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Regulation of Growth of Human Diploid Fibroblasts by Factors Elaborated by Activated Lymphoid Cells

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Fibroblast growth and synthesis activities appear to be under exquisite control. This control is mediated in part by substances present in blood plasma or released by other cells. We have studied the role of peripheral blood mononuclear cells (PBM) activated with phytohemagglutinin-P (PHA) on DNA synthesis, proliferation, and the cell cycle of human diploid fibroblasts. Culture medium from activated but not from unactivated PBM cultures inhibited fibroblast DNA synthesis and growth in a dose-dependent manner. The activity, which was designated as lymphocyte factor (LF), was very potent; it inhibited 50% of the DNA synthesis and cell growth at a dilution of 1:160. It has a molecular weight between 50,000 and 100,000 daltons and it is destroyed by trypsin digestion or by heating at 80°C for 30 minutes. The activity was not due to the presence of prostaglandin. Furthermore, using immunoprecipitation and affinity chromatography, it was shown conclusively to to be distinctly different from alpha lymphotoxin (α-LT). It was not cytotoxic, as shown by the ^{51}chromium release technique. Using flow microfluorimetry it was shown that the activity regulates fibroblast growth by preventing quiescent cells in the G_0 or G_1 stage of the cell cycle from entering the S phase. Cells already in S at the time of exposure complete DNA synthesis but cannot divide, and they accumulate in G_2. The activity also has marked effects on protein synthesis. Activated mononuclear cells may play a major role in regulating fibroblast growth and synthesis in normally healing wounds and in acute and chronic inflammatory processes.

Following traumatic injury and blood clot formation, fibroblasts residing in the neighboring viable tissues begin to undergo mitosis and to migrate into the wound site. The cells continue to proliferate until a dense population is formed; then replication slows and the cells begin to produce collagen and other matrix components (Ross, 1968; Ross et al., 1970). As the matrix is restored to normal, synthesis slows and the number of resident fibroblasts decreases. Although excessive proliferation and biosynthesis of connective-tissue components can occur, such a response is rare and abnormal. Similarly, in chronic inflammatory diseases such as rheumatoid arthritis and chronic periodontitis, while fibroblast killing occurs initially (Schroeder and Page, 1972; Kobayashi and Ziff, 1973; Simpson and Avery, 1974; Ishikawa and Ziff, 1976), the fibroblast population is restored to normal during the healing process, at least in periodontitis (Listgarten et al., 1978; Lindhe et al., 1978). These observations indicate that the regulatory mechanisms are extremely sensitive and that substances regulating fibroblast growth and synthesis activities may be present in normal tissues, at sites of traumatic injury, and in inflamed tissues. Several investigators have demonstrated that substances released from leukocytes or present in blood plasma can markedly effect fibroblast activities (Ross et al., 1974; Gospodarowicz and Moran, 1975; Carpenter and Cohen, 1976; Leibovich and Ross, 1976; Ko et al., 1977; Ross and Vogel, 1978; Wahl et al., 1978; Jiminez et al., 1979; Korotzer et al., 1980). In the present studies, we show that activated peripheral blood mononuclear cells produce and release a factor or factors which regulate the growth of fibroblasts in vitro by affecting transit of the cells through the stages of mitotic cycle.

MATERIALS AND METHODS

Fibroblast cultures

Using previously described procedures (Narayanan and Page, 1976), fibroblasts were obtained from explants of normal human gingiva and maintained in Dulbecco-Vogt medium (DV) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, NY), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells used for the experiments were between the eighth and 13th transfer in culture. These cells have been characterized morphologically and biochemically (Narayanan and Page, 1976; Engel et al., 1980).

Mononuclear cell cultures

Normal human peripheral blood mononuclear cells (PBM) were isolated by sedimentation in ficol hypaque (Litton Bionetics, Kensington, MD) and washed twice with

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Hanks' balanced salt solution (HBSS). Cells (2×10^6/ml) were cultured in RPMI-1640 medium supplemented with glutamine (2mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 1% FCS with and without 15 μg/ml Phytohemagglutinin-P (PHA) (Difco Laboratories, Detroit, MI). Cultures were incubated for 72 hours in 75-mm polypropylene tissue-culture tubes (Falcon Labware, Oxnard, CA) in a 5% CO_2-humidified atmosphere, harvested by centrifugation at 400 × g for 10 minutes, and the supernatants were exhaustively dialyzed at 4°C against HBSS. The cell free-control and experimental supernatants were stored at −20°C.

