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Decreased adherence of interleukin 6-treated breast carcinoma cells can lead to separation from neighbors after mitosis

(ZR-75-1B cells/DNA synthesis/cell division/phorbol 12-myristate 13-acetate)

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ABSTRACT **Interleukin 6 (IL-6) has been shown to inhibit the proliferation, but increase the motility, of wild-type ZR-75-1 human ductal breast carcinoma cells, a line of cells that resemble closely the malignant cells cultured from the ascitic effusion. IL-6-treated cells lose their epithelial character, become stellate or fusiform in shape, and migrate away from neighbors. In the wild-type ZR-75-1 cells, IL-6 causes cell–cell separation in preformed colonies as well as postmitotically. We have now investigated the action of IL-6 in clone B ZR-75-1 cells, which are morphologically distinct from wild-type ZR-75-1 cells. In the more polygonal rather than cuboidal clone B cells, IL-6 did not cause early inhibition of DNA synthesis and it caused little cell–cell separation in preformed colonies. However, IL-6 treatment markedly prolonged the interval between mitosis and readherence of daughter cells to their neighbors and the substratum. Supernatants from IL-6-treated cultures contained detached viable cells in increased numbers. Intermitotic intervals were prolonged in IL-6-treated cultures. IL-6-treated dividing breast carcinoma cells are characterized by an increased probability of separation from neighbors and the substratum.**

Epithelial cells are connected to each other through a set of junctional complexes (1–3). During mitosis an epithelial cell is only loosely attached to its neighbors as it rounds up and divides. The daughter cells promptly assume positions as epithelial cells within the cellular environment of the mother cell. It may be expected that, given the presence of a cytokine whose action is to favor the separation of epithelial cells from each other, dividing cells in a population might have the highest probability of completely separating from their neighbors. Thus, dividing cells may under some circumstances constitute the primary target for the cell–cell separation-inducing cytokine action.

Interleukin 6 (IL-6) in the ng/ml concentration range inhibits the proliferation, but increases the motility, of wild-type, uncloned ZR-75-1 cells, a line of ductal breast carcinoma cells (4, 5). IL-6-treated cells convert from an epithelial (polygonal cuboidal) to a fibroblastoid (fusiform, stellate) shape, separate from each other, and translocate over variable distances that can be many times greater than the cell diameter (4, 5). We have demonstrated that IL-6 inhibits DNA synthesis in wild-type ZR-75-1 cells, but that morphological conversion and increased motility are not secondary to the inhibition of DNA synthesis (5). The actions of IL-6 on DNA synthesis and cell motility appear to be independent of each other.

In the present experiments with a cloned population of ZR-75-1 cells we demonstrate that, while IL-6 does not inhibit DNA synthesis in the clone B cells, mitosis in the presence of the cytokine is commonly associated with a failure of daughter cells to reattach promptly and to assume epithelial morphology. Such newly divided ZR-75-1 clone B (ZR-75-1B) cells sometimes translocate over considerable distances. ZR-75-1B cells not engaged in mitosis show comparatively little change in cell shape or cell–cell association in response to IL-6. Thus, IL-6 can have marked effects on the adhesive behavior of postmitotic cells in a population in which it has no early effect on cellular DNA synthesis and has relatively little effect on cell–cell association among cells not engaged in mitosis.

MATERIALS AND METHODS

Cell Line and Culture Conditions. ZR-75-1B cells (6, 7) were obtained from M. E. Lippman (Vincent T. Lombardi Cancer Research Center, Georgetown University, Washington). The ZR-75-1B cells have a flatter shape than wild-type ZR-75-1 cells and do not grow in the form of convoluted three-dimensional aggregates, which characterize the wild-type ZR-75-1 cells received in our laboratory from the American Type Culture Collection in September 1988. ZR-75-1B and wild-type ZR-75-1 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and supplemented with 10 nM 17β-estradiol. Stock cultures were split 1:5 once a week and were refed once during the week.

