Energy Metabolism during Apoptosis

bcl-2 PROMOTES SURVIVAL IN HEMATOPOIETIC CELLS INDUCED TO APOPTOSE BY GROWTH FACTOR WITHDRAWAL BY STABILIZING A FORM OF METABOLIC ARREST

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We have investigated cell metabolism during apoptosis in the murine interleukin-3 (IL-3)-dependent cell line Bo and two derivative clones (B14 and B15) overexpressing human bcl-2a. On removal of IL-3, Bo cells underwent apoptosis within 8 h, whereas B14 and B15 cells were resistant for at least 24 h. Metabolically, Bo, B14, and B15 cells were indistinguishable from each other. All were insensitive to mitochondrial poisons, derived ATP entirely by glycolysis, and maintained similar mitochondrial membrane potentials measured by rhodamine-123 fluorescence with or without IL-3. All virtually ceased glycolysis and production of lactic acid on IL-3 withdrawal but maintained intracellular [ATP] until in Bo cultures the cells began to apoptose. B14 and B15 cells became glycolytically arrested but maintained stable ATP levels during protection from apoptosis. Depletion of intracellular ATP by uncoupling the mitochondrial ATPase with 2,4-dinitrophenol or carbonyl cyanide p-trifluoromethoxyphenylhydrazone induced apoptosis in Bo cells with or without IL-3, but not in B14 or B15 cells. bcl-2-overexpressing cells were recoverable with high plating efficiency even after prolonged exposure to 2,4-dinitrophenol. We conclude that IL-3 withdrawal leads to arrest of energy metabolism in which ATP levels are maintained. In Bo cells this is followed by apoptosis, whereas in bcl-2-overexpressing cells this state is stably prolonged. ATP depletion is a strong apoptotic signal which overrides IL-3 signaling in normal cells but is ineffective in bcl-2-overexpressing cells. Prolonged metabolic arrest and resistance to ATP depletion facilitated by bcl-2 are both reversible. Persistent reversible metabolic dormancy would provide cells with a survival advantage in nonsustainable environments (e.g. hypoxia or substrate lack) and suggests a mechanism for the survival advantage displayed by cells overexpressing bcl-2.

Apoptosis (programmed cell death) is thought to play a crucial role in regulating cell growth (1, 2). Many cells, including hematopoietic cells (3), are susceptible to apoptosis induced by a wide variety of agents and conditions such as serum deprivation, radiation, heat shock, cytotoxic drugs, oncogene products, or lytic viruses (4–9). Apoptosis is regulated by a number of genes, in particular those of the bcl-2 family, originally identified with follicular B-cell lymphoma (10). bcl-2 codes for a 26-kDa membrane-inserted protein that has been located in outer mitochondrial, endoplasmic reticulum, and nuclear membranes (11–13). Overexpression of the membrane-inserted form, but not the truncated free form (bcl-2b), protects many cells from apoptosis induced by numerous noxious agents (4–9) and also confers a survival advantage on cells in vivo (9). bcl-2 has therefore been considered a facilitatory gene for malignant change. Recently, a series of bcl-2 homologs has been isolated (14, 15) whose activity can be apoptosis-inducing or -suppressing, depending on their state of dimerization with other members of the family. Other genes may also play important accessory roles in apoptosis; for example, c-myc and p53 (16–18). The mechanism of action of bcl-2 is unknown. Although its location in mitochondrial membranes suggests it may influence mitochondrial function, and recent work suggests that mitochondria may contribute to early stages of apoptosis induction, (19–21) cells depleted of mitochondrial DNA or cultured in anaerobic conditions are still protected from apoptosis by bcl-2 (22–24). bcl-2 has also been suggested to protect cells from apoptosis by inhibiting lipid peroxidation induced by free radicals (25, 26).