Fibroblast DNA synthesis and proliferation

Cell growth and DNA synthesis were measured as previously described (Korotzer et al., 1980). Briefly, fibroblasts were harvested by exposure to 0.05% trypsin and washed with DV medium. Cells (25,000 cells in 0.5 ml of DV medium with 10% FCS) were plated subconfluently in Linbro wells (Linbro Scientific Inc., Manden, CT). After 12–16 hours of incubation, the attached cells were washed twice with 1.0 ml HBSS and made quiescent and synchronous by incubation for 48 hours in RPMI-1640 medium without serum. In cultures prepared under these conditions, fewer than 1.0% of the cell nuclei become labeled by autoradiography during a subsequent 24-hour pulse with tritiated thymidine. DNA synthesis and growth were activated by addition of fresh medium containing 10% FCS along with experimental or control PBM supernatants. DNA synthesis was measured by a 4-hour pulse with 0.5 μCi ^{125}I-iododeoxyuridine (^{125}IdUrd) (specific activity 2,000 Ci/mM; New England Nuclear, Boston, MA) containing 10^{-6} M 5-fluorodeoxyuridine (Aldrich Chemical Co., Milwaukee, WI) added to inhibit thymidylate synthetase. ^{125}IdUrd, an analogue of thymidine, can be used successfully to assess DNA synthesis (Asantila and Toivanen, 1974; Ko et al., 1977; Korotzer et al., 1980). We have done extensive testing and learned that ^{125}IdUrd used under the conditions of our experiments yields results identical to those obtained using labeled thymidine (data not shown). At the end of the pulse period, the cells were washed twice with HBSS, harvested with 0.05% trypsin, and transferred to Falcon 2025 tubes containing 0.2 ml FCS as carrier. The wells were then rinsed with 0.5 ml HBSS and the contents transferred to the tubes. Cold trichloroacetic acid was added to a final concentration of 5%, and the tubes were centrifuged at 1,000 × g for 30 minutes. The supernatants were discarded and the radioactivity in the precipitated material was measured in a gamma counter.

Cell growth was assessed over a 5-day period using a Coulter Counter. Quiescent, subconfluent cultures were activated with fresh medium containing 10% FCS with and without experimental or control supernatant. Every day half the medium was replaced with fresh medium containing experimental or control supernatant.

Cytotoxicity measurements

^{51}Cr release studies were performed as originally described by Spofford et al. (1974) and modified in our laboratory for studies on alpha-L-cells and human gingival fibroblasts (Sims et al., 1979).

Flow microfluorimetry (FMF)

Samples were prepared and stained and flow measurements were recorded and analyzed as described by Rabinovitch et al. (1981). Briefly, fibroblasts were harvested with 0.05% trypsin solutions, washed twice with phosphate-buffered saline, pelleted, and resuspended in 2.5 ml of saline. The cells were fixed by dropwise addition of 7.5 ml of cold 95% ethanol with mixing. The fixed samples, which were stored at 4°C prior to staining, were allowed to reach room temperature and harvested by centrifugation at 600 × g for 10 minutes. Cells ($0.25–1.5 \times 10^6$) were resuspended in 0.5 ml ethidium bromide (25 μg/ml in 0.1 M tris with 0.6% NaCl). After 10 minutes, 0.8 ml of mithramycin staining solution (50 μg/ml, 7.5 mM by $MgCl_2$, and 12.5% ethanol) was added. RNAse (0.1 ml of 1% solution in saline) was added to the stained cell suspension 30 minutes prior to flow analysis.

Samples were analyzed with the epiillumination flow system designed by Gohde (ICP 21; Phywe AG, Gretttingen, West Germany; now Ortho Instruments, Westwood, MA) equipped with a Tracor-Northern NS-600 multichannel analyzer. Flow rates were adjusted to approximately 200 cells/second and three to four histograms with more than 1×10^3 cells at the G_1 peak position were recorded for each sample. Data were stored and analyzed by a PDP 11/03 dedicated computer (Digital Equipment Corporation, Maynard, MA). Cell cycle compartments were determined by the method of Dean and Jett (1974).