IL-6 Preparation. Human recombinant IL-6 prepared in a baculovirus system was used (8). It was highly purified and had a specific activity of 1×10^8 units per mg of protein as determined in an IL-6-dependent murine hybridoma line. It was kindly provided by Masayoshi Kohase (National Institute of Health, Tokyo).

DNA Synthesis. [^{3}H]Thymidine incorporation was used to measure DNA synthesis as described (4).

Time-Lapse Cinemicrography. Cells were planted in 25-cm^2 flasks in 5.0 ml of growth medium at 2.5×10^3 cells per cm^2. The following day, medium was changed to a control or IL-6-containing solution and treatment and filming was begun as described (4). Cultures were photographed every 6 min with a ×6.3 planar phase-contrast objective.

RESULTS

IL-6 Does Not Inhibit DNA Synthesis in ZR-75-1B Cells. Table 1 shows that in a concentration range within which baculovirus-derived IL-6 markedly inhibits DNA synthesis in wild-type ZR-75-1 cells as measured by pulse-labeling with [^{3}H]thymidine after 24-h treatment with IL-6 (5), the cytokine has no significant effect on this parameter in ZR-75-1B cells. It should be noted that in the wild-type ZR-75-1 cells, the inhibitory effect of IL-6 on DNA synthesis decreases with time as measured 48 and 72 h after the beginning of treatment

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Abbreviations: IL-6, interleukin 6; PMA, phorbol 12-myristate 13-acetate.

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Table 1. Lack of effect of IL-6 on DNA synthesis in ZR-75-1B cells

Cytokine	[^{3}H]Thymidine incorporation, % control ± SD	
	ZR-75-1B*	ZR-75-1†
None	100	100
BV IL-6 (15 ng/ml)	97 ± 6.7	15 ± 2.12
BV IL-6 (1.5 ng/ml)	95 ± 4.5	17 ± 1.41
BV IL-6 (0.15 ng/ml)	94 ± 6.4	34 ± 12.7

BV, baculovirus.

*Mean of three experiments in which [^{3}H]thymidine incorporation in controls was as follows: 15,488 ± 1967; 27,393 ± 827; and 15,507 ± 1567 cpm.

†Mean of two experiments in which [^{3}H]thymidine incorporation in controls was as follows: 12,837 ± 1144 and 8833 ± 1416 cpm.

(5). However, in clonogenic assays of 2 weeks duration, IL-6 decreases the number of colonies formed.

IL-6 Prolongs the Interval Between Division and Readherence and Flattening of Cells. In three separate time-lapse cinemicrographic experiments, control untreated ZR-75-1B cells usually readhered rapidly to the substratum after mitosis, assumed flat, typically epithelial morphology, and stayed in one place. In contrast, many postmitotic IL-6-treated ZR-75-1B cells did not promptly readhere. Instead, they moved about for various periods of time before attaching and spreading on the substratum. Some of the postmitotic IL-6-treated cells had not readhered by the time the run was concluded, which was 9 or 10 days after the beginning of IL-6 treatment.

