Growth-inhibitory Activity of Lymphoid Cell Plasma Membranes. I. Inhibition of Lymphocyte and Lymphoid Tumor Cell Growth

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ABSTRACT Membranes isolated from normal spleen cells or lymphoid tumor cells were found to inhibit in vitro growth of several murine tumor cell lines including a B cell hybridoma, a thymoma, and a mastocytoma. 50% inhibition occurred at membrane protein concentrations of 60–100 μg/ml. A similar concentration dependence was found for inhibition of [^{3}H]-thymidine incorporation by tumor cells and for the lipopolysaccharide-induced mitogenic response of normal spleen cells. The inhibitory activity co-purified with the plasma membrane upon fractionation of crude membranes. Membrane solubilization with deoxycholate followed by dialysis to remove the detergent gave good recovery of inhibitory activity in the resulting reconstituted membranes. Membrane-mediated growth inhibition resulted from a decreased rate of proliferation and not from increased cell death. A toxic effect of the membranes was further ruled out by the finding that increasing the fetal calf serum content of the medium could substantially reverse the growth inhibition. Thus, the plasma membrane of lymphoid cells contains a component that can slow or stop the growth of cells in culture. This membrane component may have a role in cell contact-mediated regulation of growth.

Cell–cell interactions play a central role in initiation of many immune responses. Lymphocyte recognition of surface components on adjacent cells leads, in many cases, to proliferation and/or differentiation of the lymphocyte (1, 2). Plasma membranes isolated from cells bearing the appropriate surface components have been studied as a means of dissecting these recognition events. In many cases, the isolated membrane can replace the live stimulator cell in allowing recognition and triggering of the lymphocyte response (3). Similarly, artificial membranes (liposomes) bearing the relevant isolated membrane proteins can trigger responses that are normally initiated by cell–cell contact (3, 4).

Isolated membranes have also been useful in the study of density-dependent growth arrest of adherent cells. Although the mechanisms of density-dependent growth regulation are not completely understood, it appears likely that cell–cell contact is an important component of the growth regulation (5–7). Evidence supporting the importance of cell contact in the regulatory events includes the finding that isolated plasma membranes inhibit the growth of 3T3 cells in vitro (8, 9). Inhibitory activity can be recovered after detergent solubilization of the membranes, and some progress has been made in characterizing the relevant membrane components (10–12).

Although cell–cell interactions can clearly provide positive signals leading to the growth and differentiation of lymphocytes, evidence for such interactions resulting in negative growth inhibitory signals has not appeared. In 1971, Lerner and Hodge (13) reported that WiL 2, a human B lymphoblast cell line, stops growing at a high cell density and resumes growth when the cells are diluted. Since that time, many workers have used this "density-dependent growth arrest" (14, 15) as a method of achieving synchrony of lymphoid tumor cells, but the mechanisms mediating this inhibition have not been defined.

We have found that the same isolated membranes that can provide a positive signal leading to lymphocyte growth can, at somewhat higher concentrations, lead to profound inhibition of the growth of lymphoid tumor cells or the proliferative response of normal lymphocytes to mitogens. As shown here, the inhibitory activity co-purifies with the plasma membrane and does not appear to be due to a toxic effect of the membranes or to a nonspecific effect of adding protein or lipids to the cell cultures. These findings suggest the possibility

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that recognition of a plasma membrane component may play a negative regulatory role in control of lymphoid cell growth. The following article (16) describes the partial biochemical characterization of the growth inhibitory component of the membranes.

MATERIALS AND METHODS

Cells and Cell Growth: P815 mastocytoma cells[1] were maintained by intraperitoneal passage and were passaged in vitro for approximately 5 wk in RPMI 1640 (GIBCO Laboratories, Grand Island Biological Co., Grand Island, NY) before use in experiments. RDM-4 lymphoma and EL-4 thymoma cell lines were maintained by intraperitoneal passage in AKR and C57BL/6 mice, respectively. 14-4.4s hybridoma cells (17), BW5147 thymoma, and R1E/TL8X-1 cells were maintained in vitro in Dulbecco's modified Eagle's medium. All media were supplemented with 10% fetal calf serum (FCS),[2] 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine and all growth experiments were carried out at 37°C in a 5% CO_2 atmosphere. In experiments where cell numbers were determined, cells were cultured in Falcon 2-ml (2-cm^2) tissue culture wells (Falcon Plastics, Oxnard, CA) at the initial densities indicated in the figure legends. Membranes, if used, were added at the time of plating. Cells were counted in a hemocytometer, using trypan blue exclusion as a measure of viability. 600–1600 cells were counted from each culture well and standard deviations for triplicate or quadruplicate samples were ±10% or less.