Anti α-lymphotoxin (α-LT) antibody preparation and use

The methods for production of immunogens, immunization, and specificity of antisera have been previously reported (Lewis et al., 1977; Granger et al., 1978). In order to deplete the mononuclear cell supernatant of α-LT, it was applied to a Sepharose 4B-goat anti-α-LT column (1.2–12 cm) at 4°C. The column was equilibrated with PBS and 0.5-ml fractions were collected at 4°C. The fractions were monitored at O.D. 280 mm and appropriately pooled. The pooled fractions were diluted in RPMI-1640 medium containing 10% FCS and assayed for inhibition of DNA synthesis and alpha-L-cell cytoxocity.

RESULTS

Fibroblasts made quiescent and synchronous by serum deprivation undergo a burst or wave of DNA synthesis when incubated in serum-containing medium (Ko et al. 1977). Addition to the culture medium of supernatant fluid from PBM cultures activated by exposure to PHA results in significant inhibition of DNA synthesis (Table 1). This inhibition is dependent upon PBM activation, since the magnitude of DNA synthesis was not affected by addition of supernatant fluids from cultures to which PHA was added at the termination of the culture period. The time course of DNA synthesis (Ko et al., 1977) was not altered by supernatant fluids from control or activated lymphoid cells (data not shown). As shown in Figure 1, supernatant fluid from activated PBM cells inhibited DNA synthesis by fibroblasts by over 95% at a dilution of 1:5 and 50% at 1:160; it was still perceptibly inhibitory at 1:640. A 1:2 dilution of the supernatant prevented cell growth completely, as assessed by Coulter counter measurements over a 5-day period, and a dilution of 1:32 inhibited growth 95% (Fig. 2).

An activity similar to that which we have demonstrated has been reported previously and designated as proliferation inhibition factor (PIF) (Green et al., 1970, 1972) and inhibitor of DNA synthesis (IDS) (Namba et al., 1977).

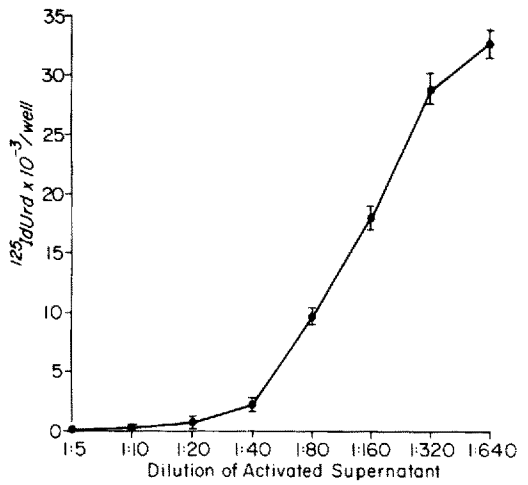

Fig. 1. The effect of varying dilutions of PHA-activated PBM culture supernatants on DNA synthesis by human gingival fibroblasts. The results are reported as the $\bar{X} \pm$ SD of ^{125}IdUrd incorporation for quadruplicate cultures.

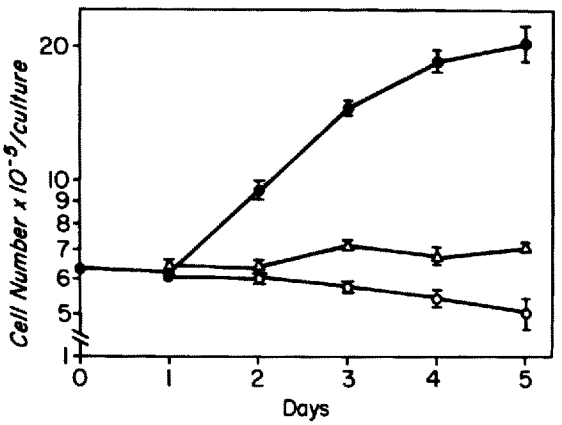

Fig. 2. The effect of PHA-activated culture supernatants on the growth of human gingival fibroblasts. Quiescent cultures were prepared and activated with medium containing 10% serum. Each day, the medium was removed and replaced with the corresponding test and control supernatant. Three replicate cultures were harvested each day with trypsin for determination of cell number, using a Coulter counter. Cultures contained: 1:2 dilution of LF (○); 1:32 dilution of LF (△); control supernatant (●).