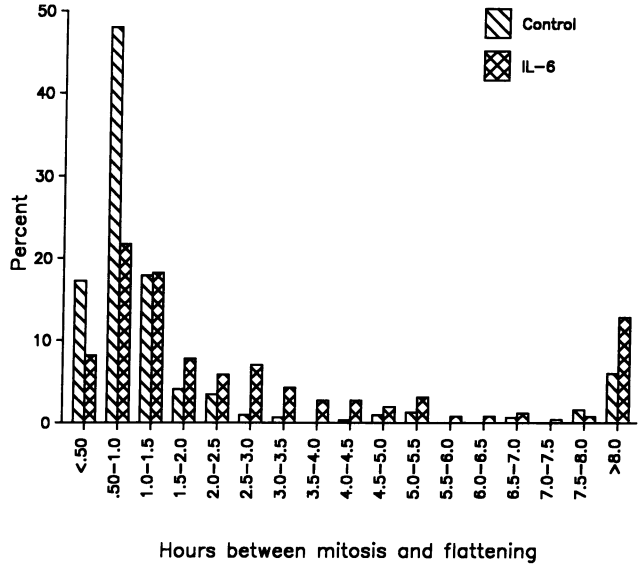

FIG. 1. IL-6 prolongs the interval between cell division and readherence and flattening of the daughter cells. ZR-75-1B cells were planted at a density of 2.5×10^3 cells per cm^2 in two 25-cm^2 flasks in 5 ml of growth medium. The flasks were gassed with 5% CO_2/95% air and incubated at 37°C. One day later, the medium was changed and one flask received IL-6 (15 ng/ml), with the other serving as a simultaneous control. Incubation was continued in constant temperature chambers and the cultures were photographed every 6 min. In the first experiment, filming was begun 1 day after medium change; in the second experiment, it was begun shortly after medium change. Both experiments ran for 9 days after medium change. For analysis, time-lapse cinemicrographs were projected onto a screen and the occurrence in individual cells of premitotic rounding, of cell division, and of postmitotic readherence and flattening was timed by means of a frame counter on the projector. Frequency distribution analysis was carried out for the following intervals: from division to flattening (see Fig. 2), from rounding to division (see Fig. 3), and from division to division (see Fig. 4). Results of the two experiments are shown in the form of weighted averages.

Time-lapse cinemicrographs of control and IL-6-treated cells from two experiments were analyzed to determine the time interval from division to readherence and spreading on the substratum. The overall results were similar in the two experiments with respect to this parameter as well as other parameters. Fig. 1 shows frequency distribution bar graphs for the mitosis to flattening interval based on the pooled data. It is evident that IL-6 prolonged the interval between mitosis and the subsequent full readherence and flattening of the daughter cells. The greatest number of events in both control and IL-6-treated cells fell into the interval length of 0.5–1.0 h, but, whereas 65% of the control cells succeeded in readhering and flattening within 1 h after mitosis, only 30% of the postmitotic IL-6-treated cells did so. Of special interest is the finding that, whereas in control cells postmitotic readherence and flattening required >8 h in only 3.1% of the events, in IL-6-treated cells 13% of the events fell into this category. Moreover, no postmitotic control cell out of a total of 322 failed to readhere and flatten within the experimental period of observation, whereas 8 of the total of 248 IL-6-treated postmitotic cells observed failed to do so and 9 other IL-6-treated cells left the field of observation.

Length of the Interval from Cell Division to Flattening as a Function of Incubation Time. The cinemicrographic data from the two experiments illustrated in Fig. 1 were analyzed with respect to the time of occurrence of mitoses followed by cell readherence and flattening. The results in Fig. 2 suggest that the mean interval between mitosis and flattening decreased somewhat with time in the control cells; however, there appears to be no systematic variation in the effect of IL-6 on this interval with time from the 4th to the 8th day of treatment (IL-6 as percent of control: 237, 219, 225, 149, 263, respectively). The apparent greater effect during the 3rd day of treatment (384%) may be a consequence of the relatively small number of observations during this early period in the experiment. Overall, the length of the interval between mitosis and flattening in IL-6-treated cultures was ≈250% of

