Inhibition of Lymphocyte Proliferation by Detergent-solubilized Mouse Liver Membranes

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ABSTRACT The role of phenomena analogous to fibroblast contact inhibition in lymphocyte growth regulation is controversial, although it is clear that direct cell-cell contact is vital to immunoregulation and accessory cell function. An extract of mouse liver plasma membrane proteins, referred to as suppressive liver extract (SLE), that suppresses the growth of 3T3 fibroblasts also inhibited the mitogen-induced proliferation of murine lymphocytes. A dose of 20 μg/ml SLE was >95% suppressive of proliferation in both mouse T and mouse B cells treated with a variety of mitogens. B cell growth factor, while increasing DNA synthesis overall in mitogen-stimulated B cells, did not change the extent of SLE suppression, which suggests that the SLE does not interfere with lymphocyte–growth factor interactions. In exploring a sequence of B cell activation events, we discovered that SLE had no effect on the early activation event of increased phosphatidylinositol turnover. Blastogenesis, however, was inhibited in mitogen-stimulated, SLE-treated B cells. The maximum suppressive effect was observed if the SLE was added within 8–12 h of the mitogenic stimulus. SLE did not affect the viability of cells in culture. These results point to a possible unity of regulatory mechanisms between contact inhibition in fibroblasts and the processes of immunoregulation.

Regulation and control of cell proliferation is poorly understood. One approach to understanding the regulation of cell growth has been the study of the density-dependent inhibition of fibroblast proliferation. Fibroblasts, when grown to confluency in culture, cease dividing while retaining viability. The challenge has been to ascertain the nature of the factor or factors responsible for this growth regulation (1). In this regard, it has been demonstrated that peptide factors that regulate cell growth are located on the cell surface. Both growth inhibitory and growth stimulatory factors have been identified in preparations of plasma membranes from cultured mouse and human fibroblasts (1–4), cultured rat hepatocytes (5), and mouse liver (6). Cells released from "normal" growth regulation (transformed cells) express these factors on their cell surfaces but do not respond to them (1, 6, 7). The growth inhibitory membrane factors appear to act by reversibly halting the progression of fibroblasts through the G1 phase of the cell cycle at the same point as serum deprivation (8, 9). These inhibitory factors also induce other responses normally associated with high cell density growth arrest, such as an increase in the number of receptors for epidermal growth factor (10) and the induction of tyrosine aminotransferase in hepatocytes (11). The contention that these factors are actually peptides is supported by the observation that their suppressive action on fibroblasts is heat and acid labile (1, 8), and the further indication that the activity of the solubilized product is not affected by total delipidation (12).

One system in which cell–cell interactions are thought to play a significant role in growth regulation is the immune system. This system requires that immunocompetent lympocytes be in a resting state (G0) until triggered by appropriate stimuli. The lymphocytes then enter the cell cycle, during which they enlarge in size, proliferate, and reach terminally differentiated functional status. Each stage of this progression is critically regulated by interactions between lymphocytes, as well as between these cells and accessory cells such as monocytes and dendritic cells. The purpose of this study was to detemine if the presence of exogenous growth inhibitory factor obtained from mouse liver membranes inhibited the mitogenic response of murine T and B lymphocytes. Mouse liver membrane extracts inhibit fibroblast growth (6); this indicates inhibitory cross-reactivity between these two different cell types. The demonstration of inhibition of lymphocyte proliferation would be consistent with the notion that this system

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of inhibitory growth regulation has general applicability across a wide range of cell types. Our results indicate that suppressive liver extract (SLE)[1] from mouse liver blocked the mitogen-induced proliferation of mouse T and B cells. This effect was labile to heat treatment and resistant to delipidation of the SLE. The maximum inhibitory effect of the SLE occurred within 12 h of mitogenic stimulus. Because of this, we also examined the effect of SLE on an early event, namely phosphatidylinositol turnover. The early activation of phosphatidylinositol metabolism, which is an early event in cell activation in both T (13, 14) and B (15) cells, was not significantly influenced by SLE. These results indicate possible similarity of mechanisms between contact inhibition and lymphocyte growth regulation.

MATERIALS AND METHODS

Mice: Male BALB/c mice were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN, and were used between 6 and 12 wk of age.

Lymphocyte Isolation and Purification: B cells were isolated from mouse spleen and purified as described previously (15). Briefly, spleens were harvested from mice treated with anti-thymocyte serum (M. A. Bioproducts, Walkersville, MD), the cells isolated, and the erythrocytes lysed. The splenocytes were then depleted of adherent cells (primarily monocytes) by incubation for 1 h at 37°C in glass petri dishes (20 ml/plate) at 2×10^6 cells/ml in RPMI 1640 plus penicillin/streptomycin, glutamine (complete medium), and 10% fetal bovine serum (FBS). The remaining T cells were depleted by treatment with cytotoxic monoclonal antibodies to T cell markers, anti-thymocyte serum, and complement. Cells thus treated were >90% positive for surface Ig and had <5% T cells as determined by flow cytometric analysis. Mouse T cells were isolated from spleens of untreated mice after erythrocyte lysis and glass plate adherence. Viabilities of cells used as determined by the method of fluorescein/ethidium bromide staining (16) were always in excess of 90%.

