

Growth-inhibitory Activity of Lymphoid Cell Plasma Membranes. II. Partial Characterization of the Inhibitor

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ABSTRACT We have shown that plasma membranes from lymphoid cells have inhibitory activity for the growth of normal lymphocytes and lymphoid tumor cells (Stallcup, K. C., A. Dawson, and M. F. Mescher, *J. Cell Biol.* 99:1221–1226). This growth-inhibitory activity has been found to co-purify with major histocompatibility complex class I antigens (H-2K and D) when these cell surface glycoproteins are isolated from detergent lysates of cells by affinity chromatography on monoclonal antibody columns. When incorporated into liposomes, the affinity-purified H-2 antigens inhibited the growth of both normal lymphocytes and tumor cells at concentrations of 1–3 $\mu\text{g/ml}$. Inhibition was readily reversed upon removal of the liposomes from the cell cultures, even after several days of exposure of cells to the inhibitor. Inhibitory activity was insensitive to protease digestion or heat treatment, indicating that it was not due to the H-2 glycoproteins. This was confirmed by the demonstration that inhibitory activity could be separated from the H-2 protein by gel filtration in the presence of deoxycholate and could be extracted from membranes or H-2 antigen preparations with organic solvents. The results demonstrate that the growth-inhibitory component(s) of the plasma membrane is a minor lipid or lipid-like molecule which retains activity in the absence of other membrane components. The findings reported here and in the preceding article suggest that this novel membrane component may have a role in control of lymphoid cell growth, possibly mediated by cell contacts.

Many cultured adherent cell lines stop growing as the cultures reach monolayer confluence. The mechanisms responsible for this density-dependent growth control may include depletion of growth factors (1, 2), release of soluble inhibiting factors (3–5), or cell-to-cell contact (6–8). Evidence in support of a role for cell contact in growth control has been provided by observations that plasma membranes isolated from 3T3 cells inhibit DNA synthesis when added to *in vitro* cultures of 3T3 cells (9, 10). This inhibitory activity can be recovered after detergent solubilization of the membranes and the inhibitor has been partially purified. The properties of the inhibitor indicate that it is a membrane protein(s) (11–13).

Lymphoid cells also stop growing at high cell densities and resume growth upon dilution into fresh medium (14). The basis of this growth regulation has not been extensively studied. Clearly, any of the mechanisms suggested above for density-dependent growth control of adherent cells may also be important in regulation of lymphoid cell growth. A role for cell contact in the growth regulation of lymphocytes is

suggested by our finding that membranes isolated from these cells will inhibit *in vitro* growth of normal and transformed lymphocytes (15) and generation of immune responses (15a).

Positive signals, leading to lymphocyte proliferation and/or differentiation, and normally resulting from cell–cell contacts, can be provided by membranes from cells bearing the appropriate surface antigens (16, 17). The same membranes, at somewhat higher doses, inhibit growth and the inhibitory activity was found to co-purify with the plasma membrane (15). Growth inhibition resulted from a decreased rate of proliferation, not from any increase in the rate of cell death. The inhibition could be partially reversed by increasing the serum concentration in the medium, even after exposure of the cells to membranes for 24 h. Further evidence that the inhibitory activity was due to a membrane component(s) was provided by the finding that it was solubilized by treating membranes with detergent and could be subsequently recovered in reconstituted membranes formed upon removal of the detergent by dialysis.

Class I major histocompatibility complex antigens, H-2K and H-2D in the mouse, are polymorphic cell surface glycoproteins whose recognition by lymphocytes can stimulate proliferation and/or differentiation of the lymphocytes (18, 19). They consist of a glycosylated heavy chain of ~47,000 mol wt, which spans the membrane, and a noncovalently associated light chain (β_2 -microglobulin) of 13,000 mol wt. These antigens have been purified from detergent solubilized membranes by affinity chromatography using monoclonal antibodies bound to Sepharose (20, 21). When incorporated into liposomes, the antigens retain activity for stimulating specific *in vitro* lymphocyte responses (21–23). This report describes evidence demonstrating that the growth-inhibitory activity of membranes co-purifies with the H-2 antigens during affinity chromatography. H-2 antigen-containing liposomes were found to inhibit growth at concentrations of 1–3 μg protein/ml. Because the liposomes can be readily separated from cells, it was possible to directly demonstrate the reversible nature of the inhibition. Further study demonstrated that the inhibitor was not the H-2 glycoprotein but another membrane component which co-purifies with it. Unlike the inhibitory component of 3T3 membranes (11–13), the inhibitor(s) is not a membrane protein. Instead, it is a minor lipid or lipidlike component that can be extracted from H-2 antigen preparations or membranes by organic solvents.