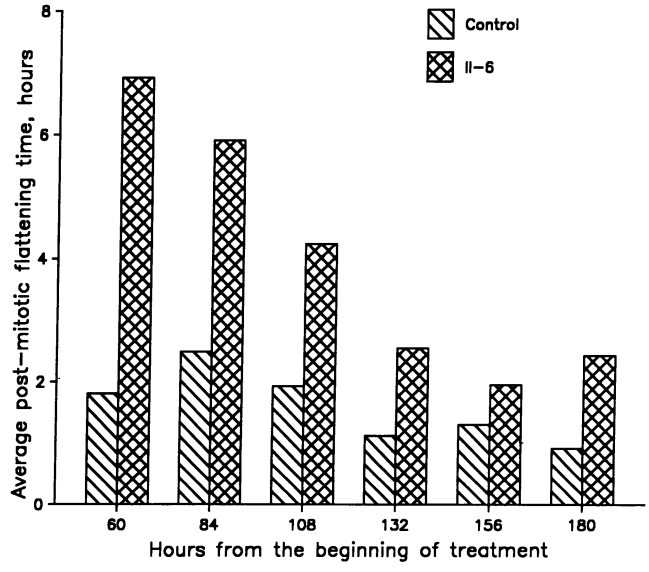

FIG. 2. Time course of IL-6 effects on the interval from division to flattening of postmitotic cells. Data from two experiments were used to calculate weighted averages for 24-h periods. The midpoints of the 24-h periods are plotted on the abscissa. Data for days 3–8 after medium change and beginning of treatment are shown. During this period, the total number of determinations of the mitosis to flattening intervals was 287 in the control cultures and 181 in the IL-6-treated cultures. The number per 24 h increased with time as the cells multiplied. See Fig. 1 legend for details.

that in control cultures. We reported previously (4) that in cultures of wild-type ZR-75-1 or T-47D cells continuously treated with IL-6 for 10 days after a single addition there was no evidence of reversion of IL-6-induced changes with time.

IL-6 Prolongs the Interval Between Rounding and Division of Cells. Fig. 3 shows that IL-6 treatment also prolonged the interval between cell rounding and division. The commonest length of this interval was 0.5–0.6 h in both control and IL-6-treated cells; 25% of the intervals for control cells and 29% of the intervals for IL-6-treated cells fell into this category. However, whereas the rounding to division interval was >0.6 h in 24% of the mitotic events in control cells, it was 42% of the events in IL-6-treated cells.

IL-6 Increases the Intermitotic Interval in ZR-75-1B Cells. Although IL-6 does not inhibit DNA synthesis after 24 h of treatment (Table 1), Fig. 4 shows that IL-6 increased the intermitotic interval in ZR-75-1B cells. In control cells, 79% of the intermitotic intervals were <45 h, with a broad distribution in frequency categories from <25 to 40–45 h. In IL-6-treated cells, the intervals were shorter than 45 h in only 46% of the cases, and there was a broad peak in frequency categories from 35–40 to 50–55 h. As shown in Figs. 1 and 2, IL-6 prolongs the intervals from division to flattening and from rounding to division. There are more detached cells in the medium in IL-6-treated than in control cultures and a greater fraction of the detached cells are viable in IL-6-treated than in control cultures (cf. Tables 2 and 3). These are clearly some of the factors underlying the observed increase in intermitotic intervals in IL-6-treated cells; however, other factors have not been excluded.

Numbers of Adherent and Nonadherent ZR-75-1B Cells in Control and IL-6-Treated Cultures. Table 2 summarizes the results of enumeration of adherent and nonadherent cells by Coulter counting at the end (day 9) of one of the time-lapse cinemicrographic experiments. As can be seen, twice as many IL-6-treated as control cells were located in the supernatant medium at the end of the run. This is consistent with the time-lapse cinemicrographic observation that postmitotic control cells generally readhere rapidly to the substratum, whereas postmitotic IL-6-treated cells do not. Similar numerical results were obtained in three additional experiments in which the seeding density was varied (5×10^3, 1×10^4, and 5×10^4 cells per cm^2) and cells were counted in a hemocytometer (first two experiments) or a Coulter counter (third experiment) 6 days after the beginning of treatment. Expressed in terms of percentages of total cells, 2.1×, 1.9×, and 1.6× as many IL-6-treated cells as control cells were located in the supernatant medium in the three experiments, respectively, after 6 days of treatment. No significant differences in the cell distribution, adherent vs. nonadherent, between control and IL-6-treated cultures were detected 3 days after the beginning of treatment.