Assay of DNA Synthesis: Mouse T cells were suspended in complete medium plus 10% FBS at 10^5 cells/0.24 ml/well in 96-well microtiter plates (Corning Glass Works, Corning, NY). Mouse enriched B cells were suspended in the same medium with 300 μM beta-mercaptoethanol at 2×10^5 cells/0.24 ml/well. Mitogens were added to cultures at time zero, (^{3}H-methyl)thymidine (^{3}H-TdR) (1 μCi/well, 6.7 Ci/mmole) (ICN K & K Laboratories Inc., Plainview, NY) was added at 62 h, and the cultures were harvested at 72 h on glass fiber filter strips. All incubations were at 37°C in a 5% CO_2 atmosphere. Total ^{3}H-TdR incorporation was determined by liquid scintillation spectrometry using 2 ml of scintillation cocktail 4a20 (Research Products International Corp., Mt. Prospect, IL). Each data point was obtained in triplicate. Percentage suppression was calculated as incorporation at a given suppressive dose divided by control incorporation (mitogen in the absence of SLE).

Assay of Phospholipid Metabolism: Phosphatidylinositol metabolism in B cells was assayed as described elsewhere (15). Briefly, enriched B cells were suspended in phosphate-free complete medium plus 2% phosphate-free FBS (FBS was dialzyed extensively against phosphate-free medium) at 2×10^6 cells/ml in 1-ml tube cultures, treated with 50 μg/ml anti-IgM, and then incubated at 37°C. 100 μCi ^{32}Pi (ICN K & K Laboratories Inc.) was added 2 h before harvest, at which time the cells were washed and the phospholipids extracted by a modified Folch extraction (17). The extracted lipids were chromatographed on oxalate-treated high performance thin layer chromatography plates (MCB Reagents, Gibbstown, NJ) in a tank equilibrated with a solvent system of chloroform, acetone, methanol, acetic acid, and water (40:15:13:12:8, vol/vol) for 3.5 h (18). Phospholipid standards (Sigma Chemical Co., St. Louis, MO) were added to cell lipid extracts and visualized in an iodine tank to confirm the equilibrium position of each phospholipid species. Autoradiograms were obtained and the spots corresponding to each phospholipid species were scraped off and counted in a liquid scintillation counter as described above.

Cell Size Characterization: Cells were cultured at 37°C in 24-well cluster plates (Corning Glass Works) at 10^6 cells/ml/well complete medium plus 10% FBS. The cells were resuspended at various times after mitogen and SLE addition, and 0.2 ml of the resuspended cells was added to 9.8 ml of Isoton for cell size analysis on a Coulter Model ZM Counter/Channelizer (Coulter Electronics Inc., Hialeah, FL). The data were analyzed and stored on an Apple IIe microcomputer.

Mitogens: For the T cells, phytohemagglutinin (Sigma Chemical Co.) and concanavalin A (Sigma Chemical Co.) were used at 10 μg/ml and 3 μg/ml, respectively. For the B cells, *E. coli* lipopolysaccharide (LPS) 055:b5 (W) (Difco Laboratories Inc., Detroit, MI) and the F(ab')2 fragment of goat anti-mouse IgM (mu chain specific) (CooperBiomedical, Inc., Malvern, PA) were each used at 50 μg/ml. B cell growth factor (BCGF) activity was obtained from conditioned media of EL-4 cells, a murine thymoma that produces BCGF (19, 20). EL-4 cells were cultured at 10^6 cells/ml in complete medium plus 1% FBS with 10 ng/ml phorbol myristate acetate. After 48 h at 37°C, the medium was centrifuged and the supernatant collected and passed over activated charcoal. This conditioned medium contained BCGF activity with no IL-2 activity, as assayed with mouse enriched B cells and the IL-2 dependent line CTLL-2 (21).

Preparation of SLE from Mouse Liver: Mouse liver membranes were prepared following the protocol devised by Aronson and Touster for the preparation of rat liver membranes (22). This procedure consists of cell homogenization by a motor-driven teflon pestle, differential low speed centrifugations to remove nuclei and whole cells, and flotation of various membrane fractions in a discontinuous high speed sucrose gradient. The original procedure described the isolation of membrane-rich fractions from both cell and nuclei: for the studies described in this paper only cellular membranes were collected. Typical membrane preparations showed a 10–15-fold enrichment for a plasma membrane marker (alkaline phosphodiesterase) with very little enrichment for any other organelle. Membrane could be stored at −70°C for up to 6 mo without any appreciable loss of growth inhibitory activity. Membranes prepared in this fashion have been shown to contain both growth stimulatory and growth inhibitory activities when added to mouse fibroblasts in culture (6).