MATERIALS AND METHODS

Mice and Cell Lines: Mouse strains¹ used in these experiments included (BALB/c \times DBA/2)F₁ (CD2F₁) from Cumberland Farms (Clinton, TN) and AKR, BALB/c, C57BL/6, (C57BL/6 \times A/J)F₁ (B6AF₁), and (AKR \times DBA/2)F₁ (AKD2F₁) from Jackson Laboratory (Bar Harbor, ME). Tumor cell lines used included P815, a mastocytoma, RDM-4, a lymphoma, the thymoma lines, EL4 and BW5147, and RIE/TL8X.1, a mutant lymphoma line (24). P815, RDM-4, and EL4 were routinely maintained by intraperitoneal passage in CD2F₁, AKR, and C57BL/6 mice, respectively. BW5147 and RIE/TL8X.1 were maintained *in vitro*. Tumor cells were grown *in vitro* at 37°C using RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin.

Assay of Cytolytic T Lymphocyte (CTL)² Responses: CD2F₁ mice were immunized by intraperitoneal injection of 2×10^7 RDM-4 tumor cells. 4–6 wk later, spleen cells were removed and placed in culture (7×10^6 cells per 2 ml of culture volume) and H-2 antigen was added. Control cultures were done in parallel without added antigen. 5 d later the cells were harvested, washed, and counted, and mixed with ⁵¹Cr-labeled RDM-4 target cells. After 4 h at 37°C, cells were pelleted, supernatants collected, and the ⁵¹Cr-release determined. Percent specific release was calculated as $(E - C)/(FT - C) \times 100$, where *E* is isotope release from tubes containing immune effector cells plus targets, *C* is isotope release from tubes containing normal spleen cells plus targets, and *FT* is maximum isotope release determined after four cycles of freezing and thawing the target cells. Details of the culture conditions and chromium release assay have been published (16).

Assay of Growth-inhibitory Activity

TUMOR CELL GROWTH: *In vitro* growth of tumor cells was done in 2-

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:* CTL, cytolytic T lymphocyte; DOC, deoxycholate; LPS, lipopolysaccharide; MAb, monoclonal antibody; TBS, Tris-buffered saline; Tdr, thymidine; TX-100, Triton X-100.

cm² Linbro wells (Linbro Chemical Co., Hamden, CT) in 2 ml of RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin. Cultures were incubated at 37°C in a 5% CO₂ atmosphere. Growth was assessed by cell counting using a hemocytometer, and trypan blue was included to allow an estimate of the number of dead cells present. Standard deviation of triplicate or quadruplicate samples was $\pm 10\%$ or less.

[³H]THYMIDINE (Tdr) INCORPORATION BY TUMOR CELLS: [³H]-Tdr incorporation by BW5147 tumor cells was measured as described in the preceding article (15). Tdr incorporation in the presence of inhibitor is expressed as percent control, where control is the amount of radioactivity incorporated in identical cultures that were not exposed to inhibitor. All samples were done in quadruplicate and standard deviations ranged from $\pm 2\%$ to $\pm 10\%$ (expressed as percent of control).

LIPOLYSACCHARIDE (LPS)-INDUCED MITOGENIC RESPONSE: The mitogenic response of normal spleen B cells to LPS was measured by [³H]Tdr incorporation as described in the preceding article (15). Unless indicated otherwise, spleen cells from BALB/c mice were used in the assays. Tdr incorporation in the presence of inhibitor is expressed as percent of control, where control is the incorporation in identical cultures that were not exposed to inhibitor. Standard deviations of quadruplicate samples averaged $\pm 10\%$ or less, expressed as percent of control. The stimulation index, a measure of the efficacy of mitogen-induced proliferation (described in the preceding article [15]) is included in the figure legend for each experiment.

Membrane Isolation: A membrane preparation, containing both plasma membrane and endoplasmic reticulum, was isolated from tumor cells as described previously (16) and in the preceding article (15). In some cases the membranes were further fractionated by sucrose density-gradient centrifugation to yield fractions enriched in either plasma membrane or endoplasmic reticulum (15, 16).

Affinity Purification of H-2K and D Antigens: H-2 antigens were affinity purified using monoclonal antibodies (MAbs) covalently coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The 11-4.1 MAb (25) was used for purification of H-2K^k from RDM-4 cells. M1/42, an antibody specific for H-2 glycoproteins but lacking haplotype specificity (21), was used for purification of H-2K^d and D^d from P815 cells. The purification procedures using these MAbs have been described in detail (20, 21, 26). Briefly, tumor cells or membranes bearing the appropriate antigen(s) were lysed by suspension in 0.5% Triton X-100 (TX-100) at 4°C. The lysate was then centrifuged to remove insoluble material, passed over a Sepharose 4B precolumn and then over the MAb-Sepharose column. After loading, the MAb column was washed with 5–10 column volumes of the lysis buffer and the bound antigen then eluted with 0.5% deoxycholate (DOC) in 0.14 M NaCl, 10 mM Tris, pH 8 (Tris-buffered saline, TBS) for 11-4.1 columns, or 0.5% DOC, 0.5 M NaCl in TBS for M1/42 columns. Because TX-100 is toxic to cells, the first two or three elution fractions containing antigen were usually not included in the final pooled antigen preparations. Although resulting in a decreased yield of H-2 antigen, this avoided the possibility of TX-100 carry-over and potential effects of the detergent in cell growth experiments. (TX-100 is readily detectable by thin-layer chromatography and was not found in the H-2 antigen preparations examined in this manner.)