Another growth inhibitor is α-LT, which can be cytotoxic at some concentrations (Jeffes and Granger, 1975, 1976; Lee and Lucas, 1977). Although the activity we have demonstrated may be related to other previously described growth inhibitors, we have designated it lymphocyte factor (LF), since it not only inhibits cell growth but also has marked effects on protein and collagen synthesis (Korotzer et al., unpublished). Activated PBM culture media also contain prostaglandins (Sims et al., 1979) and these substances, especially PGE_2, can inhibit DNA synthesis and growth of fibroblasts (Ko et al., 1977).

Several experiments were designed and executed to characterize LF and to learn its mechanism of action. The inhibitory activity was not a result of the presence of prostaglandin in the culture media. Prostaglandins can be eliminated from culture media by dialysis. The 50% inhibition of DNA synthesis by a 1:60 dilution of the activated PBM culture medium, which is typically observed (Fig. 1), was not changed by extensive dialysis of the culture medium prior to testing (data not shown). The drug indomethacin effectively inhibits prostaglandin synthetase in PBM cultures. As shown in Table 2, media obtained from activated PBM cultures containing indomethacin and those generated in its absence were equally effective in inhibiting fibroblast growth. Finally, LF exhibited a molecular weight of 50,000 to 100,000 in filtration experiments and was destroyed by trypsin digestion and by heating at 80°C for 30 minutes. Thus the activity has properties expected of a polypeptide.

Since LF, PIF, and IDS have molecular sizes similar to α-LT and since evidence has been presented that α-LT can inhibit growth (Jeffes and Granger, 1975, 1976), extensive experiments were done to determine the relationship between LF and α-LT activities.

Supernatants from activated PBM cultures known to contain potent LF activity were tested for cytotoxicity, using the ^{51}Cr release method. As shown in Table 3, the supernatants were cytotoxic to α-L-cells over a concentration range of 1:2 to 1:256 but not to fibroblasts. Additional experiments were designed to learn whether or not LF activity persists when α-LT activity is inactivated. Supernatant fluids containing high levels of LF and α-LT activity were treated with specific anti-α-LT antibody and the effects on both activities were assessed. As shown in Table 4, before addition of the antibody, the supernatant fluid induced 42.3% ^{51}Cr release from cultures of α-L-cells and reduced DNA synthesis by fibroblast 96.3%. After treatment with specific anti-α-LT antibody, ^{51}Cr release was reduced to background levels but LF activity remained at pretreatment levels. The addition of antibody alone to the fibroblast cultures had no perceptible effects on DNA synthesis. Since LF activity could reside in the α-LT molecule but at a site not inactivated by the specific antibody, additional experiments were done. Alpha-LT molecules were physically removed from the culture supernatants by passage over a column containing immobilized anti-α-LT antibody. As shown in Table 5, supernatants from activated PBM cultures prepared in this manner contained no measurable α-LT activity but they still contained pretreatment levels of LF activity.

Our attention was turned next to investigation of the manner in which LF interacts with fibroblasts to inhibit growth. Since LF prevents serum activation of quiescent synchronous fibroblasts, we suspected that it could act by preventing the initiation of DNA synthesis by cells in the G_0 or G_1 stages of the cell cycle. Flow microfluorimetry (FMF) experiments were done to learn whether or not this assumption is correct. This technique permits identification of the position of each cell in the replicative cycle on the basis of its DNA content. Cultures of fibroblasts arrested in the G_1 stage of the cell cycle by serum deprivation were treated with LF, followed by activation with serum-containing medium, and measurements were taken. As seen in Figure 3 and Table 6, LF prevented cells from making the transition from G_1 to S. Additional experiments were done in which actively growing subconfluent cultures in serum-containing medium were treated with LF-containing supernatants. We assumed, on the basis of the above observations, that the cells would accumulate in G_1,

TABLE 1. Effect of PHA-activated and unactivated mononuclear cell Supernatants on the DNA synthesis by human gingival fibroblasts

Additions to culture medium	cpm × 10^{-3}*
None	37.2 ± 3.1
Control supernatant	38.5 ± 4.7
Experimental supernatant	1.6 ± 0.1

*Reported as the $\bar{X}$ ± SD of ^{125}IdUrd incorporation for quadruplicate cultures. A 1:10 dilution of PBM supernatant was used.

TABLE 2. Effects of indomethacin on LF production by activated PBM

Dilution of active supernatant	% inhibition of fibroblast DNA synthesis	
	+ indomethacin[1]	− indomethacin
1:10	> 99	> 99
1:20	> 99	> 99
1:40	97	95
1:80	90	83
1:160	77	64
1:320	49	33
1:640	41	25

[1]One microgram/ml indomethacin added to PBM cultures.