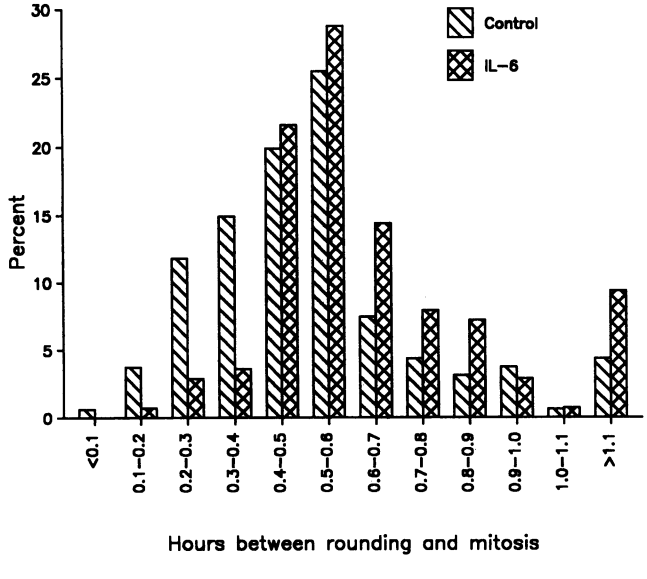

FIG. 3. IL-6 prolongs the interval between cell rounding and division. See Fig. 1 legend for details.

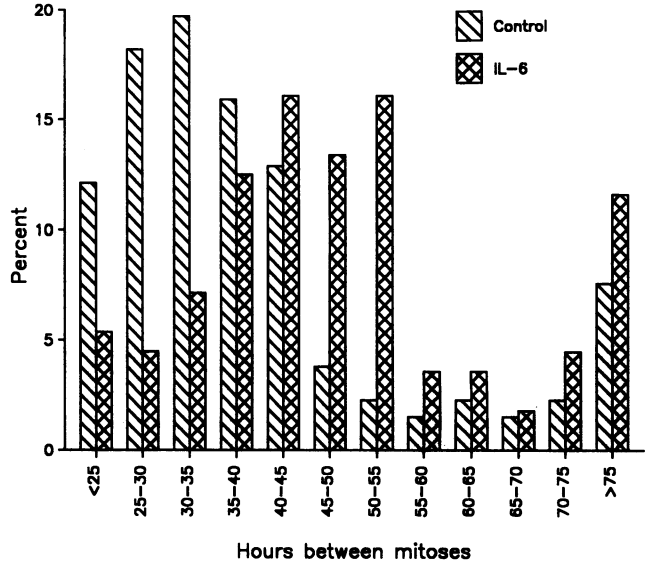

FIG. 4. IL-6 prolongs the inter-mitotic interval. See Fig. 1 legend for details.

We have determined the viability of detached cells by erythrocin B exclusion 4 and 6 days after the beginning of IL-6 treatment in an experiment in which ZR-75-1B cells were planted at a density of 5.4×10^4 cells per cm^2 and the medium was changed and IL-6 (15 ng/ml) was added the following day. Four days later, 29% of the cells in the supernatant of the control culture were viable, whereas in the IL-6-treated culture 43% of the detached cells were viable (Table 3). Six days after medium change and the beginning of IL-6 treatment, the corresponding numbers were 14% and 33%. Thus, at both 4 and 6 days after the beginning of IL-6 treatment, a considerably higher proportion of detached cells were viable in IL-6-treated than in control cultures.

Morphological Characteristics of IL-6-Treated ZR-75-1B Cells. In contrast to our previous observations on colonies of wild-type ZR-75-1 cells (2), preformed colonies of clone B cells did not generally show IL-6-induced cell–cell separation attended by cell translocation. Rather, the appearance of scattered ZR-75-1B cells is in most instances linked to the behavior of postmitotic cells. Fig. 5 illustrates the morphology of control (*A–C*) and IL-6-treated (*D–F*) ZR-75-1B cells and cell colonies photographed 10 days after the beginning of a time-lapse cinemicrography experiment. Overall, the cells in control colonies are more polygonal and tightly packed than in the IL-6-treated colonies, in which the cells are more

Table 2. IL-6 increases the fraction of detached ZR-75-1B cells

Treatment	Number of cells × 10^{-3} per cm^2		% of total
	Adherent	Detached	
Control	3.6	0.68	16
BV IL-6 (15 ng/ml)	2.7	1.3	33

Flasks (25 cm^2) were seeded with ZR-75-1B cells at a density of 2.5×10^3 cells per cm^2 in 5 ml of medium. The medium was changed and IL-6 was added 1 day after seeding. The cultures were observed by time-lapse cinemicrography for 9 days, after which cells were counted in a Coulter Counter. BV, baculovirus.