Assay of BW5147 Cell Proliferation: BW5147 cells were taken from stock cultures that were in log-phase growth and plated in Linbro flat-bottomed microwells (0.28 cm^2) (Linbro Chemical Co., Hamden, CT). Cells were seeded at densities of 3.2–7 × 10^4 cells/ml, using 0.2 ml/well. When used, membranes were immediately added to the experimental cultures and the cells were incubated at 37°C. Cultures were radioactively labeled by a 6-h exposure to [^{3}H]thymidine (Tdr) (final concentration = 5 μCi/ml). At the end of the labeling period, the cells were harvested in a Bellco Microharvester (Bellco Glass, Inc., Vineland, NJ) and the incorporated radioactivity was precipitated onto glass fiber filters with 5% trichloroacetic acid. Filters were placed in Omnifluor (New England Nuclear, Boston, MA) and radioactivity was determined using a Packard Tri-carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Tdr incorporation in the presence of membranes is expressed as percent control, where control is the amount of radioactivity incorporated by identical cultures that were not exposed to membranes. All samples were in quadruplicate and standard deviations of samples ranged from ±2 to ±8% (expressed as percent of control).

Assay of Lipopolysaccharide (LPS)-induced Mitogenic Response: Spleen cells from normal mice were cultured in flat-bottomed 0.28 cm^2 microtiter wells (Linbro) in RPMI 1640 (GIBCO Laboratories) containing 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 0.5% mouse serum. Cells were plated at a density of 1 × 10^6 cells/well in 0.2 ml of the above media, and if present, 20 μg/ml LPS (DIFCO Laboratories, Detroit, MI). After 2 d in culture at 37°C, 1 μCi [^{3}H]Tdr (New England Nuclear) was added to each well for a 6-h incorporation period. Wells were harvested and incorporated radioactivity was precipitated onto glass fiber filters using a Bellco Microharvester. Dried filters were counted in a Packard Tri-Carb scintillation counter using Omnifluor scintillation cocktail. Stimulation indexes were calculated using the formula $(E - C)/C$, where E = Tdr incorporation in the presence of LPS and C = Tdr incorporation in the absence of LPS. Standard deviations were calculated for the incorporation values of the quadruplicate samples and averaged 5% or less, expressed as percent thymidine incorporation in the absence of membranes.

Membrane Preparation: Membranes from tumor cells and normal spleen cells were prepared as described (18, 19). Briefly, cells were lysed by N_2 cavitation, nuclei were removed by low-speed centrifugation and a particulate fraction, crude membranes (CMs), was pelleted by centrifugation at 22,000 *g*. This fraction includes plasma membrane (PM) and endoplasmic reticulum (ER) and accounts for ~8% of the total cellular protein. The crude membranes could be further fractionated by centrifugation on a sucrose step gradient. PMs were obtained as an interfacial band on the gradient, whereas the ER, together with some of the PMs, formed a pellet (18, 19). The average yield of CMs was 160 μg/10^7 cells from P815 and 40 μg/10^7 from RDM-4. The average yield of PM was 10 μg/10^7 cells from P815 and 5 μg/10^7 cells from RDM-4. Recovery of PM marker enzyme activity was ~50% in the CM fraction and 20–30% in the PM fraction (19). Protein content of membrane fractions was measured by the method of Lowry et al. (20), in the presence of 1% SDS using bovine serum albumin as the standard.