Soluble growth inhibitory fractions were prepared by first extracting the membranes three times with 0.13 M sodium pyrophosphate/0.1 mM dithiothreitol, pH 7.4, for 60 minutes at 4°C. Such extractions remove a growth stimulatory activity from the membrane (6) while leaving the inhibitory component in the membrane. The pyrophosphate-treated membranes were then extracted with dimethyl maleic anhydride as described by Raben et al. (12) for solubilization of a 3T3 cell growth inhibitory factor. Briefly, membranes at 160 μg/ml in 0.7 mM Tris-HCl, 0.07 mM EDTA, had dimethyl maleic anhydride added to 2 mg/ml final concentration after adjusting the pH to 8.5 with 1 N NaOH. The pH was kept at 8–8.5 by the addition of 1 N NaOH as the reaction proceeded. When acid evolution stopped (after ~45 min), the membranes were recovered by centrifugation and solubilized by the addition of 30 mM octylglucoside in 10 mM potassium phosphate, 0.1 mM dithiothreitol, pH 7.4 (octylglucoside buffer), and incubated for 45 min at 4°C at a protein concentration of 0.63 mg/ml. Centrifugation at 50,000 *g* for 45 min then clarified the extract, and the supernatant was treated with 0.1 vol of 1 M sodium acetate, pH 4.0. This procedure precipitates the growth inhibitory activity as well as removes any dimethylmaleyl groups which may be on the membrane. Centrifugation (50,000 *g* for 45 min) recovered the pellet which was solubilized a second time with octylglucoside as described above. The resultant supernatant was then dialyzed extensively against 5 mM HEPES, 0.1 mM dithiothreitol, pH 7.4 (buffer A), and stored at −70°C until use. Such preparations contain both protein and some lipid, and remain active for as long as 12 mo under these conditions (6). Fractions that contained soluble inhibitor were brought to isotonicity by addition of 10 × medium and added to the cells in microtiter wells.

Delipidation of the Solubilized Inhibitor: Lipid was removed from the solubilized material by the procedure of Dean and Tanford (23) as described by Raben et al. (12). Soluble inhibitor, obtained as described above and still in the presence of octylglucoside, was precipitated by the addition of 0.1 vol of 1 M sodium acetate, pH 4.0, and the resulting pellet was resuspended in octylglucoside buffer at a concentration of 1 mg/ml. We added [^{3}H]-cholesterol (250 nmol at 2 μCi/μmol) to this solution as a lipid marker. The inhibitor was then diluted with an equal volume of 30% (wt/vol) polyethylene glycol 6000, 40% (vol/vol) glycerol in ocytlglucoside buffer. After 30 min at 4°C with occasional mixing the suspension was centrifuged at 104,000 *g* for 30 min. The pellet was suspended in the original volume of octylglucoside buffer and the precipitation with polyethylene glycol was repeated. The final pellet was then resuspended in 0.5 ml buffer A and used for growth inhibition studies. Typically, 30% of the protein was recovered, whereas >99% of the added [^{3}H]-cholesterol was found in the polyethylene glycol supernatants.

RESULTS

Effect of SLE on Proliferation of Lymphocytes

SLE had a significant and dose-dependent suppressive effect

[1] *Abbreviations used in this paper:* BCGF, B cell growth factor; FBS, fetal bovine serum; ^{3}H-TdR, (^{3}H-methyl)thymidine; LPS, lipopolysaccharide; SLE, suppressive liver extract.

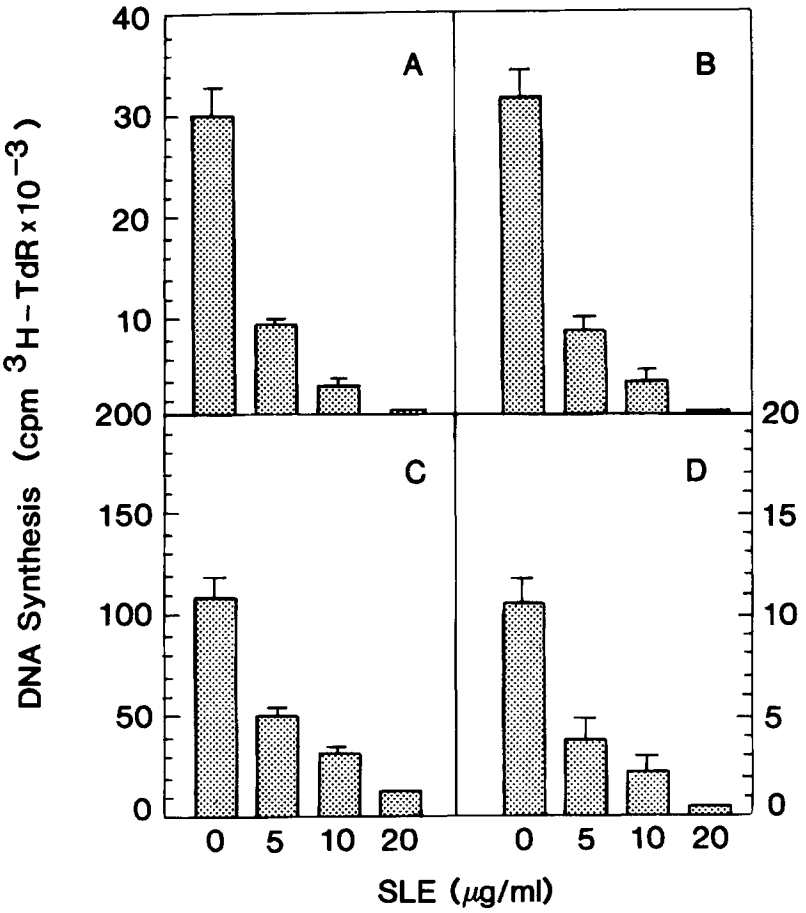

FIGURE 1 DNA synthesis is suppressed in mitogen-activated mouse lymphocytes exposed to SLE. B cells were stimulated with 50 μg/ml anti-IgM (*A*) and 50 μg/ml LPS (*B*). These data are representative of five experiments. T cells were stimulated with 3 μg/ml concanavalin A (*C*) and 10 μg/ml PHA (*D*). These data are representative of three experiments. DNA synthesis was assayed as described in Materials and Methods. Both SLE (0, 5, 10, and 20 μg/ml) and the mitogen were added to the cells at time 0. Means ± SEM of triplicates.