TABLE 3. Cytotoxicity of supernatant from activated PBM cultures to fibroblasts and α-L-cells

Dilution of active supernatant	% Specific release ^{51}Cr	
	HGF_4	α-L-cells
1:2	2.6	34.4
1:4	1.4	31.0
1:8	1.9	28.3
1:16	2.1	22.3
1:32	2.0	16.9
1:64	2.3	13.0
1:128	1.3	9.0
1:256	1.5	6.9

TABLE 4. Effect of PHA-activated lymphoid supernatant treated with anti-α-lymphotoxin antibody on ^{51}Cr release by α-L-cells and ^{125}IdUrd incorporation by human gingival fibroblasts

Additions to culture medium	Human gingival fibroblasts (cpm ^{125}IdUrd incorporation)	α-L-cells (% specific release ^{51}Cr)
Nothing	40,140	–
Activated supernatant	1,512	42.3
Activated supernatant + anti-α-LT	1,852	1.6
Anti-α-LT	34,276	–

TABLE 5. Effect of immunoadsorption of PHA-activated PBM culture supernatant by immobilized goat anti-αLT, on LF and LT activities

Treatment	LF activity (cpm × 10^{-3})[1]	α-LT activity (% specific ^{51}Cr release)[2]
Preadsorption		
active supernatant	1.7 ± 0.2	36.5
control supernatant	48.3 ± 3.4	1.8
Postadsorption		
active supernatant	8.0 ± 1.1	2.1
control supernatant	46.7 ± 3.9	2.3

[1]Reported as the X ± SD of ^{125} IdUrd incorporation for quadruplicate cultures of human gingival fibroblasts.
[2]Reported as percentage specific ^{51}Cr release for quadruplicate cultures of α-L-cells.

but, as seen in Figure 3 and Table 6, this did not occur; the cells accumulated in G_2 plus M. In control cultures of actively growing cells, 12% of the cells were present in G_2 plus M, while the proportion in cultures exposed to LF was increased more than threefold, with a corresponding decrease in the proportion of cells in G_1 and S. Thus, some cells, probably those past a given point in the G_1 stage of the cell cycle, are permitted to complete DNA synthesis. The FMF technique does not permit differentiation between cells in G_2 and those in M. The cells accumulating in these fractions may have completed DNA synthesis and nuclear division but may not be able to under cytokinesis Alternatively, they may be arrested after completing DNA synthesis but prior to spindle formation and separation of the chromosomes, or during chromosomal separation. The correct possibility was assessed by exposing cultures of growing fibroblasts on glass coverslips to LF followed by fixation, staining, and examination under the light microscope. The mitotic index reported as the percentage of cells in mitosis was 10.3 and 9.7 for control cultures but only 0.7 for those exposed to LF-containing medium (Table 7). No binucleate cells were seen. Thus, in actively growing cultures, LF appears to arrest growth after completion of DNA synthesis but prior to spindle formation and chromosomal separation.

DISCUSSION

Fibroblasts are subjected to very complex and exquisite control mechanisms. In normal adult tissue these cells are relatively quiescent (Chu, 1960; Bullough and Laurence, 1960; Hopps and Johnson, 1976); but when tissues are wounded, proliferation of fibroblasts occurs in the surrounding viable tissue and cells migrate, presumably by chemotaxis, into the wound site where they undergo rapid mitosis and synthesis activity to restore the affected tissue. Subsequently, the size of the fibroblast population decreases and levels of growth and synthesis return to normal (Ross, 1968). During healing the cells maintain an exquisite balance among various tissue components they produce, such as various collagens and proteoglycans.

In aberrant wound healing and in some forms of inflammation, fibroblast growth and synthesis activities are abnormal and their activity, or lack thereof, contributes in important ways to the resulting connective-tissue alterations. For example, resident fibroblasts in the synovial tissues of patients with rheumatoid arthritis are killed (Kobayashi and Ziff, 1973; Ishikawa and Ziff, 1976). A similar phenomenon occurs in the gingival tissues at an early stage of periodontitis (Schroeder and Page, 1972; Simpson and Avery, 1974), while at other stages there is extensive proliferation (Hopps and Johnson, 1976). In chronic periodontitis, where detailed studies have been done, there is a net reduction in the total amount of collagen present in the affected tissue, the rate of protein and collagen synthesis and degradation are greatly accelerated, and an entirely new collagen molecule, the α-I trimer, appears (Narayanan et al., 1978). As the disease progresses, severe fibrosis of the marginal gingival tissue may occur (Page and Schroeder, 1976; Page and Narayanan, 1981). In such situations, mechanisms regulating fibroblast growth and synthesis appear to go awry.