In most purifications, a small number of cells ($5\text{--}10 \times 10^7$) were surface labeled with ¹²⁵I by lactoperoxidase-catalyzed iodination, lysed with TX-100, and added to the bulk cell lysate to allow monitoring of the purification procedure (26). Preparations were normally done using 10^{10} or more cells and yields were ~0.8 mg H-2K^k per 10^{10} RDM-4 cells and 0.1–0.2 mg H-2K^d from P815 cells. In some cases, both H-2K^k and H-2D^k were purified from RDM-4 by serial passage of the lysate over an 11-4.1 column (H-2K^k) followed by an M1/42 column (H-2D^k) (21).

Incorporation of H-2 Antigens into Liposomes: Lipids used for formation of liposomes were obtained by chloroform/methanol (2:1) extraction of P815 cells (22). The extract was washed with 0.3 vol of water, dried under an N₂ stream, suspended in 0.5% DOC in TBS, and stored at –20°C until used. Lipid concentrations were determined as organic phosphate content. Yield of extracted lipid was ~10 μmol phospholipid per 10^9 cells. A detergent-insoluble matrix from membranes was included in some liposome preparations (22). It was prepared by extracting membranes (at 1 mg/ml) with 0.5% TX-100 in TBS for 20 min at 4°C, centrifuging at 100,000 g for 45 min, and washing the pellet once in PBS. The washed pellet was then resuspended in 0.5% DOC in TBS. This insoluble material accounts for ~20% of the membrane protein and consists of a protein matrix present in the form of structures having the same size distribution as the original membrane vesicles (27).

Preparation of H-2 antigen-containing liposomes by detergent dialysis and their characterization have been previously described (22). Briefly, liposomes were prepared by mixing affinity-purified H-2 antigen (in 0.5% DOC, TBS) with lipid at a ratio of 1 μg protein to 5–10 nmol phospholipid (~1 mol protein/

300–600 mol phospholipid). In some experiments, liposomes were prepared using dimyristoyl phosphatidyl choline (Sigma Chemical Co., St. Louis, MO) at a ratio of 1 μ g H-2 antigen to 1 μ g dimyristoyl phosphatidyl choline, instead of lipids extracted from P815 cells. Mixtures were then dialyzed versus 4 liters of TBS for 24 h and then versus 4 liters TBS, 4 mM CaCl_2 for an additional 24 h. The resulting liposomes were concentrated by centrifuging the dialyzed mixture for 90 min at 100,000 *g*. Under these conditions, ~50–70% of the lipid and H-2 antigens are recovered in the pellet in the form of small unilamellar vesicles and most of the protein is exposed to protease at the liposome surface (22).

In cases where the membrane matrix was included, the starting mixture in 0.5% DOC contained H-2, matrix, and lipid in the proportions of 1 μ g H-2, 10 μ g matrix, and 13 nmol lipid. Dialysis and concentration was done as described above. It appears that a bilayer, with incorporated H-2, reforms on the membrane matrix under these conditions (22). The resulting liposomes are larger and more irregular in shape than those obtained using just lipid and protein.

Heat and Protease Treatment of H-2K^k: In experiments to examine stability of the growth inhibitory activity, affinity-purified H-2K^k (containing a trace of ¹²⁵I-H-2) in 0.5% DOC, TBS was heat or protease treated before formation of liposomes. Heat treatment was done by incubating a 15 μ g/ml solution at 100°C (boiling water bath) for 10 min, followed by cooling on ice. Protease treatment was done by incubating 22 μ g/ml H-2K^k with 56 μ g/ml pronase (Calbiochem-Behring Corp., San Diego, CA) in 0.5% DOC, TBS with 10 mM CaCl_2 for 24 h at 37°C. Fresh pronase (56 μ g/ml) was then added and incubation continued for 19 h at 37°C. Liposomes were prepared from untreated and treated H-2K^k using 7.5 nmol lipid per 1 μ g of starting protein (before treatment). 80% of the ¹²⁵I-H-2 radioactivity was recovered after dialysis of the untreated and heat-treated samples. Less than 5% of the radioactivity was recovered with the pronase-treated sample.

Gel Filtration of H-2 Antigens and Solubilized Membranes: Gel filtration was done on a 120 \times 0.75-cm Bio-Gel A, 0.5 M (Bio-Rad Laboratories, Richmond, CA) column equilibrated with 0.25% DOC in TBS. Column calibration was done using Blue Dextran (excluded volume), bovine serum albumin (68,000 mol wt), ovalbumin (45,000 mol wt), ribonuclease A (13,700 mol wt), and fluorescein malaemide (included volume). The column was eluted at a flow rate of ~20 ml/h and fractions of 2.8 ml were collected. Affinity-purified ¹²⁵I-H-2 in 0.5% DOC, TBS was applied directly to the column. Membranes were solubilized in 0.5% DOC, TBS at a detergent to protein ratio of 5:1 for 20 min at 4°C. The sample was then centrifuged at 100,000 *g* for 45 min to pellet insoluble material (~20% of the total protein) and the soluble fraction applied to the column.