on mitogen-induced proliferation of both T and B cells isolated from mouse spleen (Fig. 1). DNA synthesis by B cells treated with 50 μg/ml anti-IgM or 50 μg/ml LPS was suppressed by 99% upon concurrent addition of 20 μg/ml SLE. T cells treated with 10 μg/ml phytohemagglutinin or 3 μg/ml concanavalin A were also >95% inhibited at a similar dose of SLE. A trivial explanation of this suppression would be a toxic effect of SLE on these cells. This was ruled out by the observation that 20 μg/ml SLE had no effect on cell viabilities 24 h after addition of SLE to anti-IgM-stimulated B cell cultures (Fig. 2). It is important to note that the unstimulated cells lost viability quickly, whereas stimulated cells, even in the inhibitory presence of SLE, retained viability comparable to the unsuppressed condition up to 24 h after mitogen addition. A further concern was that the suppressive effect of SLE was not protein mediated, as the extract does contain lipid. Stallcup and various co-workers (24, 25) have demonstrated that a lymphocyte lipid component will inhibit lymphocyte proliferation. As an indication of the fact that the suppressive component is not a lipid, SLE incubated for 15 min at 80°C loses its suppressive activity against fibroblasts (2, 6). To compare the behavior of SLE with a suppressive lipid, we chose 25-hydroxycholesterol, which is highly suppressive for lymphocytes (26, 27). While heat-treated SLE lost its suppressive ability, heat treatment of sterol and heat treatment of the combination of SLE and sterol did not affect their ability to inhibit lymphocyte growth (Fig. 3). Though heat treatment of the SLE ablated its inhibitory capabilities, delipidation did not (Table I). Furthermore, lipids extracted from SLE by a method similar to that of Stallcup et al. (24) had no suppressive effect (Table I). Indeed, both the lipid extract and the heated SLE actually had some similar stimu-

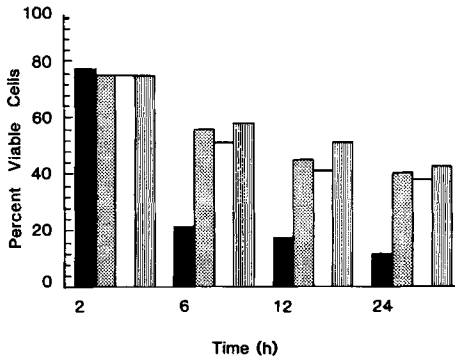

FIGURE 2 SLE is not cytotoxic. B cells were cultured in 24-well plates at 10^6 cells/ml per well for 2-24 h. Cells were either unstimulated (control, ■) or stimulated with 50 μg/ml anti-IgM in the presence of either 0 (□), 10 (□), or 20 (▥) μg/ml SLE. Percent viable cells were determined by the method of fluorescein/ethidium bromide staining.

latory activity. It is possible that this stimulatory component is the same in both cases, and that it is masked by the inhibitory protein in SLE. When the SLE is heated, the inhibitory component is inactivated and the stimulatory component reveals itself. In any case, the data indicate in the lymphocyte system that the SLE suppression is not lipid mediated.

Timing of SLE Effect and Relationship to Early Activation Events

To determine the effective temporal window of SLE's regulatory action, we added SLE to anti-IgM-stimulated B cell

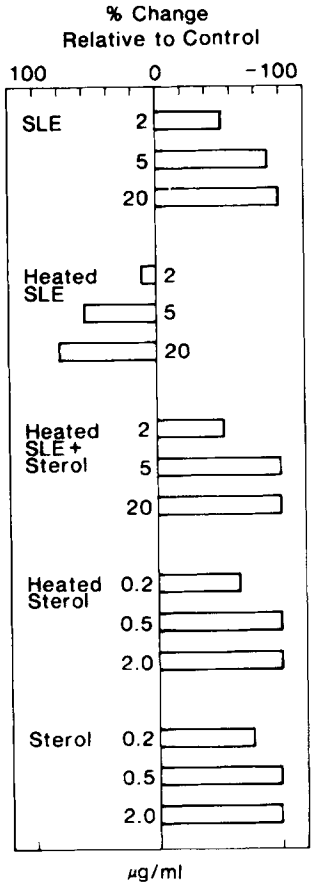

FIGURE 3 The suppressive component of SLE is a protein, not a lipid. B cells were stimulated with 50 μg/ml anti-IgM and treated as indicated, with 2, 5, and 20 μg/ml of the following: SLE, heat-inactivated SLE, heat-inactivated SLE + 25-hydroxycholesterol (sterol) (with the sterol at a 10-fold lower concentration than the SLE), and 0.2, 0.5, and 2 μg/ml heated sterol and unheated sterol. All data are expressed as percent change relative to stimulated, untreated control (5,000 cpm). These data are representative of three experiments.