Recent work has shown that tumor cells expressing bcl-2 or mutant p53 have a survival advantage in hypoxic conditions (27), raising the question whether bcl-2 confers a survival advantage by regulating metabolism and the synthesis or use of ATP. In murine interleukin-3 (IL-3)1-dependent cells, ATP is derived entirely from glycolysis (28), and apoptosis induced by IL-3 withdrawal is preceded by drastic reductions in lactic acid production without changes in glucose transport (29, 30). These cells thus provide a useful model for metabolic regulation induced by oncogenes including bcl-2. We examined the possibility that in these cells bcl-2 might either regulate ATP production independently of the IL-3 signaling pathway or sustain them in a minimal but metabolically active state. We found that bcl-2 expression makes no difference to the cessation of energy metabolism normally leading to apoptosis but facilitates a prolonged metabolically dormant state from which cells recover with high efficiency.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The murine IL-3-dependent pro-B line Bo and two independent derivative clones B14 and B15 overexpressing human bcl-2 were kindly made available by Dr. Mary Collins, Institute for Cancer Research, London. Whereas Bo cells apoptose within 8 h after IL-3 withdrawal, both B14 and B15 cells arrest growth but remain viable for several days and are refractory to further damage by radiation or cytotoxic drugs (3). Cells were maintained as described previ-

1 The abbreviations used are: IL-3, interleukin-3; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MOPS, 4-morpholinopropanesulfonic acid; TUNEL, terminal deoxyoligonucleotide end labeling.
bcl-2 and Metabolism in Hematopoietic Cells

RESULTS

Neither IL-3 Withdrawal nor bcl-2 Overexpression Induces Mitochondrial Oxidative Metabolism—Bo, B14, and B15 cells all possess rapid population doubling times of 10–14 h in the presence of IL-3 (data not shown). We wished first to exclude that bcl-2 overexpression may induce mitochondrial respiration, thereby significantly increasing efficiency of ATP generation per unit of glucose metabolized. Rhodamine-123 fluorescence was employed as a marker of mitochondrial energization (35). Data are shown in Fig. 1 for Bo and B15 cells. There was no difference in basal fluorescence between Bo and B15 exponential cells nor between cells deprived of IL-3 and reincubated with or without IL-3 for several hours. However, Bo, B14, and B15 cells depleted of IL-3 underwent rounding of shape and volume contraction at 30 min. Bo cells commenced to apoptose between 4 and 6 h, which coincided with a slight relative decrease in rhodamine fluorescence compared with bcl-2-expressing B15 cells (Fig. 1). This reduction in mitochondrial membrane potential (Fig. 1) is consistent with changes in permeability transition (21), but we were unable to demonstrate any effect of cyclosporin A, an inhibitor of mitochondrial pore opening (data not shown). Also, a significant proportion of Bo cells (5–10%) became permeable to trypan blue at about 8 h, suggesting alternatively that the dye change could be due to a general increase in cell permeability. Comparison of B14 with B15 cells showed closely similar profiles (Fig. 2). We noted, however, that all cells, including Bo, kept undisturbed by further addition of materials or washing showed a similar progressive decrease in rhodamine-123 retention over 6 h, but this was still unaffected by IL-3. Confocal and fluorescence microscopy demonstrated that rhodamine-123 was located in the cytoplasmic region surrounding the nucleus in Bo, B14, and B15 cells, consistent with mitochondrial localization, there being no significant differences in whether or not IL-3 was present (data not shown). To confirm that bcl-2 protection did not involve mitochondrial activation, we incubated cells with cyanide (500 μM–50 mM) or antimycin A (50 ng/ml–5 μg/ml); cyanide inhibits cytochrome oxidase, antimycin A electron flow through the cytochrome bc1 complex. Neither induced nor accelerated apoptosis after 24 h, with or without IL-3, as reported for other IL-3-dependent cells (30 and data not shown), suggesting that induction of oxidative phosphorylation was not involved in the regulation of apoptosis by bcl-2.