Table 3. More of the detached cells are viable in IL-6-treated than in control cultures

Duration of treatment, days	Treatment	Number of cells × 10^{-4} per cm^2			% detached cells that are viable
		Adherent	Detached	Total	
4	Control	7.5	1.0	8.5	29
	IL-6 (15 ng/ml)	7.3	1.4	8.7	43
6	Control	13.0	2.9	15.9	14
	IL-6 (15 ng/ml)	11.3	3.4	14.7	33

Six-well plates were seeded with ZR-75-1B cells at a density of 5.4 × 10^4 cells per cm^2 in 2 ml of medium. The medium was changed and IL-6 was added the following day. Cell viability was determined by erythrocin B exclusion.

angular. The proportion of scattered cells is considerably greater in IL-6-treated than in control cultures. The shape of scattered cells ranges from round to stellate or fusiform. These static observations at the termination of the run are consistent with the time-lapse cinemicrographic record of the dynamics of IL-6-induced changes, which have established the postmitotic cells as the predominant reservoir for cells that scatter after IL-6 treatment.

5-Fluorouracil Does Not Increase the Limited Effects of IL-6 on the Morphology of Preformed Colonies of ZR-75-1B Cells. We have investigated the question of whether chemical inhibition of DNA synthesis in ZR-75-1B cells leads to an increase in the morphological and motility effects of IL-6 in the cloned cell population. ZR-75-1B cells were seeded at 2.5 × 10^4 cells per cm^2 in 25-cm^2 flasks and incubated for 5 days before medium change and addition of IL-6 (15 ng/ml), or 5-fluorouracil (32 ng/ml) or IL-6 (15 ng/ml) plus 5-fluorouracil (32 ng/ml). 5-Fluorouracil-treated cultures underwent progressive cell loss after the 4th day, but at no time was there evidence of epithelial–fibroblastoid conversion or of cell scattering. Treatment for 4 days or longer with IL-6 in the presence of 5-fluorouracil did not detectably alter the morphological features of ZR-75-1B cells as compared to their features when incubated with IL-6 alone, except for the 5-fluorouracil-induced growth inhibition and cell loss. Thus, chemical inhibition of DNA synthesis in variant ZR-75-1B cells does not itself cause cell–cell separation and does not enhance the effects of IL-6.

Limited Effects of Phorbol 12-Myristate 13-Acetate (PMA) on ZR-75-1B Cell Morphology. We have recently found that treatment of wild-type ZR-75-1 cells with 2–20 nM PMA induces loosening of the tightly packed cell colonies attended by some cell–cell separation (5). These changes in wild-type ZR-75-1 cell colony organization are similar to those caused by IL-6; however, the PMA-treated cells are generally flatter than IL-6-treated cells. Combined treatment of wild-type ZR-75-1 cells with IL-6 and PMA leads to very marked and rapid morphological changes and scattering of the cells, indicating synergistic interaction (5). Experiments with anti-IL-6 antibodies have shown that PMA action is not mediated