Detergent Solubilization and Reconstitution of Membranes: Membranes (CM) from P815 were solubilized with deoxycholate (DOC, Sigma Chemical Co., St. Louis, MO) by mixing equal volumes of CM suspension and 1% DOC, and then adding 0.5% DOC to give a final detergent/protein ratio of 5:1 (wt/wt). All DOC containing solutions were made in Tris-buffered saline (10 mM Tris, 140 mM NaCl, pH 8). After standing for 15 min on ice, the detergent-soluble and -insoluble fractions were separated by centrifugation at 100,000 *g* for 45 min. An aliquot of the detergent-solubilized material was taken for protein determination (9), and the remainder of the material was dialyzed for 48 h at 4°C against 2 × 4 liter of Tris-buffered saline containing 4 mM $CaCl_2$. The detergent-insoluble material was resuspended in 1.5 ml of 0.5% DOC, an aliquot was taken for protein determination, and the remaining material was dialyzed as above. After dialysis the samples were centrifuged at 100,000 *g* for 45 min and the resulting pellets were resuspended in RPMI 1640 at protein concentrations of 3.8 mg/ml (based on the amount of protein present at the beginning of dialysis).

RESULTS

Membrane-mediated Inhibition of Tumor Cell Growth

CMs at concentrations of 5–25 μg/ml will stimulate generation of an allogeneic cytolytic T lymphocyte response by spleen cells, an immune response that requires spleen cell recognition of cell surface glycoproteins (transplantation antigens) on the membranes (3, 19). At the same time, two- to fivefold higher concentrations of CMs inhibited generation of the response (20a). The potential complexity of this effect on a response by a whole spleen cell population, including the possibility of specific immune mechanisms being involved, prompted an examination of the effects of membranes on the growth of homogeneous lymphoid tumor cell populations.

A CM fraction was prepared from RDM-4 lymphoma cells and examined for its effect on in vitro growth of P815 cells (a mastocytoma) or 14-4.4s cells (a B cell hybridoma). Cells were put into culture at low density and allowed to grow for 3 d in the absence or presence of CMs. At the end of this time, the number of live cells in the culture was determined by counting the trypan blue-excluding cells. In all cases, the number of dead cells was <20% of the total cells and the percent of dead cells did not differ significantly between control and inhibited cultures. As shown in Fig. 1, the growth of both types of cell was profoundly inhibited in the presence of membranes, with 50% inhibition occurring at doses of 60–100 μg of membrane protein/ml. Higher doses of membrane (125–300 μg/ml) gave virtually complete growth inhibition.

The membrane-mediated growth inhibition seen in these experiments could occur by one of several mechanisms. Cell division might proceed normally in the presence of membranes but with an increased rate of cell death to yield lower numbers of live cells. Alternatively, membranes or a toxic component in the preparations could kill susceptible cells in the culture, allowing a subpopulation of unaffected cells to grow up and finally dominate the culture. Finally, membranes might slow the growth of cells by increasing the time between cell divisions. It was possible to distinguish between these

[1] Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education, and Welfare publication No. [National Institutes of Health] 78–23, revised 1978).

[2] *Abbreviations used in this paper:* CM, crude membrane; DOC, deoxycholate; ER, endoplasmic reticulum; FCS, fetal calf serum; LPS, lipopolysaccharide; PM, plasma membrane; Tdr, thymidine.

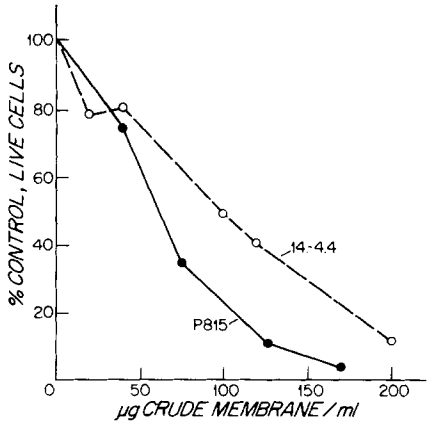

FIGURE 1 Inhibition of P815 and 14-4.4s cell growth by membranes (CM) from RDM-4 lymphoma cells. P815 mastocytoma and 14-4.4s hybridoma cells (17) were adjusted to a density of 1.5 × 10^4 cells/ml and plated in 2-ml (2 cm^2) tissue culture wells in the absence or presence of the indicated concentrations of RDM-4 crude membranes. Cells were counted after 3 d of culture at 37°C. Dead cells were <20% of the total (live plus dead) cells in all cases. The densities of the control (nonmembrane treated) cultures on day 3 were 7.9 × 10^5 cells/ml for P815 and 6.8 × 10^5 cells/ml for 14-4.4s.