bcl-2-protected Cells Undergo a Decline in Glycolysis Identical to That of Parental Cells Undergoing Apoptosis—Fig. 3 shows lactic acid production from two representative complete experiments using Bo and B15 cells with measurements taken at different time intervals. Two h after IL-3 withdrawal from both Bo and B15 cultures, lactate output declined to approximately 50% of controls supplemented with IL-3 and stopped almost completely at about 8 h. At 8 h, most Bo cells were visibly apoptosing, whereas B15 cells maintained almost full viability over the subsequent 24 h. However, no further production of lactic acid occurred from B15 cells after 8 h, indicating that glycolytic flux in these cells was arrested. Identical results were obtained from B14 cells (Table I). Since Bo, B14, and B15 cells displayed identical declines in glycolytic flux on IL-3 withdrawal, and B14 and B15 cells established prolonged glycolytic arrest, bcl-2 could not function by generating ATP through glycolysis in the absence of IL-3. Since we also excluded generation of ATP by mitochondrial respiration, we analyzed whether bcl-2 expression might activate or sustain ATP-consuming pathways, for example “housekeeping” or repair functions. Alternatively, enhanced survival could be due to decreased consumption of ATP allowing cellular [ATP] to be maintained. We therefore measured changes in cellular [ATP].
FIG. 1

bcl-2 and Metabolism in Hematopoietic Cells
bcl-2 Does Not Affect Intracellular [ATP] or [ADP] — Data from seven individual experiments using B15 cells are summarized in Table II, each data point being from least three experiments. Values are given as percentages of initial values (t = 0) since (i) cells in IL-3 maintained proliferation, whereas those without IL-3 ceased, and (ii) the fractional increase in numbers after short periods of time could not be determined accurately. The basal ATP content of the cells was similar in both Bo and B15 cells (Table II) and comparable to values determined previously in other IL-3-dependent cells (30). In IL-3-supplemented Bo and B15 cells (Table II) and comparable to values determined previously in other IL-3-dependent cells (30). In IL-3-supplemented Bo and B15 cells [ATP] continued to rise commensurate with continued growth and increased cell number. In IL-3-starved Bo and B15 cells, intracellular [ATP] followed early rises similar to that observed in IL-3-supplemented cells until Bo cells commenced to apoptose (at about 6 h). After this point ATP levels in Bo cells fell dramatically, which was attributable to general cell leakage and apoptotic fragmentation. However, in IL-3-starved B15 cells, cellular ATP content plateaued after 8 h and remained almost unaltered over the next 20 h. bcl-2 protection therefore does not appear to induce, activate, or maintain ATP-depleting mechanisms for cell survival, but rather maintains [ATP]. The early rise in [ATP] during IL-3 withdrawal while lactic acid production (and therefore glycolysis) declines, and its subsequent plateauing in B15 cells, suggest also that IL-3 signaling regulates ATP demand, not its generation. Thus glycolytic down-regulation appears to follow reduction in ATP demand when IL-3 is withdrawn and [ATP] is maintained. Later, when ATP demand is abrogated altogether, glycolysis ceases. In preventing apoptosis, bcl-2 thus enables cells to establish a stable state of glycolytic and therefore energy arrest.

Cellular ADP content was also determined in parallel with ATP, but the lower intracellular concentration of this nucleotide made its accurate determination difficult. However, we obtained no evidence for consistent changes in ATP:ADP ratios accompanying IL-3 stimulation or bcl-2 overexpression.
ATP Depletion Induces Apoptosis—One explanation for the extended survival of bcl-2-protected cells might be that it inhibits ATP-consuming metabolic pathways whose unbalanced activity signals apoptosis but whose impact on gross [ATP] is too small to be measurable. If so, then imposition of an ATP-depleting reaction might be expected to accelerate apoptosis and overcome the protective effects of bcl-2. In the absence of oxidative phosphorylation, mitochondrial uncouplers can still lower intracellular ATP through reversal of the mitochondrial F0-F1-ATPase, thereby causing hydrolysis of glycolytically derived ATP. Such ATP depletion would also lead to up-regulation of glycolytic flux through feedback regulation and therefore produce an increase in output of lactic acid. We therefore induced such a state by adding mitochondrial uncouplers: DNP, which has been used in many studies to specifically lower intracellular ATP (e.g., 36), or FCCP, a potent uncoupler of the mitochondrial F0-F1-ATPase (37).