TABLE I. *Lipids Extracted from SLE Fail To Suppress Proliferation**

Inhibitor	Concentration (μg/ml)				
	0.2	*0.5*	*2*	*5*	*20*
Sterol	−79%	−96%	−99%	ND	ND
SLE	ND	ND	−78%	−90%	−98%
Delipidated SLE‡	ND	ND	−57%	−94%	−99%
SLE lipids§	ND	ND	+54%	+23%	+86%

* All data are expressed as percent change relative to control stimulation (9,000 cpm) of DNA synthesis (^{3}H-TdR incorporation after a 10-h pulse with harvest at 72 h—mitogen and inhibitor added at time 0). These data are representative of three experiments.

‡ SLE delipidated with polyethyleneglycol as indicated in Materials and Methods.

§ Lipids were extracted from SLE as described by Stallcup et al. (25). 6 ml of chloroform:methanol (2:1) were added to 0.6 ml of SLE (120 μg). After vigorous mixing and 30 min at 4°C, the phases were separated by centrifugation, and the organic layer was obtained. This organic layer was mixed with 3 ml of buffer A, vortexed, and the organic layer obtained once again. The organic layer was then dried down under N_2, and resuspended in buffer A using bath sonication at the same volume as the original SLE. Equivalent dilutions to those used in the SLE dose-response curve were then used. *ND*, not determined.

cultures at varying times after the addition of the mitogen (Fig. 4). The suppressive action of SLE (10 μg/ml) was greatly reduced if the addition occurred later than 8–12 h after mitogen addition. SLE added 12 h after mitogen addition had only a 22% suppressive effect. This contrasted to 85% suppression when SLE was added with the mitogen at time 0. Since no differences in incorporation relative to control were observed when SLE was added with ^{3}H-TdR at 62 h, we ruled out another trivial explanation: that SLE interferes with ^{3}H-TdR transport or incorporation. The period during which SLE exerts its suppressive effect (0–12 h) is fairly early in the cell cycle. The maximum rate of DNA synthesis occurs 72 h after mitogen addition. Thus, for SLE to inhibit mitogenesis, it must be present early in the cell cycle, a time during which a number of significant metabolic changes are occurring in mitogen-activated cells. Such events, which are associated with the commitment of the cell to enter G1, include increased synthesis of phosphatidylinositol (15) and an increase in cell size (lymphoblast formation). We examined these events for an effect of SLE to determine whether a specific early response was inhibited. Two different mitogens were used in these experiments. Anti-IgM stimulation of B cells results in a phosphatidylinositol response, whereas LPS treatment does not (15). The lack of a phosphatidylinositol signal from LPS has been attributed (15) to the ability of LPS to bypass the need for a phosphatidylinositol signal by directly activating protein kinase C (28). Thus, for mitogens which can directly activate protein kinase C, phosphatidylinositol turnover is not an obligatory event; however for those mitogens which do not directly activate protein kinase C, phosphatidylinositol turnover is required to activate this enzyme. Accordingly, anti-IgM was used to monitor each of the activation events: phosphatidylinositol turnover, blastogenesis, and DNA synthesis. LPS was used to monitor only the latter two.

The incorporation of ^{32}Pi into phosphatidylinositol was tested at 2 and 4 h after mitogen addition using a 2-h pulse of ^{32}Pi before cell harvest. During this time period, the metabolism of phosphatidylinositol is the most active of any of the phospholipids (15). ^{32}Pi activity incorporated into phosphatidylinositol increased three- to fivefold in cells stimulated with 50 μg/ml anti-IgM as compared to unstimulated cells. This level of incorporation in the stimulated cells was not altered by the concurrent addition of 20 μg/ml SLE (Table II). In contrast, cells treated with LPS and SLE did not substantially enlarge, as compared to control cells treated with LPS alone. As is shown in Fig. 5, the LPS-stimulated cells had enlarged by 48 h, whereas the cells treated with LPS and 20 μg/ml SLE had a size profile comparable to cells treated with no mitogen. Aliquots (0.2 ml) of the same cells assayed for cell size enlargement were also assayed for DNA synthesis (data not shown). These same cells treated with 20 μg/ml SLE were, in addition to their lack of a blast response, also >90% suppressed in the later event of DNA synthesis. Though the data shown involves LPS lymphoblasts, the data obtained using anti-IgM stimulated cells was equivalent.

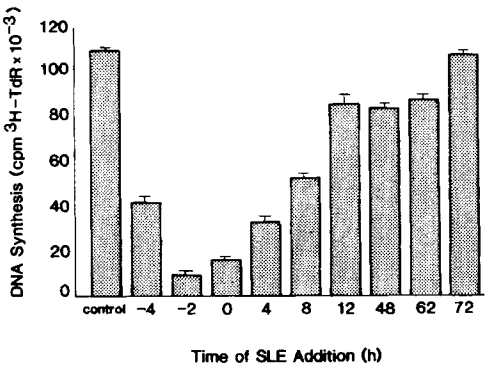

FIGURE 4 Time course of SLE effect on proliferation. Mouse B cells were stimulated with 50 μg/ml anti-IgM at time 0. 10 μg/ml SLE was added at time indicated, relative to mitogen addition. SLE was added at 62 h concurrently with ^{3}H-TdR, and at the final time point just before harvesting. These are representative results from one of two experiments. Means ± SEM of triplicates.

