IFN-β. IL-6 inhibits breast carcinoma cell proliferation, but increases cell motility, whereas IFN-β inhibits both cell proliferation and motile functions in human fibroblasts (38, 39) and cervical carcinoma cells (38, 40). Furthermore, treatment with IL-6 causes a decrease in microfilament bundles, whereas IFN-β increases the abundance of these structures in fibroblasts (39) and of the submembrane microfilaments in cervical carcinoma cells growing in suspension (41). IFN-β (500-2,400 U/ml) caused only slight inhibition of [^{3}H]thymidine incorporation in T-47D cells and at 500 U/ml had no significant effect on [^{3}H]thymidine incorporation in T-47D cells and at 500 U/ml had no significant effect on colony formation (42; unpublished results).

Epithelial cell junctions not only fulfill the requirements for an effective permeability barrier and for communication between cells, but together with intracellular cytoskeletal components they contribute to the organization and architecture of cells in tissues. The "adherens" (adhering)-type junctions (20) comprise two types of plaque structure with different associated filaments and play an important role in the establishment of an architectural framework (22, 23). One junction-filament complex provides anchorage structures for actin-containing microfilaments and is characterized by a plaque in the form of a loosely woven mat that contains microfilaments, α-actinin, and vinculin (21, 43). This type of junction can be belt-like, streak-like, or small-plate-like. The other complex provides anchorage for intermediate filaments of diverse types and contains a rather rigid plaque, the desmosome, composed largely of an exclusive set of proteins, among which desmoplakin I and II are prominent (22, 23, 44).

Our results show that IL-6 treatment of ductal breast carcinoma cells decreases both types of adherens junctions or structures in these cells, and these changes correlate with altered morphology and increased motility of IL-6-treated cells.

Time-lapse cinemicrography reveals that contiguous ductal carcinoma cells within a colony in a culture that has not been treated with IL-6 can undergo separation and rejoining and that this activity can be marked in different parts of a colony at different times. The question arises whether IL-6 or a substance with IL-6-like activity is made and released in relatively small amounts by groups of cells in a colony, and is responsible for the transient shape change and increased local movement of groups of cells within a colony. Constitutive expression of the IL-6 gene has been demonstrated in a number of tumor cell lines, including T24 renal carcinoma and cardiac myxoma (2, 45). Strong IL-6 immunostaining has been observed in T-47D cells in culture (46). Depending on variations in the rate of production by groups of cells, there could be local concentration differences in IL-6 or another motility-promoting cytokine, which would account for the heterogeneity in motile activity of cells within a sheet. The transient nature of the increases in the motility of groups of cells could reflect fall in local concentration of the cytokine due to diffusion or other factors followed by reversal of the state of increased motility and reestablishment of junctions between cells. In one preliminary time-lapse cinemicrographic experiment we did not detect a major difference in the basal motile activity of ZR-75-1 cells incubated with anti-IL-6 immune serum. A small difference in basal activity would be difficult to establish by this technique. As T-47D cells lend themselves better to observations of morphologic and kinetic changes than ZR-75-1 cells, it will be of interest to explore the question further with T-47D cells. Quantitation of cell migration on a population basis using Boyden chambers (29, 47, 48) offers an alternative approach.

The essential finding is that cells within an epithelial sheet are in a dynamic state that can be influenced by cytokines. Epithelial cells engage in locomotion during embryogenesis, normal differentiation in postembryonic tissues, wound healing, and in some neoplastic tissues. Locomotion and proliferation of epithelial cells can occur separately, as exemplified during wound healing, when migration precedes the proliferative response of cells and migratory and proliferative cellular compartments are spatially separate (reviewed in reference 49). Little is known about the existence or function of motility-promoting cytokines in tissue morphogenesis or neoplasia. At the cellular level, the stage-wise process of formation of desmosomes in response to cell-to-cell contact, involving recruitment of desmoplakins I and II from a soluble to an insoluble pool and redistribution to the plasma membrane in areas of cell–cell contact has recently been described (50, 51). It remains to be determined whether IL-6 inhibits the synthesis of desmoplakins and vinculin or their associative interactions and distribution in cells. The recent reports that IL-6 decreases fibronectin synthesis and increases collagenase production in different cells (52–54) raise the possibility that IL-6 may also have significant effects on the extracellular matrix and basement membrane integrity.