In the presence of IL-3, DNP lowered ATP by an average of 40% after 2 h compared with controls in both Bo and B15 cell lines (Table II) and also raised ADP (data not shown). DNP also

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**TABLE I**

| Time (h) | B14 cells | B15 cells |
|---------|-----------|-----------|
|         | +IL-3 | -IL-3 | +IL-3 + DNP | -IL-3 + DNP | +IL-3 + FCCP | -IL-3 + FCCP |
| 2       | 0.55  | 0.3   | 0.74       | 1.09       | 1.3       | 1.3       |
| 4       | 0.9   | 0.39  | 1.4        | 1.8        | 2         | 1.9       |
| 6       | 1.3   | 0.5   | 2.2        | 2.5        | 2.9       | 2.8       |
| 8       | 3.1   | 0.68  | 3.8        | 4.6        | 5.5       | 5         |
| 22      | 7.3   | 0.67  | 5.9        | 6          | 8.8       | 8         |

**FIG. 3. Effects of IL-3 withdrawal and uncoupler on lactic acid production in Bo and B15 cells.** L-Lactate concentrations in the supernatant from incubations of Bo (left panels) or B15 (right panels) cells were measured at the times indicated as described under "Experimental Procedures." Two separate experiments are shown, with each data point representing the mean of duplicate determinations. ■, with IL-3; ▲, without IL-3; ◀, with IL-3 with DNP; ★, without IL-3 with DNP. The cell concentration was 2 × 10⁶/ml.

**Glycolytic arrest in B14 and B15 cells and comparison of effects of DNP and FCCP on glycolytic flux**

DNP was used at 3 mM, FCCP at 4 μM. Data are for mean values from a typical complete experiment. All errors were within 5% of means. Time = 0 values showed no lactate in culture medium. Note cessation of glycolysis in cells incubated without IL-3 only (compare 8 h with 22). Both DNP and FCCP maintain lactate production in the absence of IL-3 in B14 and B15 cells. Nonlinearity at 22 h is due to medium exhaustion.
increased production of lactic acid by up to nearly 4-fold with almost identical kinetics in both lines (Fig. 3), confirming that uncoupling the mitochondrial ATPase stimulated glycolytic flux as predicted. Almost identical results were obtained using a different uncoupler, FCCP. Comparing the effects of FCCP with DNP showed that FCCP was more effective in early uncoupling (Table I) but that both lines responded almost identically to both uncouplers and produced almost identical levels of lactate on a per cell basis. In IL-3-starved Bo cells, enhanced lactic acid production stopped when cells apoptosed. DNP and FCCP induced or accelerated apoptosis in Bo cells even in the presence of IL-3. Such cells displayed earlier typical membrane blebbing and nuclear/DNA fragmentation shown by microscopy, TUNEL assays (Fig. 4), and DNA ladder gels (Fig. 5, B, D, F, and H). TUNEL assays detect in situ fragmented DNA through fluorescent end labeling of fragmented DNA in intact nuclei. DNP increased the proportion of TUNEL-positive cells from 10% in controls incubated without IL-3 for 6 h to nearly 50% (Fig. 4). In contrast, both B14 and B15 exhibited identical resistance to apoptosis with DNP and FCCP. Whereas DNP decreased the ATP content in B15 cells to an extent similar to that seen in Bo cells (Table II) they retained increased viability with significantly less DNA fragmentation (Fig. 5, C, E, and G), even in the absence of IL-3. Both B14 and B15 cultures continued to generate increased lactic acid throughout the 24-h period (Fig. 3 and Table I); but even after 24-h exposure to DNP in the absence of IL-3, DNA was still significantly intact compared to with cells (Fig. 5, I).

The prolonged survival of bcl-2-overexpressing cells treated with DNP correlated with survival of clonogenic cells determined by agar colony assays (Table III). Whereas Bo cells from cultures preincubated for 24 h in DNP but without IL-3 generated no colonies at 5 days, B15 cultures similarly treated generated 40% of colonies of control cultures. Similarly, whereas almost no Bo cells survived DNP treatment in the presence of IL-3, B15 cells maintained nearly 70% of the control clonogenic cells. Colonies from DNP-treated B15 cultures were also the same size as controls, showing that any lag phase in recovery must have been very short. Thus our results show that although lowering ATP, even by a relatively modest amount, generates a strong apoptotic signal in Bo cells, it does not generate this signal in bcl-2-transfected cells, suggesting that focal ATP depletion may initiate apoptosis in Bo cells and that bcl-2 inhibits this step. Taken together, our results also imply that glucose and lactate transport and the glycolytic pathway are not disrupted by IL-3 withdrawal or uncouplers; furthermore, glycolysis is not operating at maximum capacity during growth stimulated by IL-3. Together with the data on maintenance of ATP, it appears that bcl-2 does not act globally on energy metabolism, and bcl-2-overexpressing cells need neither generate nor use ATP to prolong their survival.