alternatives by examining the detailed growth kinetics of cells in the absence and presence of membranes. A cloned hybridoma cell line, 14-4.4s (17), was used for these experiments to minimize the possible presence of variant subpopulations. Cells were placed in culture with various concentrations of RDM-4 CM and cell counts were done at daily intervals. The results (Fig. 2*A*) demonstrated that cell division was slowed in the presence of membranes from one division every 12 h in control cultures to one division every 17 h in cultures exposed to 127 μg of CM/ml. Although the growth curves do not appear dramatically different when plotted on a log scale (Fig. 2*A*), the inhibition caused by membranes was very significant and comparable to that shown in Fig. 1. Thus, by day 3 of the experiment, cultures containing 127 μg of CM/ml had only 40% as many cells as the controls. Fig. 2*B* shows the number of dead cells, as determined by trypan blue exclusion, as percent of the total (live and dead) cells in the culture. During the first 4 d, the time of most active growth, the ratio of live to dead cells was the same for control and inhibited cultures. The percentage of dead cells in the control culture begins to increase dramatically at saturation density. A similar effect was seen with the inhibited cultures on days 6 and 7, as they too, reached maximum densities. Thus, growth inhibition showed no correlation with an increase in number of dead cells. These results indicate that membranes are not inhibiting by a cytotoxic mechanism, but instead act to slow the rate of cell division.

Membranes from several cell lines (P815, RDM-4, R1E/TL8X.1) have been examined for their ability to inhibit the growth of P815, RDM-4, 14-4.4s, EL-4, and BW5147 tumor cells. Inhibition was seen in all cases and the concentration of CM protein required for 50% inhibition ranged from 50 to 300 μg/ml. No significant or reproducible differences have been noted in the inhibitory activity of membranes from different cell lines or in the sensitivity of different cell lines to inhibition. As in the experiments shown above, no increase in the number of dead cells was seen in the inhibited cultures when compared to controls.

Inhibition of DNA Synthesis and Co-purification of Inhibitory Activity with Plasma Membranes

Membrane-mediated growth inhibition, as measured by direct cell counting, was apparent within 24 h (Fig. 2*A*). Inhibition of DNA synthesis was detectable at earlier times after membrane addition if [^{3}H]Tdr incorporation was measured. Significant inhibition of incorporation was observed after a 6-h exposure of BW5147 cells to CM and [^{3}H]Tdr (data not shown). The inhibition had the same membrane concentration dependence as was seen when growth was measured by cell counts over longer periods of time. The lower cell numbers and smaller culture volumes (0.2 ml) needed for assay of [^{3}H]Tdr incorporation, made it feasible to further examine the subcellular localization of the inhibitory activity.

CMs can be further fractionated by centrifugation on a sucrose step gradient to yield two fractions, one enriched in PM and one enriched in ER. The PM fraction is enriched four- to eightfold in PM-localized enzyme markers in comparison to the ER-enriched fraction (18, 19). Examination of the effects of these fractions on [^{3}H]Tdr incorporation by BW5147 cells during a 6-h pulse showed a similar enrichment of inhibitory activity in the PM fraction in comparison to the ER (Fig. 3*A*). Similar levels of PM-mediated inhibition were also seen when cells were cultured in the presence of membranes for 24 h and then pulsed with [^{3}H]Tdr for 6 h (Fig. 3*B*). Comparison of a number of purified PM preparations from RDM-4 cells showed that the concentration needed to achieve 50% inhibition ranged from 15 to 70 μg of membrane protein/ml (Fig. 3*B*). In each case, a higher concentration of the ER-enriched fraction was needed to achieve 50% inhibi-