Overall, the dose-response relationships for the suppressive effects of IL-6 on DNA synthesis, colony number, and the proportion of epithelioid colonies, are similar. Inhibition of [^{3}H]thymidine incorporation by IL-6 is evident in subconfluent cultures after only 20–24-h treatment (18; and present results), whereas changes in cell and colony morphology become detectable after treatment for 2–3 d. The apparent relationship between inhibition of growth and loss of the epithelioid character and increased cell motility is of great interest and merits further study as it defines a

distinctive behavorial phenotype on the part of transformed breast ductal epithelial cells. TNF-α causes 50% inhibition of [^{3}H]thymidine incorporation in T-47D cells at a concentration of 16 ng/ml, and its effect can be blocked by anti-TNF immune serum, but not by antibodies against IL-6 or IFN-β (unpublished results). It will be of interest to determine whether TNF-α does or does not cause cell scattering, and similarly, whether methotrexate, an inhibitor of DNA synthesis, has any effects on cell–cell association and motility.

Previous investigations in other laboratories have led to the tentative identification of a 50,000 M_r substance as the scatter factor for Madin-Darby canine kidney (MDCK) and other epithelial cells, which is produced by embryonic fibroblasts (28), and a 55,000 M_r substance as an autocrine motility factor for melanoma cells (29). These factors have properties characteristic of proteins. Stoker's scatter factor lacks activity in breast carcinoma cells and we have so far not detected scatter factor activity with IL-6 in MDCK cells (unpublished results). CHO cell–derived IL-6 (15–150 ng/ml) also had no apparent effect on cell–cell association and morphology of normal human keratinocytes, which, in the presence of IL-6, formed large colonies indistinguishable from control colonies (unpublished results). IL-6 is the first fully identified and extensively characterized molecule capable of increasing local motility of breast cancer cells, and should prove useful in the analysis of the detailed mechanism whereby motility of tumor cells can be increased by cytokines.

Cytological examination of tissue aspirates has shown that benign cells are characterized by excellent adhesiveness (except in lymph nodes, spleen, and bone marrow) and cancer cells by poor adhesiveness (55). Aggregates of ductal breast carcinoma cells in aspirates are made up of loosely arranged cells, with partly or fully detached cells at the periphery of the clusters (55). As revealed by time-lapse cinemicrography of breast cancer cells in culture, cell–cell association represents a dynamic state with cells in a colony separating from each other and often, but not always, rejoining. Alterations in intercellular junctions have been observed in a variety of cancers, but no conclusive evidence has been obtained linking such defects with invasion or metastasis (56). Invasion may require changes in intercellular adhesion, but these may be both focal and transient (57). A conventional microscopic examination of junctions in tumor samples does not provide information on dynamic aspects of junction formation or dissolution in tumor cells, in particular those neoplastic cells that escape from the primary tumor. A variety of approaches, including intensive study of enzymes acting on the extracellular matrix proteins, will be important in the evaluation of cell–cell interactions and cell motility in studies of invasiveness and metastasis. Such approaches will clearly need to take account of motility-promoting cytokines that may be produced by tumor cells themselves, by mesenchymal cells, or by other cells.

Summary

Treatment of transformed breast duct epithelial cells with IL-6 produces a unique cellular phenotype characterized by diminished proliferation and increased motility. Human ductal carcinoma cells (T-47D and ZR-75-1 lines) are typically epithelioid in shape and form compact colonies in culture. Time-lapse cinemicrography shows that some untreated cells can transiently become fusiform or stellate in shape and separate from each other within a colony, but they usually rejoin their neighbors.

While IL-6 suppresses the proliferation of these carcinoma cells, the IL-6–treated cells generally become stellate or fusiform and show increased motility. These changes persist as long as the cells are exposed to IL-6. This results in the dispersal of cells within colonies. The effects on cell growth, shape, and motility are reversible upon removal of IL-6. IL-6–treated T-47D cells display diminished adherens-type cell junctions, as indicated by markedly decreased vinculin-containing adhesions and intercellular desmosomal attachments. The effects on ZR-75-1 cell shape, colony number, and DNA synthesis are dependent on IL-6 concentration in the range from 0.15 to 15 ng/ml. Higher concentrations are required in T-47D cells for equivalent effects. Anti-IL-6 immune serum blocks IL-6 action. IL-6 represents a well-characterized molecule that regulates both the proliferation and junction-forming ability of breast ductal carcinoma cells.

We thank Dr. Steven C. Clark for generously providing CHO cell–derived human recombinant IL-6 and Ms. Celia K. Graham for expert preparation of manuscript copy.

Received for publication 8 June 1989 and in revised form 31 July 1989.

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