**DISCUSSION**

In Bo cells, apoptosis induced by IL-3 withdrawal is preceded by rapid rounding of cells and decline in lactic acid production. However, this is not accompanied initially by intracellular ATP depletion. This suggests that removing IL-3 reduces the demand for ATP which in turn leads to a reduction in glycolytic flux and therefore lactate production. The constraint on glycolysis imposed by reduced ATP demand can, however, be reversed by activating the mitochondrial F0F1-ATPase with uncoupler. This reduces cellular ATP levels, stimulates glycolytic flux (probably regulated at the level of phosphofructokinase), and also accelerates apoptosis. IL-3 withdrawal therefore cannot directly affect glucose or lactate transport or enzymes regulating glycolysis; indeed, these cannot be saturated during normal growth as shown by the significant and immediate up-regulation of lactate production by ATP depletion. We also found no evidence that metabolic down-regulation induced by IL-3 withdrawal increases intracellular free radical concentrations; antioxidants in fact accelerated apoptosis. This would,

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**TABLE II**

**Intracellular ATP content of Bo and B15 cells after incubation in the presence and absence of IL-3 and DNP**

Conditions of incubation were the same as described in the legend to Fig. 3. Data for intracellular ATP content are presented as a percentage of the value found at time zero for each incubation. Mean values for ATP content of Bo and B15 cells were (±S.E.) 100 ± 18 and 121 ± 28 pmol/10^6 cells, respectively. Each data point is the mean ± S.E. from at least three separate experiments; the table summarizes seven different experiments. The increase in ATP at 2 h represents the cells recovering their metabolic status after washing in ice-cold buffer, the increase between 8 and 22 h reflects the doubling of cell number over this time period. ND, none detected.

| ATP content of cell as a percentage of that at time zero | 2 h | 4 h | 6 h | 8 h | 22 h |
|---------------------------------------------------------|-----|-----|-----|-----|------|
| Bo                                                      | 174 ± 16 | 202 ± 13 | 197 ± 27 | 229 ± 10 | 56 ± 29 |
| Bo + IL-3                                               | 183 ± 12 | 213 ± 4 | 226 ± 36 | 243 ± 52 | 623 ± 183 |
| Bo + DNP                                                | 125 ± 5 | 143 ± 10 | 102 ± 12 | 94 ± 35 | ND |
| Bo + IL-3 + DNP                                         | 141 ± 25 | 146 ± 6 | 155 ± 35 | 117 ± 36 | ND |
| B15                                                     | 184 ± 24 | 196 ± 12 | 226 ± 13 | 289 ± 36 | 211 ± 17 |
| B15 + IL-3                                              | 188 ± 31 | 220 ± 18 | 237 ± 11 | 323 ± 18 | 629 ± 129 |
| B15 + DNP                                               | 122 ± 15 | 105 ± 21 | 237 ± 39 | 187 ± 3 | 61 ± 9 |
| B15 + IL-3 + DNP                                        | 133 ± 15 | 108 ± 6 | 239 ± 18 | 157 ± 7 | 104 ± 15 |

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**Table II**: Intracellular ATP content of Bo and B15 cells after incubation in the presence and absence of IL-3 and DNP.
However, be consistent with the recent observation that dATP or dADP may regulate apoptosis (40); antioxidants would be expected to increase reduced glutathione and thereby increase dNTPs via ribonucleotide reductase. We have also found that depletion of reduced glutathione inhibits some of the early stages of apoptosis in these cells.2

Surprisingly, we have found that in B14 and B15 cells overexpressing bcl-2, none of the preapoptotic characteristics of parental Bo cells undergoing apoptosis (cell rounding, maintenance of [ATP], down-regulation, and cessation of glycolysis) is altered. We obtained no evidence that bcl-2 initiates mitochondrial respiration nor that IL-3 signaling or its deprivation significantly affects mitochondrial membrane potential as proposed for a role in apoptosis induction (40). However, bcl-2-overexpressing cells are resistant to prolonged ATP depletion induced by mitochondrial uncouplers even in the absence of IL-3; although uncoupler produces identical sustained and increased glycolytic flux, apoptosis is still inhibited, and such cells can recover with high efficiency as revealed by clonal assays. B14 and B15 cells maintained in the absence of IL-3 therefore appear to have no requirement for ATP generation nor for significant ATP usage and appear to survive in an extended energetically arrested state.