Mechanisms regulating fibroblast growth and synthesis activities in healthy tissues and the ways in which these go awry in disease are not understood. There is reason to suspect that factors present in the blood and substances re-

TABLE 6. Effect of PHA-activated and unactivated mononuclear cell supernatants on the cell cycle distribution of quiescent and actively growing human gingival fibroblasts

State of cells	Additions to culture medium	Time of incubation (hours)	Percentage of cells in		
			G_1	S	G_2 + M
Quiescent	Nothing	0	96.9	1.1	2.0
Quiescent	Experimental supernatant	30	98.0	1.0	1.0
Quiescent	Control supernatant	30	69.6	21.0	9.4
Actively growing	Nothing	0	62.0	26.0	12.0
Actively growing	Experimental supernatant	36	48.9	14.0	37.1
Actively growing	Control supernatant	36	66.0	22.0	12.0

TABLE 7. Effect of PHA-activated and unactivated mononuclear cell supernatants on gingival fibroblasts

Additions to culture	Mitotic index[1]
None	10.3 ± 1.2
Control supernatant	9.7 ± 1.5
Experimental supernatant	0.7 ± 0.6

[1]Actively growing cultures were treated with experimental and control supernates for 36 hours. The cells attached to coverslips were fixed in Bouin's solution and stained with hematoxylin and eosin. The index was determined by counting three groups of 1,000 cells in randomly selected microscopic fields. Data expressed as $\bar{X} \pm SD$.

leased by neighboring cells may play important roles. For example, we have shown recently that the native C1 component of complement, a protein not normally encountered in significant concentrations by fibroblasts except at sites of injury and inflammation, activates a subset of diploid fibroblasts to undergo replication (Korotzer et al., 1980). Factors affecting fibroblast growth are released by blood platelets (Ross et al., 1974; Ross and Vogel, 1978; Scher et al., 1979) and by activated macrophages (Leibovich and Ross, 1976; Wahl et al., 1977; Löning et al., 1980). Activated lymphocytes are of special interest. In chronic periodontitis lymphocytes, many of which are blast-transforming cells, populate the affected gingival tissue prior to and during the periods of connective tissue alteration (Page and Schroeder, 1976). Activated lymphoid cells produce a host of lymphokines, some of which appear to affect growth and synthesis of other cells (Greene et al., 1970, 1972; Granger, 1972; Granger et al., 1973; Jeffes and Granger, 1975; 1976; Johnson and Ziff, 1976; Namba et al., 1977; Lee and Lucas, 1977; Wagshal et al., 1978; Wahl et al., 1978; Jimenez et al., 1979; Korn et al., 1980).

Our experiments have been directed at gaining a better understanding of the role of lymphocyte factors in regulating fibroblast activities. Our observations show that activated but not unactivated peripheral blood mononuclear cells release a factor or factors (LF) that inhibit DNA synthesis and growth of human diploid fibroblasts maintained in culture. The culture fluids are extremely rich in LF activity, with 50% inhibition of DNA synthesis occurring at a dilution of 1:160. LF is not a prostaglandin: It is not dialyzable and it is produced by mononuclear cell cultures containing indomethacin. The active material is a polypeptide between 50,000 to 100,000 daltons which is destroyed by heat and by protease digestion. LF activity is separable from α-LT activity. It inhibits DNA synthesis and growth by preventing activation of DNA synthesis by quiescent synchronous cells, thereby precluding their transition from G_1 to the S stage of the cell cycle, and by causing cells in actively growing cultures to accumulate in the G_2 stage.