TABLE II. *SLE Treatment Does Not Suppress the Early Event of Phosphatidylinositol Turnover*

	Incubation*	
	2 h	*4 h*
No mitogen	2,280 ± 285	8,000 ± 658
50 μg/ml anti-IgM	6,806 ± 1,509	28,090 ± 3,166
50 μg/ml anti-IgM + 10 μg/ml SLE	7,753 ± 1,514	30,432 ± 5,093
50 μg/ml anti-IgM + 20 μg/ml SLE	7,956 ± 789	31,976 ± 2,450

* Cells were incubated for the times indicated after mitogen and SLE addition and pulsed with ^{32}Pi 2 h before harvest as indicated in Materials and Methods. All data are cpm ^{32}Pi incorporated into phosphatidylinositol—mean of triplicates ± SEM. These data are representative of three experiments.

B Cells Require B Cell Growth Factor for Optimal Mitogenesis

BCGF is thought to be required by the B cell at 4–6 h (19) after mitogen stimulation, which is during the time of the suppressive extract's greatest inhibitory activity. Enriched B cells treated with 30 μg/ml anti-IgM and 10% EL-4-conditioned medium incorporated 32,000 cpm ^{3}H-TdR at 72 h, 170% greater than the 12,000 cpm incorporated by cells stimulated with anti-IgM alone. Though DNA synthesis was greater across the SLE dose-response curve in the BCGF-supplemented cells, the inhibition caused by SLE was comparable at each dose of the inhibitor. 5 μg/ml SLE was 60% suppressive in cultures containing 10% BCGF, as compared to 62% in unsupplemented cells. The percentage suppression by 20 μg/ml SLE was essentially 100% in both instances (<200 cpm).

DISCUSSION

We have examined the suppressive effect of a mouse liver membrane extract on the mitogen-induced proliferation of mouse lymphocytes. This extract inhibited the proliferation of mouse fibroblasts (6). Four conclusions have emerged from our work. (*a*) SLE had a strong, dose-dependent, suppressive effect on mitogen-induced proliferation of mouse T and B lymphocytes. Addition of 20 μg/ml (expressed as protein concentration) of SLE caused a 95–99% suppression of DNA synthesis in both T and B cells, regardless of the mitogen used. (*b*) Loss of suppressive activity upon heat treatment of SLE, together with retention of this activity upon delipidation, indicates that the suppressive factor in SLE is a protein, and not a lipid. (*c*) The suppressive effect of SLE on mouse lymphocytes extended to movement through G1, as indicated by a failure of B cells treated with both LPS and SLE to undergo blastogenesis. Postponing the addition of the SLE 8–12 h after mitogen addition decreased the suppressive effect significantly. The suppressive effect did not extend to the early event of increased phosphatidylinositol metabolism, which was increased in stimulated cells over unstimulated control cells at 2 and 4 h, but unaffected by concurrent SLE addition. (*d*) Addition of B cell growth factor did nothing to relieve the extent of SLE suppression. This experiment suggests that the inhibitory effect of SLE is not a result of a protein in the mixture competing with lymphocyte growth factors for access to the growth factor receptors.

The question of a wider applicability of this suppressive phenomenon led us to the experiments with lymphocytes described here. The very fact that a hepatocyte membrane extract is suppressive for fibroblast growth raises the possibility that some cell surface–mediated mechanisms of growth regulation are comparable across a range of cell types including, now, lymphocytes. When SLE was added 12 h after mitogen addition it had little effect on DNA synthesis 60 h later; this observation and the data on viability would seem to rule out a simple toxic effect of the extract on lymphocytes. This is also supported by the fact that SLE had little suppressive effect on human lymphocytes (data not shown). Interestingly, lack of a toxic effect of SLE is further borne out in tests on the one transformed cell line for which we have examined the effect of SLE. EL-4 cells, a murine thymoma line (20), are completely indifferent to SLE addition (data not shown). This further differentiates our suppressive activity from that of Stallcup et al. (24, 25), as that lipid inhibitor suppresses the proliferation of both normal and transformed lymphocytes. Mouse fibroblast membranes have been shown to block