Inhibitory activity in the fractions was determined by removing an aliquot of each fraction, adding lipid, and dialyzing to form liposomes. Each liposome sample was then tested at four concentrations over an eightfold concentration range for inhibition of an LPS-induced mitogenic response. The amount of material needed for 50% inhibition of the response was defined as one inhibitory unit and the total units in each fraction was calculated as $T/[(S/L)(X)]$, where *T* is the total volume of the fraction, *S* is the volume of aliquot used to make liposomes, *L* is the volume of medium used to resuspend the liposome pellet, and *X* is the volume of liposome suspension needed for 50% inhibition of the response.

Organic Extraction and Thin-layer Chromatography: Organic extractions were done using 2:1 1-butanol/6 M pyridinium acetate, pH 4.2. Affinity-purified H-2 antigens (at 15–30 μ g/ml) and gel filtration fractions containing inhibitor were dialyzed overnight versus 4 liters of 50 mM NaCl before extraction (to remove DOC). To control for possible introduction of inhibitory material from the organic solvents, we extracted equal volumes of buffer and processed them in the same way as the samples. Extraction was done by mixing equal volumes of sample and butanol/6 M pyridinium acetate and incubating for 30 min at room temperature with occasional vortexing. The mixtures were then centrifuged and the organic and aqueous phases collected and dried under an N₂ stream at 40–45°C. 0.5% DOC, TBS was added to dissolve the dried films, lipid added (5 nmol/ μ g starting H-2 Ag) and liposomes prepared. Membranes were suspended in 50 mM NaCl at 0.75 mg/ml and extracted and dried in the same way. Serum-free RPMI 1640 medium was then added, and the dried films were resuspended using a bath sonicator.

Thin-layer chromatography was done on silica gel G plates (Analtech, Inc., Newark, DE) using a solvent system of chloroform/methanol/H₂O, (60:35:8). Samples were applied directly to the plates in aqueous buffer and dried. Lipid standards (Supelco Inc., Bellefonte, PA) were applied in chloroform/methanol (1:1). After developing and drying the plates, lipids were visualized by exposure of the plate to iodine vapor.

Analytical Methods: Protein concentration was determined by the method of Lowry et al. (28) in the presence of 1% SDS using BSA as the standard. Organic phosphate was determined as described by Ames and Dubin

(29). SDS PAGE was done using the buffer system described by Laemmli (30) with a 3% stacking gel and a running gel consisting of a 5–15% gradient of polyacrylamide. Before electrophoresis, samples were reduced with 5% β -mercaptoethanol and heated for 3 min at 100°C in sample application buffer. After electrophoresis proteins were visualized by staining with Coomassie Blue and autoradiography was done to visualize ¹²⁵I-labeled protein using Kodak XR5 film (Eastman Kodak, Inc., Rochester, NY).

RESULTS

Co-purification of H-2 Antigens and Growth-inhibitory Activity

Purification of H-2 antigens by affinity chromatography on MAb–Sephrose columns results in good yields (70–90%) of highly purified antigen (20, 21, 26). When examined by SDS PAGE, the only major bands detected by Coomassie Blue staining are the glycosylated heavy chain of ~47,000 mol wt and the noncovalently associated β_2 -microglobulin of 13,000 mol wt (not shown). Minor contaminants can sometimes be detected but are not reproducibly seen, and in many preparations are undetectable.

The purified H-2K and D glycoproteins can be incorporated into small unilamellar liposomes by dialysis to remove detergent from a mixture of the protein and lipid in DOC (22). In this form, the antigens are active in stimulating generation of an allogeneic CTL response (21, 22), a response that depends upon lymphocyte recognition of H-2 antigens via cell surface receptors. Recognition of the antigen was found to be more effective if a detergent-insoluble matrix fraction isolated from plasma membrane vesicles was included during liposome formation (22). Stimulation of a CD2F1 (H-2^d) anti-H-2^k CTL response by H-2K^k containing liposomes is shown in Fig. 1A. Consistent with previous results, a response was generated when antigen was added to the cultures at 0.05–0.5 μ g/ml. However, the level of response decreased dramatically when antigen concentration was increased to 1–2 μ g/ml (Fig. 1A).

A variety of mechanisms, including specific immune responses, might account for a decreased CTL response to a high dose of antigen. However, as described in the preceding article (15), we had found that plasma membranes of lymphoid cells contain an inhibitor of normal lymphocyte and lymphoid tumor cell growth. This raised the possibility that the growth-inhibitory activity was co-purifying with the H-2 antigens and that it was this inhibitor that was acting to decrease the CTL response at the higher antigen concentrations. We therefore examined the effect of H-2 antigen-containing liposomes on the *in vitro* growth of lymphoid tumor cell lines and the mitogenic response of spleen cells to LPS, both of which are inhibited by membranes (15).