Green et al. (1970, 1972) first demonstrated that supernatant fluids of activated lymphoid cells can inhibit DNA synthesis and proliferation of other cells. The activity they described was referred to as proliferation inhibition factor, or PIF. A substance with similar biologic activity, inhibitor of DNA synthesis (IDS), was obtained from rat lymphocytes and shown to have a molecular weight of 80,000 (Namba et al., 1977). Walker and Lucas (1972) and Granger et al. 1973) have described an 80,000-dalton polypeptide which is produced by activated B and T lymphocytes and is cytotoxic to some cells. This factor has been designated as alpha-lymphotoxin (α-LT). The relationship between PIF and α-LT has been controversial. Jeffes and Granger (1975, 1976) have provided evidence that α-LT is cytotoxic at high concentrations but at lower concentrations it suppresses DNA synthesis and growth, rather than causing cell death. They suggested that α-LT and PIF activities may reside in the same molecule. In contrast, Lee and Lucas (1977) isolated one polypeptide of 80,000 daltons which is cytotoxic for α-L-cells but which does not suppress growth of HeLa cells and another separate component of approximately the same size which is not cytotoxic but which does suppress growth of HeLa cells. Our observations appear to resolve these differences.

Using specific anti-α lymphotoxin antibody, we were able to neutralize α-LT activity completely without affecting LF activity. This experiment indicates but does not definitively prove that the two activities reside in different molecules. For example, a single molecule could have one site responsible for α-LT activity, which is neutralized by the antibody, and another site responsible for LF activity, which may not be affected by the antibody. For these reasons, we did additional experiments in which molecules having α-LT activity were removed from the samples, using a column of immobilized anti-α-LT antibody. Samples passed over the column contained pretreatment levels of LF activity, but α-LT activity could not be detected. Taken together, the data show rather clearly that different molecules are responsible for α-LT and LF activities. However, these observations do not rule out the idea that α-LT at low concentrations may also inhibit cell growth. LF may be comparable to the activity previously designated as PIF and IDS, although we have refrained from using these designations. LF has biologic activities which may or may not reside in PIF and IDS. For example, LF not only suppresses cell growth but also has marked effects on protein and collagen synthesis by fibroblasts maintained in vitro (Korotzer et al., unpublished).

The mechanism by which factors from lymphocytes interact with fibroblasts to inhibit growth have not been elucidated. Lee and Lucas (1977) presented evidence that the lymphoid cell factor they studied may inhibit DNA synthe-