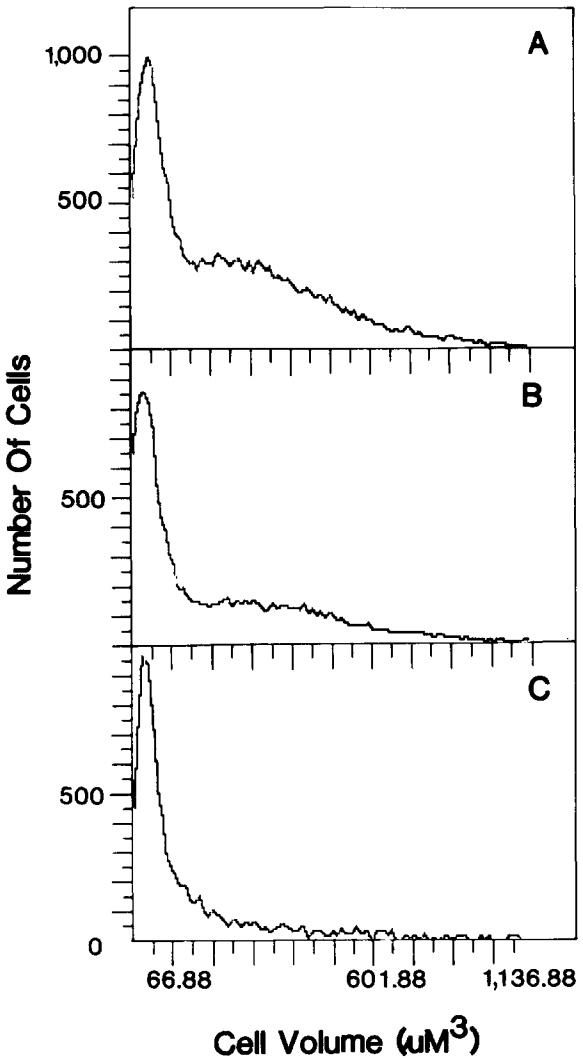

FIGURE 5 SLE suppresses mitogen-induced cell enlargement. Mouse enriched B cells were cultured for 48 h at 10^6 cells/ml in 24-well cluster plates in the presence of 50 μg/ml LPS (*A*), 50 μg/ml LPS, and 20 μg/ml SLE (*B*), and no mitogen (*C*). The cells were resuspended and cell size determined on a Coulter Counter/Channelizer. The histogram depicts the number of cells at each cell volume. The results were similar for both anti-IgM and LPS in each of four experiments performed.

growth factor-induced mitogenesis in quiescent cells (29), a situation quite analogous to what we have reported here.

The fact that SLE-treated cells failed to enlarge upon mitogenic stimulus, combined with the fact that the greatest suppressive effect on DNA synthesis occurred when SLE was provided within 12 h of the stimulus, indicates to us that the action of SLE is either early in or before G1, well before the time when the cells have proceeded far enough along the cell cycle to enlarge significantly. The small amount of suppression that did occur upon addition of SLE at a time later than 12 h may be attributable to nonsynchronous lymphocyte growth: at later times these cells are spread out along the cell cycle to some extent. Extending this examination of activation landmarks, we found no effect of SLE on mitogen-induced activation of phosphatidylinositol turnover. This allows us to narrow the target of the growth inhibitor's effect to a time after entry of the cell into G1 (marked by increased phosphatidylinositol turnover), but before the later G1 event of cell enlargement.

The importance of cell-cell communication among the cells of the immune system is indisputable, but what of the applicability of our findings to this system? Suppression of lymphocyte proliferation by the T suppressor cell is not considered to be an analogue of contact inhibition. Rather, mechanistic explanations have focused on a variety of soluble suppressor factors secreted by suppressor T cells into their environment (30). A system more analogous to contact inhibition has been described by Lerner and Hodge (31) in the WiL 2 B lymphoblast cell line. These cells stop growing at high cell density and resume growth upon dilution. As mentioned above, Stallcup et al. (24) have described the preparation of a lymphoid cell membrane extract which inhibits both normal and transformed cell growth. This inhibitor is distinct from the inhibitory component of SLE, as it is a lipid or lipidlike component which also acts on transformed cells (25).

The extremely crowded environment of a lymphocyte in a lymph node is a very different one than found in the bloodstream. Though cell culture conditions of necessity resemble the latter, it is in the former that most immune functioning actually takes place. It is also in such a crowded environment that one might expect direct cell contact phenomena, both stimulatory and inhibitory, to be of greatest physiological relevance. In this regard, we note that SLE inhibits progress along the cell cycle well before the cells have accomplished the great internal reorganization that is represented by cellular enlargement. A growth regulator that functions to keep cells in a quiescent state would be expected to act most efficiently by stopping cells early in the cycle.

In summary, we have found a fibroblast growth regulator produced from mouse liver membranes to have significant inhibitory cross-reactivity with cells of the murine immune system. The suppressive effect of SLE is manifested in a late (DNA synthesis) and an intermediate (blastogenesis) event, but not in an early (increased phosphatidylinositol metabolism) event. Experiments in progress include further characterizations of this inhibitor, together wtih explorations into the mechanism by which the factor stops lymphocyte proliferation early in G1.