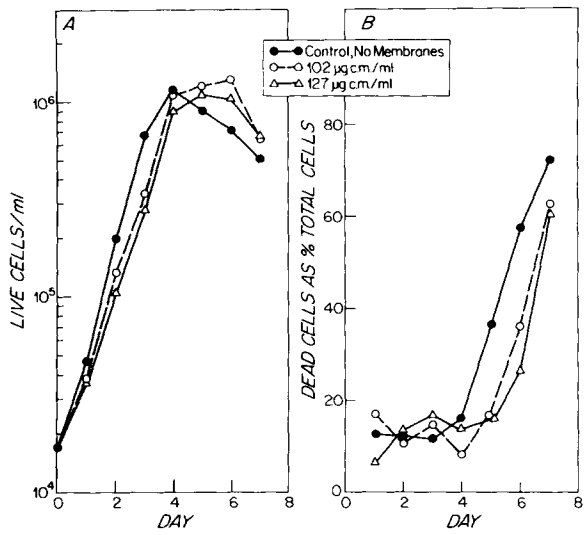

FIGURE 2 Growth of 14-4.4s cells in the absence or presence of RDM-4 cell membranes. 14-4.4s cells were placed in culture at an initial density of 1.5 × 10^4 ml, as described in the legend to Fig. 1. At the time of plating, RDM-4 cell membranes at concentrations of 102 or 127 μg of membrane protein/ml were added to appropriate wells whereas the control culture received no membranes. Cultures were maintained at 37°C and aliquots of cells were taken for counting at ~24-h intervals. The number of live (trypan blue-excluding) and dead (trypan blue-including) cells were determined by counting a cell sample that contained 600–1,600 live cells. Concentrations of crude RDM-4 membranes used were 0 μg/ml (●), 102 μg/ml (○), and 127 μg/ml (△). (*A*) Cell growth; (*B*) dead cells in cultures, expressed as percent of total (live and dead cells).

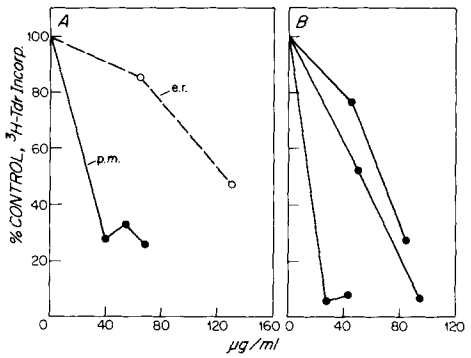

FIGURE 3 Purified PMs inhibit Tdr incorporation by BW5147 thymoma cells. (*A*) Inhibitory activity of RDM-4 plasma membrane (*p.m.*) and endoplasmic reticulum (*e.r.*) enriched fractions. BW5147 cells were plated at a density of 9.2×10^4 cells/ml in 0.2 ml (0.28 cm^2) microwells in Dulbecco's modified Eagle's medium supplemented with 10% FCS, glutamine, and antibiotics as described in Materials and Methods. [^{3}H]Tdr and the indicated concentrations of PM- or ER-enriched fractions were added and cells were cultured for 6 h at 37°C in a 4% CO_2 atmosphere. Incorporation of radioactivity was then measured as described in Materials and Methods. 100% incorporation (controls without added membrane) was 9.8×10^3 cpm. (*B*) Inhibitory activity of several RDM-4 PM preparations. BW5147 cells were plated at 2.4×10^4 cells per well as described above. PMs from three independent preparations were added and the cells were cultured for 24 h. Cultures were then pulsed for 6 h with [^{3}H]Tdr and incorporation of radioactivity was determined. 100% incorporation (controls without added membrane) was 18×10^3 cpm.

tion. Variation in the inhibitory activity present in different PM preparations might result from differences in the content of growth inhibitory molecule(s), differences in physical characteristics of the preparations (size of vesicles, degree of aggregation, content of inside-out vesicles, etc.) or both.

Co-purification of the inhibitory activity with the PM fraction and the corresponding decrease in activity in the ER fraction argue strongly against inhibition resulting from nonspecific toxic affects of adding protein or membranes to the cultures. In addition, it was previously shown that incubation of ^{51}Cr-labeled tumor cells for several hours in the presence of CM or PM at concentrations up to 1 mg/ml caused no detectable chromium release from the cells (21, 22). More direct evidence for a reversible, nontoxic mechanism of membrane-mediated inhibition was obtained in experiments examining the effects of FCS on inhibition.