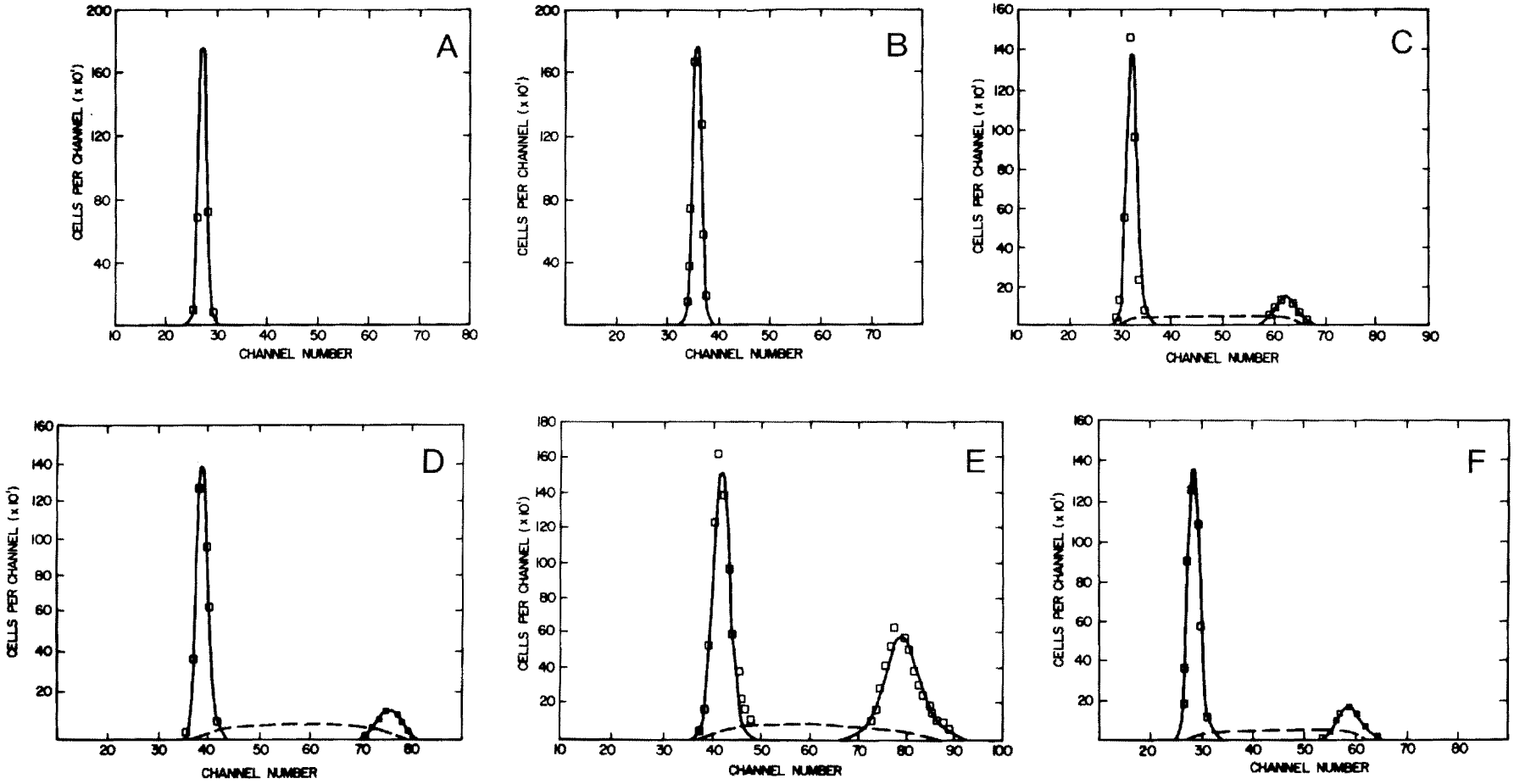

Fig. 3. Flow microfluorimetric cell cycle analysis: G_1, G_2 (solid lines), and S phase (dashed lines) compartments of the cell cycle are indicated. A. Quiescent culture prior to activation. B. Quiescent culture following activation in the presence of LF-containing supernatant. C. Quiescent culture following activation in the presence of supernatant from nonactivated PMN cultures. D. Actively growing culture. E. Actively growing culture following exposure to LF-containing supernatant. F. Actively growing culture following exposure to supernatants from nonactivated PBM cultures.

sis by inhibition of DNA polymerase activity. LF does not appear to behave in this manner. Inhibition of the activity of DNA polymerase could prevent the transition from G_1 to S, as occurs in cultures of quiescent cells exposed to LF, followed by incubation in serum-containing medium; but in actively growing cultures exposed to LF, DNA synthesis appears to continue to completion in cells which have already left G_1 and they accumulate in the G_2 stage of the cell cycle. Another possible mechanism was pointed out by Korn et al. (1980), who demonstrated that a lymphocyte-derived factor which inhibits fibroblast growth does so by stimulating prostaglandin production by the cultured fibroblasts. A mechanism of this type cannot be responsible for the inhibition of growth that we observed. LF does not induce production of growth-inhibiting concentrations of prostanglandin by the treated fibroblasts, since addition of indomethacin to the cultures had no effects on the presence or magnitude of growth inhibition (data not shown). The factor studied by Korn et al. was produced by unactivated as well as by activated lymphoid cell cultures, while LF production requires cell activation.

LF may act by causing alterations in the levels of intracellular cyclic nucleotides. Wagshal et al. (1978) have described a lymphocyte-derived inhibitor of DNA synthesis which causes the cells to accumulate in the G_1 stage of the cell cycle and induces an elevation in the levels of cyclic AMP. In addition, Bullough and Laurence (1964) have studied a tissue factor which can arrest cells in G_2 and prevent them from entering mitosis. In these cells too, there is an elevation of the levels of cyclic AMP (Remington and Klevecy, 1973). Nucleotide levels in fibroblasts exposed to LF have not been measured.

Fibroblasts are a key element in wound healing and restoration of damaged tissues. Following wounding, cells in the surrounding viable tissues enter the wound chemotactically, grow rapidly, and synthesize new connective-tissue components. As healing occurs, mitosis slows and the fibroblast population density decreases. The lymphocyte-derived activity we have described may play an important role in controlling cell growth and population size. In addition, it may permit accumulation of a subpopulation of cells which contain two complete sets of chromosomes and consequently have the capacity to respond much more rapidly to subsequent mitotic stimuli than resident cells in the G_0 or G_1 stages of the cell cycle.

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