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REFERENCES

1. Lieberman, M. A., and L. Glaser. 1981. Density-dependent regulation of cell growth: an example of a cell-cell recognition phenomenon. *J. Membr. Biol.* 63:1–11.
2. Whittenberger, B., and L. Glaser. 1977. Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes. *Proc. Natl. Acad. Sci. USA.* 74:2251–2255.
3. Natraj, C. V., and P. Datta. 1978. Control of DNA synthesis in growing BALB/c 3T3 mouse cells by a fibroblast growth regulatory factor. *Proc. Natl. Acad. Sci. USA.* 75:6115–6119.
4. Lieberman, M. A. 1983. Mitogenic proteins of the 3T3 plasma membrane. *J. Cell. Physiol.* 114:73–76.
5. Nakamura, T., K. Yoshimoto, Y. Nakayama, N. Tomita, and A. Ichihara. 1983. Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. *Proc. Natl. Acad. Sci. USA.* 80:7229–7233.
6. Lieberman, M. A. 1984. The presence of both growth inhibitory and growth stimulatory factors on membranes prepared from mouse liver. *Biochem. Biophys. Res. Commun.* 120:891–897.
7. Peterson, S. W., and V. Lerch. 1983. Inhibition of DNA synthesis in SV3T3 cultures by isolated 3T3 plasma membranes. *J. Cell Biol.* 97:276–279.
8. Whittenberger, B., D. Raben, and L. Glaser. 1979. Regulation of the cell cycle of 3T3 cells in culture by a surface membrane-enriched cell fraction. *J. Supramol. Struct.* 10:307–327.
9. Datta, P., and C. V. Natraj. 1980. Fibroblast growth regulatory factor inhibits DNA synthesis in BALB/c 3T3 cells by arresting in G1. *Exp. Cell Res.* 125:431–439.
10. Lieberman, M. A., T. F. Deuel, and L. Glaser. 1981. Increased epidermal growth factor binding to G0-arrested 3T3 cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 126:45–48.
11. Nakamura, T., Y. Nakayama, and A. Ichihara. 1984. Reciprocal modulation of growth and liver functions of mature rat hepatocytes in primary culture by an extract of hepatic plasma membranes. *J. Biol. Chem.* 259:8056–8058.
12. Raben, D., M. A. Lieberman, and L. Glaser. 1981. Growth inhibitory protein(s) in the 3T3 cell plasma membrane. Partial purification and dissociation of growth inhibitory events from inhibition of amino acid transport. *J. Cell. Physiol.* 108:35–45.
13. Fisher, D., and G. Mueller. 1968. An early alteration in the phospholipid metabolism of lymphocytes by phytohemagglutinin. *Proc. Natl. Acad. Sci. USA.* 60:1366–1402.
14. Akeson, A. L., D. W. Scupham, and J. A. K. Harmony. 1984. The phosphatidylinositol response and proliferation of oxidative enzyme activated human T lymphocytes: suppression by plasma lipoproteins. *J. Lipid Res.* 25:1195–1205.
15. Grupp, S. A., and J. A. K. Harmony. 1985. Increased phosphatidylinositol metabolism is an important but not an obligatory early event in B lymphocyte activation. *J. Immunol.* 134:4087–4094.
16. Ugai, K., M. Ziff, and P. E. Lipsky. 1979. Gold-induced changes in the morphology and functional capabilities of human monocytes. *Arthritis Rheum.* 22:1342–1360.
17. Folch, J., M. Lees, and G. Stanley. 1957. A simple method for the isolation and purfication of total lipids from animal tissue. *J. Biol. Chem.* 226:497–509.
18. Jolles, J., H. Zwiers, A. Dekker, K. W. A. Wirtz, and W. H. Gispen. 1981. Corticotropin-(1-24)-tetracosapeptide affects phosphorylation and polyphosphoinositide metabolism in rat brain. *Biochem. J.* 194:283–291.
19. Howard, M., and W. E. Paul. 1983. Regulation of B-cell growth and differentiation by soluble factors. *Annu. Rev. Immunol.* 1:307–333.
20. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. E. Paul. 1982. Identification of a T cell-derived B-cell growth factor distinct from interleukin-2. *J. Exp. Med.* 155:914–923.
21. Smith, K., M. Favata, and S. Oroszlan. 1983. Production and characterization of monoclonal antibodies to human interleukin 2: strategy and tactics. *J. Immunol.* 131:1808–1815.
22. Aronson, N. N., and O. Touster. 1974. Isolation of rat liver plasma membrane fragments in isotonic sucrose. *In* Methods of Enzymology, Vol 31. S. Fleischer and L. Packer, editors, 90–102. Academic Press, Inc. New York.
23. Dean, W. L., and C. Tanford. 1977. Reactivation of lipid-depleted Ca^{++}-ATPase by a non-ionic detergent. *J. Biol. Chem.* 252:3351–3353.
24. Stallcup, K. C., A. Dawson, and M. F. Mescher. 1984. Growth-inhibitory activity of lymphoid cell plasma membranes. I. Inhibition of lymphocyte and lymphoid tumor cell growth. *J. Cell Biol.* 99:1221–1226.
25. Stallcup, K. C., S. J. Burakoff, and M. F. Mescher. 1984. Growth-inhibitory activity of lymphoid cell plasma membranes. II. Partial characterization of the inhibitor. *J. Cell Biol.* 99:1227–1234.
26. Cuthbert, J., and P. E. Lipsky. 1980. Sterol metabolism and lymphocyte function: inhibition of endogenous sterol biosynthesis does not prevent mitogen-induced human T lymphocyte activation. *J. Immunol.* 124:2240–2246.
27. Cuthbert, J., and P. E. Lipsky. 1981. Sterol metabolism and lymphocyte responsiveness: inhibition of endogenous sterol synthesis prevents mitogen-induced human T lymphocyte proliferation. *J. Immunol.* 126:2093–2099.
28. Wightman, P., and C. R. H. Raetz. 1984. The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. *J. Biol. Chem.* 259:10048–10052.
29. Lieberman, M. A., P. Rothenberg, D. Raben, and L. Glaser. 1980. Effect of 3T3 plasma membranes on cells exposed to epidermal growth factor. *Biochem. Biophys. Res. Commun.* 92:696–702.
30. Tada, T. 1984. Help, suppression, and specific factors. *In* Fundamental Immunology. W. Paul, editor. 481–518, Raven Press, New York.
31. Lerner, R., and L. Hodge. 1971. Gene expression in synchronized lymphocytes: studies on the control of synthesis of immunoglobulin peptides. *J. Cell Physiol.* 77:265–276.