This is the first study to show that prevention of apoptosis by bcl-2 facilitates a stable state of prolonged metabolic arrest in previously rapidly proliferating cells (cycle time approximately 11 h) within hours of the removal of growth factor stimulus (IL-3). The mechanism closely coupling IL-3 signaling to glycolysis is unknown. Prolonged and stable inertness may generate resistance to many different forms of stress (4–9) and explain the known survival advantage of tumor cells protected by bcl-2, particularly in hypoxic environments (27). In solid tumors, vasculature is disorganized and creates hydrostasis (41–43). This in turn leads to hypoxia and reduction in nutrient flows as well as inhibiting diffusion of cytotoxic drugs. Cells in

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2 J.M. Garland, K. Sondergaard, and J. Jolly, submitted for publication.
such conditions are likely to be severely stressed. Possibly both IL-3 removal and ATP depletion could create a stress response in the cells used here, regulating cascades involving stress-activated kinases that are activated by diverse stresses such as heat shock, arsenite, and hyperosmotic stress (44–46) and also implicated in apoptosis induced by ceramide (47). However, taken together, our results with these cells are more consistent with a model in which apoptosis is dynamically inhibited by IL-3 signaling and related to an ATP-consuming inhibitory pathway, which, when switched off (as in IL-3 withdrawal), induces apoptosis. DNP mimics IL-3 withdrawal by making ATP unavailable locally to sustain this inhibitory pathway, whereas bcl-2 facilitates prolongation of an ATP-independent state wherein cellular [ATP] is maintained. Inhibition of apoptosis in B15 cells during ATP depletion therefore suggests that bcl-2 targets the apoptosis initiator. Tumor cells similarly maintained by bcl-2 in a stable metabolically dormant state would also be able to survive under adverse conditions but would readily revive when environments improved or when the cells became disseminated. Metabolic quiescence could therefore explain the survival advantage of tumor cells and how some metastatic deposits can remain dormant for prolonged periods of time. Although our cells are not directly comparable to those in solid tumors, and these findings may not be applicable to other cell types, we have shown that a mechanism does exist, facilitated by bcl-2, for significantly increasing survival via extending metabolic arrest.

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FIG. 5. Induction of apoptotic DNA ladders by DNP. Incubation conditions were the same as for Fig. 2 A, control cells at commencement of experiment, Bo, left; B15, right. Lanes in groups B through I, read from left to right, with IL-3 without IL-3, with IL-3 with DNP, without IL-3 with DNP. Bo, after 2 h; C, B15 after 2 h; D, Bo after 4 h; E, B15 after 4 h; F, Bo after 8 h; G, B15 after 8 h; H, Bo cells after 20 h. From left to right, without IL-3, with IL-3 with DNP, without IL-3 with DNP. I, as for H, B15 cells (controls with IL-3 showed no apoptotic ladders and have been omitted from H and I). Molecular weight marker = λ HindIII digest.

TABLE III
Survival of clonogenic cells in the presence of DNP

| Colonies/10⁴ input cells | +IL-3 | −IL-3 | +IL-3 + DNP | −IL-3 + DNP |
|-------------------------|------|-------|-------------|-------------|
| Bo                      | 2,524 ± 200 | 63 ± 23 | <10         | 0           |
| B15                     | 888 ± 84    | 515 ± 36 | 271 ± 67   | 151 ± 71   |

FIG. 6. Induction of apoptotic DNA degradation by N-acetylcysteine. Cells were washed and recultured with or without IL-3 or N-acetylcysteine for 8 h. From left to right: control, 1 mM, and 30 mM N-acetylcysteine. A–C, without IL-3; D–F, with IL-3.
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