Effects of Serum Concentration on Membrane-Mediated Inhibition

Whittenberger and Glaser (8) have found that 3T3 cell membranes can arrest the growth of sparse 3T3 cell cultures, and this inhibition is partially reversed when cells are cultured in medium with a higher serum concentration. Similarly, [^{3}H]Tdr incorporation by BW5147 cells was more effectively inhibited by membranes in the presence of 10% FCS than in the presence of 20% FCS (Table I, lines *A* and *D*). Furthermore, cells cultured in the presence of membranes for 8 h (line *C*) or 24 h (line *B*) in 10% FCS and then brought to 20% FCS were less inhibited during a subsequent 6 h [^{3}H]Tdr pulse than cells cultured with membranes and 10% FCS for 30 h. These results suggest that inhibition, even after 24-h exposure to membranes, is at least partially reversible.

One explanation for the serum effect would be that membranes act to deplete the medium of a component essential for growth. To examine this possibility, we incubated inhibitory concentrations of membranes in complete medium for 24 h under the same conditions used for the growth experiments. The samples were then centrifuged to pellet the membranes and the supernatants examined for their ability to support cell growth (as measured by [^{3}H]Tdr incorporation), in comparison to medium that had been incubated in the same way in the absence of membranes. No significant differences were observed between growth in control versus membrane-pretreated medium (not shown). This was the case when medium containing either 10% FCS or 0.5% mouse serum was used. Thus, the membranes appear to inhibit growth by a direct effect on the cells, and not via a medium depletion effect.

Attempts to more directly demonstrate the reversibility of inhibition by culturing cells in the presence of membranes, followed by removal of the membranes, have yielded variable results. The variability is probably due to the difficulty of effectively separating the cells from membranes by centrifugation. Similar experiments done using partially purified inhibitor incorporated into liposomes have further demonstrated the reversibility of inhibition and are described in the following article (16).

Inhibitory Activity of Normal Spleen Cell Membranes

The results described above demonstrate that lymphoid tumor cell plasma membranes have an inhibitory activity for tumor cell growth. Membranes (CM) prepared from normal murine spleen cells were found to have a similar inhibitory activity for tumor cell growth (Fig. 4) and inhibition occurred in the same concentration range as seen for tumor cell membranes. We also examined the effects of membranes on a normal lymphocyte response, the B lymphocyte mitogenic response to LPS (23). As with tumor cell growth, membranes from lymphoid tumor cells inhibited the mitogen-induced proliferative response (Fig. 5). The effect of CM on the LPS-induced response of spleen cells from five different strains of mice was also examined. In every case, a similar dose response

TABLE I

*Effect of Serum Concentration on Membrane-mediated Growth Inhibition**

Culture conditions	Inhibition in the presence of membranes‡
	%
(*A*) 30 h in 10% FCS	96
(*B*) 24 h in 10% FCS: 6 h in 20% FCS	86
(*C*) 8 h in 10% FCS: 22 h in 20% FCS	78
(*D*) 30 h of 20% FCS	48

* BW5147 cells were placed in culture at a density of 3.2×10^4 cells/ml alone or with RDM-4 CMs at 290 μg/ml. The concentrations of FCS in the media are indicated in the table, and increases in FCS concentration were accomplished by adding an appropriate amount of undiluted serum to the cultures at the indicated times. After incubation for 24 h at 37°C, the cultures were pulsed for 6 h with [^{3}H]Tdr and incorporation of radioactivity determined.

‡ Calculated as $E/C \times 100$ where E is incorporation in test cultures and C is incorporation in cells cultured under identical conditions but in the absence of membranes. Control values are: $A = 30 \times 10^3$ cpm; $B = 24 \times 10^3$ cpm; $C = 21 \times 10^3$ cpm; $D = 32 \times 10^3$ cpm.