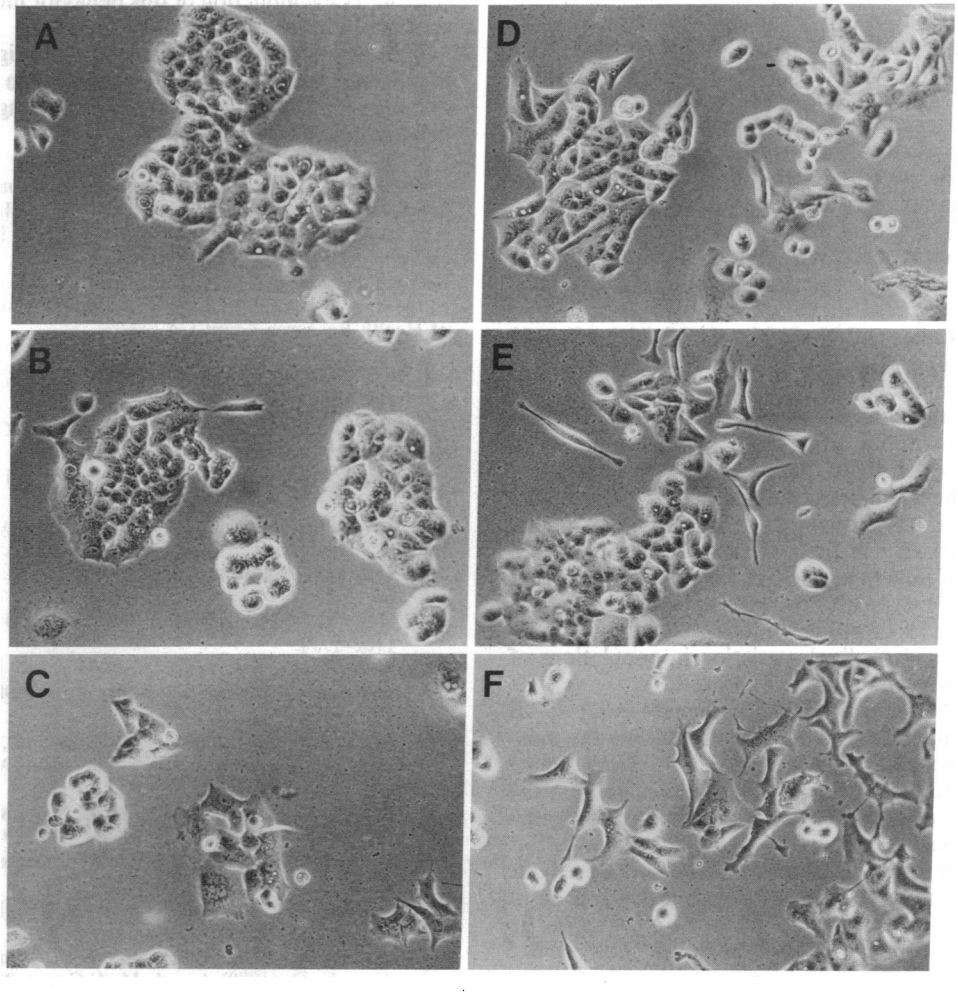

FIG. 5. IL-6 effects on the morphology of ZR-75-1B cells. Flasks (25 cm^2) were seeded with ZR-75-1B cells at a density of 2.5 × 10^3 cells per cm^2 in 5 ml of medium. The medium was changed and IL-6 (15 ng/ml) was added 1 day after seeding. The cultures were observed by time-lapse cinemicrography for 10 days, after which photomicrographs were taken with a ×16 phase-contrast objective. (*A–C*) Control cells. (*D–F*) IL-6-treated cells. (×70.)

through induction of IL-6 by PMA. As would be expected, staurosporine, a protein kinase inhibitor with some selectivity for protein kinase C, blocked PMA action. Staurosporine did not block IL-6 action, suggesting that PMA and IL-6 use different signal transduction pathways in exerting their effects on cells (5).

PMA at 20 nM caused loosening of colony structure and flattening of ZR-75-1B cells (data not shown). Simultaneous treatment of clone B cells with both IL-6 and PMA had a greater effect than treatment with either agent alone; however, it did not have as great an effect as we have observed in wild-type ZR-75-1 cells (5), which may be largely due to the lesser responsiveness of clone B cells to IL-6. Anti-IL-6 antibody blocked the effects of IL-6 but not those of PMA on ZR-75-1B cell morphology and distribution (data not shown).

