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.
of inhibitory growth regulation has general applicability across a wide range of cell types. Our results indicate that suppressive liver extract (SLE) from mouse liver blocked the mitogen-induced proliferation of mouse T and B cells. This effect was liable 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 spleen cells 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.

Mitogens: For the T cells, phytohemagglutinin (Sigma Chemical Co.) and concanavalin A (Sigma Chemical Co.) were used at 10 μg/ml and 5 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) (CoperBiomedical, 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 liver membranes from mouse livers. The procedure involved 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 little contamination 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 M 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 mg/ml in 0.7 M Tris-Cl, 0.07 M 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 8.5-8.8 by the addition of 1 N NaOH as the reaction proceeded. When acude 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), 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 μl medium and added to 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 [3H]-cholesterol (250 nmol at 2 μC/μmol) to this solution as a lipid marker. The inhibitor was then diluted with an equal volume of 30% (wt/vol) polyethylene glycol glycol 6000. 40% (vol/vol) glycerol in octylglucoside 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 >95% of the added [3H]-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
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 stimulatory 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

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**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.

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

| Inhibitor | 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 (3H-TdR incorporation after a 10-h pulse with 3000 cpm). These data are representative of three experiments.

The suppression 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.

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The incorporation of 32Pi into phosphatidylinositol was tested at 2 and 4 h after mitogen addition using a 2-h pulse of 32Pi before harvest. During this time period, the metabolism of phosphatidylinositol is the most active of any of the phospholipids (15). 32Pi activity incorporated into phosphatidylinositol increased three- to fivefold in cells stimulated with 50 μg/ml anti-IgM as measured by incorporation into phosphatidylinositol (Pi) before cell harvest. During this time period, the metabolism of phosphatidylinositol is the most active of any of the phospholipids (15). 32Pi 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 2 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.
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

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**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 WI.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 lymph node environment (30), a system more analogous to contact inhibition is provided within 12 h of the stimulus, indicates to us that the inhibitory component of SLE, as it is a lipid or lipidlike component which also acts on transformed cells (25).

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