Liposomes containing the membrane matrix and either H-2K^k (purified using the 11-4.1 MAb) or H-2K^d and D^d antigens (purified using the M1/42 MAb) were added to low density cultures of P815 cells and the number of live cells determined 3 d later. Both types of liposomes caused a dose-dependent inhibition of cell growth (Fig. 1B), with 50% inhibition occurring between 0.5 and 1 μ g/ml. Liposomes prepared with identical lipid and membrane matrix but in the absence of purified H-2 antigens were also examined. These liposomes had no effect on the cell growth (Fig. 1B). H-2 antigen-containing liposomes (using a number of independent antigen preparations) were also found to inhibit *in vitro* growth of EL-4, CH-1, and RDM-4 lymphoid tumor cells, and 14-4.4 and 10-2.16 hybridoma cells (not shown). In every

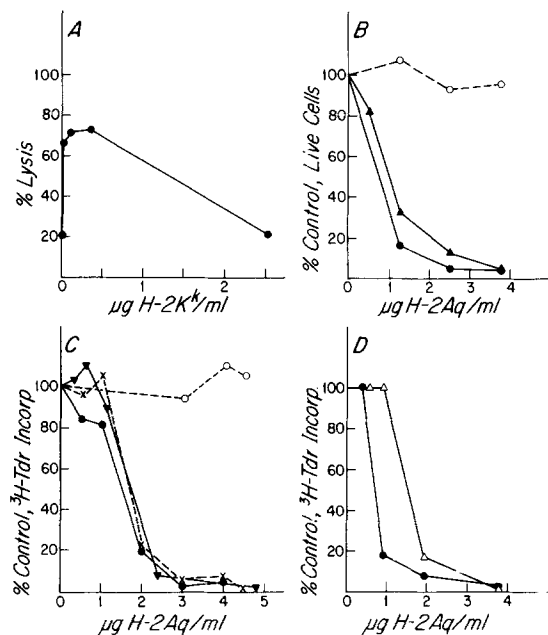


FIGURE 1 Inhibitory activity of purified H-2 antigens (H-2 Ag) in liposomes. (A) Stimulation and inhibition of a CTL response. H-2K^k was incorporated into liposomes using lipid and membrane matrix. Liposomes were added to cultures of CD2F₁ spleen cells from mice previously immunized with RDM-4 (H-2^k) cells. Cells were removed from culture 5 d later, washed, and assayed for lytic activity using ⁵¹Cr-labeled RDM-4 target cells. Data shown were obtained at an effector/target ratio of 60:1. Spleen cells maximally stimulated with 6 × 10⁶ x-irradiated AKR (H-2^k) cells gave 80% specific release. (B) Inhibition of P815 tumor cell growth. Liposomes were prepared from lipid, membrane matrix, and either H-2K^k (●) or H-2K^dD^d (▲), or without H-2 antigen (○). P815 cells were put into culture at 1.25 × 10⁴ cells/ml in 2 ml of medium with or without liposomes and counted 3 d later. Control cultures had 8.8 × 10⁵ ± 5% cells/ml (100% control). (C) Inhibition of mitogen-induced proliferation of BALB/c B cells. Spleen cells from BALB/c mice were stimulated in vitro with LPS in the presence or absence of H-2 antigen as described in Materials and Methods. [³H]Tdr incorporation in the absence of inhibitor was 39,000 ± 2,000 cpm and the stimulation index was 9.8. Liposomes were prepared using only lipid (○), lipid and H-2K^k (●), or H-2D^k (▼). Affinity-purified H-2K^k was also dialyzed to remove DOC in the absence of added lipid. The final dialysis was done versus PBS and the dialyzed sample was added directly to the cultures (x). (D) Inhibition of mitogen-induced proliferation of B10.Br B cells. Spleen cells from B10.Br mice were stimulated in vitro with LPS in the presence or absence of H-2 antigen as described in Materials and Methods. [³H]Tdr incorporation in the absence of inhibitor was 24,000 ± 3,000 cpm and the stimulation index was 10.7. Liposomes were prepared using dimyristoyl phosphatidyl choline and H-2K^k (●) or H-2^d (Δ).

case, the dose response was similar to that shown in Fig. 1B, with 50% inhibition occurring at 0.5–1.5 µg/ml H-2 antigen. In all of these experiments, the proportion of trypan blue including (dead) cells was the same in control and inhibited cultures.

Liposomes containing either H-2K^k, H-2D^k or H-2K^dD^d were also examined for their effect on the mitogenic response of B lymphocytes from two different strains of mice, BALB/c and B10.BR. In every case, the H-2 antigen-containing liposomes were inhibitory, while liposomes lacking the antigen were not (Fig. 1, C and D). Similar results have been obtained in experiments examining the response of cells from

CD2F₁ and C57BL/6 mice (not shown). Thus, the growth-inhibitory activity does not appear to be genetically restricted, as it is neither haplotype nor strain specific. Small differences in the dose-response curves for different H-2 antigen preparations were sometimes seen in individual experiments (Fig. 1D) but these differences were not consistently reproducible. In experiments examining many independent H-2 antigen preparations, 50% inhibition of the LPS-induced response occurred in the range of 0.5–6 µg/ml.