DISCUSSION

The present findings demonstrate that IL-6 can cause scattering of ZR-75-1B ductal breast carcinoma cells by preventing adherence of postmitotic cells to neighboring cells and the substratum. These findings provide support for the view that cells undergoing mitosis are in a state in which a cytokine with cell–cell separation-inducing action may have its greatest effect. This may be because cell junctions to neighboring cells and to the substratum are largely lost as part of the complex set of changes that a cell undergoes when it divides. The precise mechanism whereby IL-6 markedly delays or prevents adherence of postmitotic cells remains to be determined. IL-6 could suppress the expression of proteins required for junction formation or the assembly of such proteins into appropriate macromolecular complexes. Involved may be intracellular proteins such as the desmoplakins and vinculin, which participate in the formation of adherens-type junctions, as well as extracellular proteins such as fibronectin and thrombospondin, which contribute to the formation of the extracellular matrix. We have previously reported that IL-6 treatment of T-47D line ductal breast carcinoma cells decreases desmosomes and focal contacts as revealed by immunofluorescence staining for desmoplakins and vinculin (4).

In the previous studies with the wild type ZR-75-1 cells and the cloned T-47D cells, IL-6 inhibited the synthesis of DNA and the proliferation of cells (4, 9, 10) while enhancing their motility (4, 5). In the present experiments with ZR-75-1B cells, we have shown that IL-6 does not decrease DNA synthesis in these cells. This finding is not unexpected as subcloning of another IL-6-sensitive ductal breast carcinoma line, the T-47D cells, has shown that the population contains cells that vary markedly in their sensitivity to the growth inhibitory effects of IL-6 (11). We had noted previously that the population of wild-type ZR-75-1 cells appeared to contain some cells that were less responsive to IL-6 than most of the cells in the population (4). Such variation in populations of aneuploid carcinoma cells is to be expected.

The fact that IL-6 decreases cell adhesiveness in ZR-75-1B cells without inhibiting DNA synthesis establishes the independence of the mechanisms that underlie IL-6 effects on the two parameters. Our results show that IL-6 effects on the shape and motility of cells not engaged in mitosis are considerably less marked in clone B than in wild-type ZR-75-1 cells. This could be either because the clone B cells may be generally less sensitive to IL-6 than the wild-type cells, because the wild-type cells are genetically predisposed to undergo epithelial–fibroblastoid conversion, or because IL-6 has some other effect in the wild-type cells that enhances the responsiveness of these cells to the fibroblastoid conversion-inducing action of IL-6. Inhibition of DNA synthesis by IL-6 does not appear to be such a contributory factor, as chemical inhibition of DNA synthesis in IL-6-treated clone B ZR-75-1 cells did not enhance the fibroblastoid conversion-inducing action of IL-6. It is of interest that IL-6 substantially prolongs the intermitotic interval in ZR-75-1B cells. The mechanism underlying this effect is not clear. It appears that elongation of the interval between mitosis and readherence and flattening in IL-6-treated cells cannot account for more than a part of the effect of IL-6 on the intermitotic interval.

To determine whether IL-6 effects in human breast carcinoma cells may be mediated through the action of another cytokine, we have recently examined acidic and basic fibroblast growth factor, tumor growth factors α and β, epidermal growth factor, insulin-like growth factor, and tumor necrosis factor for effects on cell shape, motility, and proliferation that would mimic the effects of IL-6 in ZR-75-1 or T-47D cells. None of these factors mimics the action of IL-6 (5). Their diverse effects on motility and proliferation in other cell types have recently been reviewed (12). Thus, to the extent that other cytokines have so far been examined, it appears that the action of IL-6 in the human breast carcinoma cell system is unique. Whether IL-6 induces epithelial–fibroblastoid conversion in any other type of cell remains to be determined. Such a conversion could be an early step in starting a cancer cell on the path of forming a metastasis (4). It is of great interest that in the course of embryological development, specific groups of epithelial cells separate from their neighbors, assume a mesenchymal character, and become motile (13, 14). Is the induction of this behavior mediated by IL-6 or another cytokine?

These results point to mitotic cells as a significant reservoir of potential metastasis-forming cells given the presence of a cytokine that blocks adherence of the daughter cells to their neighbors after completion of mitosis.

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