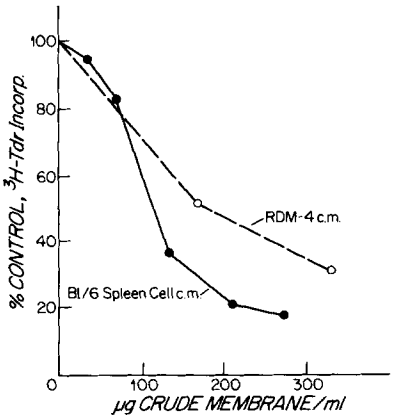

FIGURE 4 Normal spleen cell membranes inhibit [^{3}H]Tdr incorporation by BW5147 cells. Normal cells were obtained by removing and teasing apart the spleens of 100 normal adult C57BL/6 mice and crude membranes (c.m.) were prepared as described in the text. BW5147 cells at 7 × 10^4 cells/ml, membranes, and [^{3}H]Tdr were incubated for 6 h and incorporation was determined. 100% incorporation was 12 × 10^3 cpm.

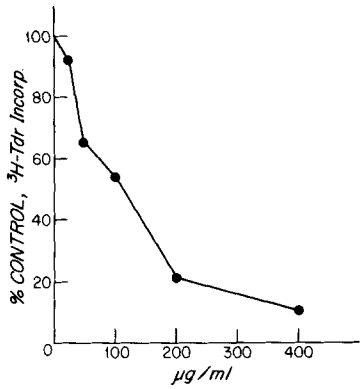

FIGURE 5 Inhibition of the LPS-induced mitogenic response of normal spleen cells by P815 tumor cell membranes. Spleen cells from BALB/c mice were cultured in the presence of LPS, as described in Materials and Methods with the indicated concentrations of CM from P815 cells. After 48 h, cultures were pulsed with [^{3}H]-Tdr for 6 h and incorporation of radioactivity was determined. 100% incorporation (control cultures with added LPS but without membranes) was 154 × 10^3 cpm. Incorporation in cultures without added LPS was 19 × 10^3 cpm.

for inhibition was found. CM isolated from normal spleen cells similarly inhibited the LPS-induced response (not shown). Thus, membranes of both normal and transformed lymphocytes have a growth inhibitory activity that can affect the proliferation of either normal lymphocytes or lymphoid tumor cells.

Solubilization and Reconstitution of the Inhibitory Activity

Co-purification of the growth inhibitory activity with the PM fraction strongly suggests that the inhibitor is a membrane component. Further evidence for this was provided by experiments examining solubilization and reconstitution of the inhibitory activity. P815 membranes were solubilized with deoxycholate at a detergent to protein ratio of 5:1 (wt/wt) and material insoluble in the detergent was pelleted by centrifugation at 100,000 *g* for 45 min. Under these conditions, 20–25% of the membrane protein remains insoluble (24). The solubilized membrane fraction was then reconstituted by dialysis to remove the DOC and the resulting reconstituted membranes pelleted by centrifugation at 100,000 *g* for 45 min. The detergent-insoluble fraction was resuspended in DOC, dialyzed, and pelleted in the same way and both fractions were examined for inhibitory activity. As shown in Table II, the inhibitory activity was effectively solubilized by detergent treatment of the membranes and ~75% of the activity was recovered in the sedimentable, reconstituted membranes. Reconstituted membranes also retained inhibitory activity as assessed by measuring [^{3}H]Tdr incorporation by BW5147 cells (data not shown). Thus, the inhibitor(s) is solubilized by detergent, is not lost during dialysis, and is incorporated in active form into the reconstituted membrane vesicles. These findings, together with co-purification with the PM, very strongly indicate that the inhibitor(s) is a component of the membrane.

DISCUSSION

Isolated PMs can replace intact stimulator cells in providing positive signals leading to lymphocyte growth and differentiation (3, 4), signals normally delivered via cell–cell contact. The results described here demonstrate that membranes isolated in the same way can, at somewhat higher concentrations, also deliver a negative, growth inhibitory signal to lymphoid tumor cells or normal lymphocytes responding to LPS. Inhibition appears to occur as a result of a decreased rate of cell growth, not from any increase in cell death. The inhibitory activity co-purifies with the PM and is present in membranes of both lymphoid tumor cells and normal spleen lymphocytes. Further evidence that the inhibitory activity is due to a membrane component was provided by the demonstration that the inhibitor(s) could be solubilized by detergent treatment of the membranes and subsequently reincorporated into sedimentable reconstituted membranes.