While including the detergent-insoluble membrane matrix in liposomes increases the efficiency of H-2 antigen recognition by CTL (22), this was not the case for growth-inhibitory activity. The liposomes used in the experiments shown in Fig. 1, C and D were made using only lipid and purified H-2 antigens, and the control liposomes from lipid alone. Furthermore, addition of lipid to the H-2 antigen preparation to form liposomes was not required. Removal of detergent by dialyzing of the H-2 antigen solution in the absence of lipid resulted in good recovery of the growth-inhibitory activity (Fig. 1C).

Growth Inhibition Is Reversible

Experiments done using membranes provided strong evidence that growth inhibition occurred as a result of a decreased rate of cell growth and was not due to an increased rate of cell death (15). Increasing the concentration of fetal calf serum in membrane-inhibited cultures resulted in some reversal of inhibition (15). However, attempts to more directly demonstrate reversal by removing the inhibitor were only partially successful (unpublished results), probably due to the difficulty of separating the membranes from the cells. A relatively homogeneous population of small (0.03–0.1 µm) unilamellar liposomes results from dialysis to remove detergent (22) and these liposomes can be readily separated from cells by differential centrifugation (31). It was therefore possible to directly examine the reversibility of inhibition mediated by H-2 antigen-containing liposomes.

P815 tumor cells were placed in culture at low density with and without H-2K^k-containing liposomes. After 3 d, cultures in the presence of liposomes contained 80% fewer cells than controls (Fig. 2A). At this time cells from inhibited cultures were washed by differential centrifugation to remove the liposomes, placed back into culture, and examined 2 d later. Removal of the liposomes resulted in an increased growth rate, and by day 5 the washed cells had reached the same density as control cultures. In contrast, cells left in the presence of liposomes continued to grow very slowly and, by day 5, had ~70% fewer cells than in control or washed cultures. Thus, cells exposed to inhibitor for 3 d, with 80% inhibition occurring, remained viable and resumed normal growth upon removal of the inhibitor.

The dose response for inhibition occurs over a very narrow concentration range, with a twofold increase in inhibitor concentration resulting in a 60–90% increase in inhibition (Fig. 1). Because of this sharp dose response, it was also possible to demonstrate reversal by simply lowering the liposome concentration in inhibited cultures by dilution with medium. In the experiment shown in Fig. 2B, RDM-4 tumor cell growth was almost completely inhibited by 1.2 µg of H-2 antigen/ml in liposomes, but the cells resumed a normal growth rate upon lowering the concentration of liposomes to 0.6 µg protein/ml by dilution. Thus, as with P815 cells, RDM-4 cells remained viable in the presence of inhibitor for days

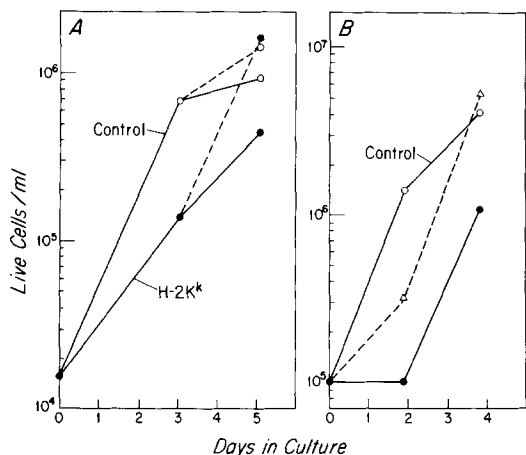


FIGURE 2 Growth inhibition by H-2 antigen-containing liposomes is reversible. (A) Reversal of inhibition by removal of liposomes. P815 cells were put into culture at a density of 1.6×10^4 cells/ml in the presence or absence of liposomes. Liposomes were prepared using dimyristoyl phosphatidyl choline, membrane matrix, and H-2K^k and were used at 5 μ g of H-2/ml. Cells were counted on days 3 and 5. At day 3, parallel cultures were either left under the same conditions or the cells were removed, washed once by centrifugation (1,600 rpm in a CRU centrifuge for 10 min) to remove liposomes and placed back into culture. Controls without liposomes, either unwashed (—○—) or washed (---○---). H-2K^k liposomes added and cells unwashed (—●—) or washed (---●---). (B) Reversal of inhibition by dilution of liposomes. RDM-4 cells were put into culture at 1×10^5 cells/ml in the presence or absence of liposomes. Liposomes were prepared using lipid and H-2K^k and were added at an initial concentration of 0.8 or 1.2 μ g of H-2/ml. On day 2, 1 ml of the 2-ml cultures was removed and the cells were counted. 1 ml of fresh medium was then added to the remaining cultures and the cells were counted again on day 4. Controls in the absence of liposomes (○); liposomes present initially at 0.8 μ g of H-2/ml and diluted to 0.4 μ g/ml on day 3 (Δ); liposomes present initially at 1.2 μ g/ml and diluted to 0.6 μ g/ml on day 3 (●).

but resumed active growth when the liposome concentration was diluted below the threshold level needed for inhibition. These results further confirm that inhibition is not due to a toxic effect resulting in cell death but instead acts by slowing the growth rate of the cells.

Growth Inhibitor(s) Is Not the H-2 Glycoprotein

Although H-2 antigens isolated by affinity chromatography on MAb-Sepharose columns are highly purified, the possibility existed that the growth-inhibitory activity was not due to the H-2 glycoprotein but to another membrane component which co-purified with the H-2 antigens. We therefore examined the effects of heat and protease treatment on the inhibitory activity. The activity was found to be heat stable, even after incubation of the H-2 antigen preparation in a boiling water bath for 10 min (Fig. 3). Furthermore, most of the inhibitory activity was recovered after papain (not shown) or pronase treatment (Fig. 3). To monitor the effectiveness of the pronase treatment, we used 125 I-H-2K^k in these experiments. Pronase treatment of the sample before liposome formation resulted in loss of all of the radioactivity from the dialysis bag during DOC removal and liposome formation. Thus, the resulting liposomes retained inhibitory activity (Fig. 3) despite extensive degradation of the H-2 glycoprotein. The small (and variable) loss of inhibitory activity after protease

treatment may result from a decreased recovery of liposomes in the absence of protein.

Further evidence that the H-2 glycoprotein was not the inhibitor was provided by examination of the inhibitory activity of membranes from a mutant cell line, R1E/TL8X-1 (24), which does not express H-2 antigens on the cell surface. Membranes from these cells had growth-inhibitory activity comparable to that found for membranes from RDM-4 (not shown) and other cells bearing H-2 antigens.

These results strongly suggested that the inhibitor was neither the H-2 glycoprotein nor dependent on the protein for activity. This was directly confirmed by the finding that H-2 antigens and the inhibitor could be separated by gel filtration in DOC (Fig. 4A). Affinity-purified 125 I-H-2K^k was applied to a Bio-Gel A 0.5 M column and elution done using 0.25% DOC in TBS. The H-2 glycoprotein eluted at a position corresponding to an apparent mol wt of $\sim 110,000$. While the mol wt of H-2 antigens is $\sim 60,000$, these proteins elute at higher apparent molecular weights on gel filtration in detergent (32), probably as a result of interaction with the detergent. In order to locate the inhibitor, equal volume aliquots of each fraction were then mixed with lipid, dialyzed to form liposomes, and tested for inhibitory activity in a B cell proliferative response. The growth-inhibitory activity was recovered as a single peak at an apparent mol wt of 35,000, well separated from the H-2 glycoprotein.

The inhibitory activity present in P815 membranes was also examined by gel filtration in DOC (Fig. 4B). Membranes were solubilized in 0.5% DOC in TBS and centrifuged at 100,000 g for 45 min to remove insoluble material. This insoluble fraction contains <5% of the inhibitory activity (15) and was discarded. The soluble fraction was applied to the gel filtration column and elution done using 0.25% DOC in TBS. Fractions were assayed for protein content (A_{280}) and for growth-inhibitory activity. A small peak of activity was found in the void volume of the column but most of the activity was recovered in a peak eluting at the same position as that found for inhibitor present in H-2 antigen preparations. DOC solubilized RDM-4 membranes were also examined by gel filtration and the inhibitory activity was recovered in the same region. A trace amount of 125 I-H-2K^k was added to the solubilized membrane samples before gel filtration. It eluted in the same position (not shown) as H-2 antigen run in the absence of other membrane proteins (Fig. 4A).

These results clearly demonstrated that the growth inhibitor

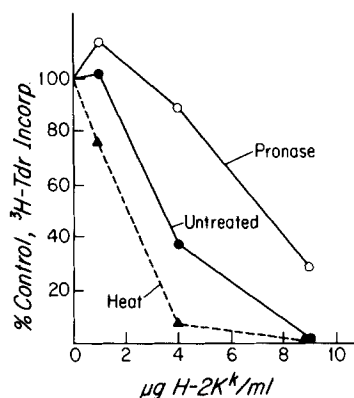


FIGURE 3 Inhibitory activity is resistant to heat or protease treatment. Affinity-purified H-2K^k was left untreated, heat treated for 10 min at 100°C, or pronase treated for 43 h at 37°C prior to formation of liposomes. Details are described in Materials and Methods. Liposomes were made using lipid and H-2 antigen and were tested for inhibitory activity in an LPS-induced mitogenic response. Controls without added liposomes incorporated $71,000 \pm 6,000$ cpm [3 H]Tdr (100% control) and the stimulation index was 8.5. Concentration of H-2K^k is based on starting amount before treatment.