The inhibition observed in these experiments is very unlikely to result from nonspecific effects of adding proteins or membranes to the cultures. >50% inhibition was obtained using concentrations of 30–70 μg/ml PM protein in medium

TABLE II

Detergent Solubilization and Reconstitution of the Inhibitory Activity in Crude Membranes

Fraction*	Protein‡	Amount required for 50% inhibition§	Inhibitory activity‖ U/mg	Inhibitory activity‖ Total units
	mg	*mg/ml*		
Crude membrane	4.30	0.119	8.4	36
Detergent soluble (reconstituted)	3.14	0.114	8.8	27
Detergent insoluble (reconstituted)	0.99	0.253	4	4

* CMs from P815 cells were solubilized in DOC and reconstituted by dialysis as described in the text.

‡ Values for soluble and insoluble fractions are amounts recovered in each fraction obtained from detergent treatment of 4.3 mg of CM.

§ Inhibitory activity was assayed in an LPS-induced response by BALB/c spleen cells (See legend to Fig. 5). Dose responses were determined for each fraction using protein concentrations ranging from 25 to 400 μg/ml. Control incorporation (in the absence of membranes) was 20 × 10^3 cpm.

‖ One unit of inhibitory activity is defined as milligrams of protein per milliliter needed to cause 50% inhibition of the spleen cell response to LPS.

containing FCS protein concentrations of ~7 mg/ml. Furthermore, enrichment of inhibitory activity in the PM fraction, and corresponding decrease in activity in the ER fraction, would not be expected if inhibition were due to nonspecific effects of membrane addition. Consistent with this conclusion is the fact that it has been possible to partially purify a minor membrane component which appears to account for most, if not all, of the growth inhibitory activity present in the membranes (16).

Examination of the kinetics of cell growth in normal and inhibited cultures by direct cell counting indicated that inhibition occurs as a result of a decreased rate of proliferation, and not as a result of an increased rate of cell death. Cultures inhibited >50% had no higher proportion of trypan blue including-cells than did control cultures at any time (Fig. 2*A*). Inhibition of proliferation occurred rapidly and could be detected by a decrease in [^{3}H]Tdr incorporation within 6 h of exposure of cells to membranes. Toxic effects of membranes are further ruled out in these short-term assays by the previous observations that incubation of ^{51}Cr-labeled lymphoid tumor cells with 5–10-fold higher membrane concentrations caused no chromium release over several hours (21, 22). It was also previously observed (21, 22) that exposure to high concentrations of membranes over several hours did not affect the ability of cytotoxic T lymphocytes to bind and lyse target cells (an event that requires metabolically active lymphocytes). Additional evidence against a toxic mechanism is provided by the demonstration that the membrane-mediated inhibition was at least partially reversed by increasing the serum content of the medium (Table I).

Whittenberger and Glaser (8) have found that plasma membranes from 3T3 cells can inhibit DNA synthesis of these cells. In contrast to our findings that both normal and transformed lymphocytes are inhibited by membranes, they found that SV-40 transformed 3T3 cells (SV3T3) were not inhibited. Evidence suggesting that SV3T3 cells may lack the receptor that mediates inhibition has appeared recently (9). Whether lack of sensitivity to the inhibitor present in 3T3 membranes is unique to the SV-40 transformed line or is a general property of transformed fibroblasts has not been reported.

The inhibitory activity present in lymphocyte membranes also appears to differ from that of 3T3 cells with respect to the nature of the inhibitory molecule(s). The available evidence indicates that the inhibitor(s) present in 3T3 membranes is a membrane protein (10–12). In contrast, the inhibitor(s) present in the lymphocyte membrane is protease insensitive and has the properties of a lipid or lipid-like molecule, as described in the following article (16).

The finding that PMs of lymphoid cells have a growth inhibitory activity raises the possibility that cell–cell interactions in the immune system may lead not only to proliferative responses but, in some cases, to inhibition of growth. Thus, cell contacts may have both positive and negative regulatory roles. Consistent with this suggestion, we have found that membranes will inhibit antigen-specific responses of normal lymphocytes in the same concentration range as seen in the experiments described here (20a). The fact that lymphoid cells retain sensitivity to this inhibition after transformation suggests the possibility that a better understanding of the mechanism of this inhibition might lead to means of controlling the growth of transformed cells.

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