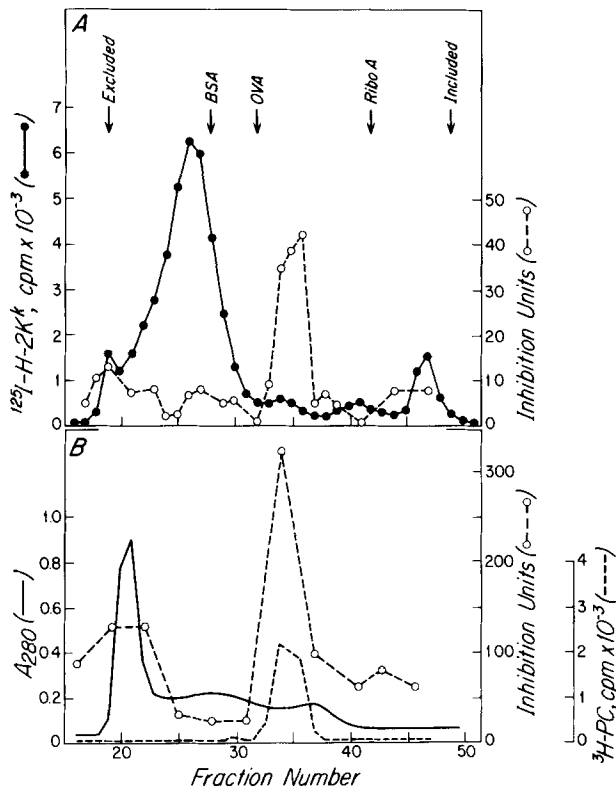


FIGURE 4 Separation of growth inhibitor from H-2 antigen by gel filtration. Gel filtration was done on a Bio-Gel A 0.5 M column in 0.5% DOC, TBS. Details of sample preparation, gel filtration, and assay of inhibitory activity are described in Materials and Methods. Elution positions of standard proteins are indicated at the top of A. (A) Gel filtration of affinity-purified H-2K^k. 125 μ g of H-2K^k, including a trace of ¹²⁵I-H-2K^k, was applied to the column and fractions were assayed for ¹²⁵I-H-2 (●) and growth-inhibitory activity (○). (B) Gel filtration of DOC-solubilized membranes from P815 cells. The DOC-soluble fraction from 10 mg of membranes was applied to the column and fractions assayed for A₂₈₀ (—) and growth-inhibitory activity (○—○). Also shown is the elution profile of [³H]-phosphatidyl choline (---) determined in a separate experiment using the same column and conditions.

present in H-2 preparations was not the H-2 glycoprotein nor dependent on it for activity. They also provided additional evidence that the inhibitor that co-purifies with the H-2 antigens is the same as the one that accounts for the growth-inhibitory activity of membranes. Co-purification of the inhibitor and H-2 antigen upon affinity chromatography using MAbs specific for determinants on the H-2 glycoprotein may indicate an association between the antigen and the inhibitor. If such association occurs, it appears that it is retained in the presence of TX-100, used for cell solubilization and binding to the column, but not in the presence of DOC. The basis for co-purification of H-2 antigen and the inhibitor is being investigated.

Lipid-like Nature of the Growth Inhibitor

The heat and protease insensitivity of the inhibitor (Fig. 3) suggested that it might be a lipid or lipid-like component of the membrane. This suggestion is not inconsistent with the elution of inhibitory activity at an apparent mol wt of 35,000 on gel filtration in DOC. [³H]phosphatidyl choline was found

to elute at the same position (Fig. 4B), indicating that this is the elution position of mixed micelles of DOC and lipid. Behavior of the inhibitor upon extraction with organic solvents was therefore examined.

Affinity purified H-2K^k was dialyzed to remove DOC and extracted with 2:1 butanol/pyridinium acetate, pH 4.2. The aqueous and organic phases were dried under an N₂ stream, the residues were dissolved in 0.5% DOC in TBS, lipid was added, and liposomes were formed by dialysis. The resulting liposomes were tested for the ability to inhibit B cell proliferation in comparison with an equal amount of unextracted H-2K^k in liposomes. The growth-inhibitory activity was quantitatively recovered in the organic phase (Fig. 5A). Membranes from RDM-4 cells were extracted in the same way, and the inhibitory activity was again quantitatively recovered in the organic phase (Fig. 5B). Similar results were obtained when membranes from P815 cells were extracted with butanol/pyridinium acetate and when inhibitor-containing fractions from gel filtration of membranes or H-2 antigen were extracted (not shown).

Extraction of H-2 antigen preparations and membranes has also been done using chloroform/methanol/H₂O (2:1:0.3). Again, the inhibitory activity was recovered in the organic phase in both cases. Using H-2 antigen preparations, it was shown that the inhibitor did not have to be incorporated into liposomes by addition of exogenous lipid to be active (Fig. 1C). Consistent with this observation, we have found that the inhibitory activity can be recovered from the dried organic extract either by adding DOC and lipid and forming liposomes, or by adding culture medium or buffer and sonicating in a bath sonicator to disperse the residue.

It is important to note that the P815 whole cell lipid extract used to make the liposomes for the experiments shown in

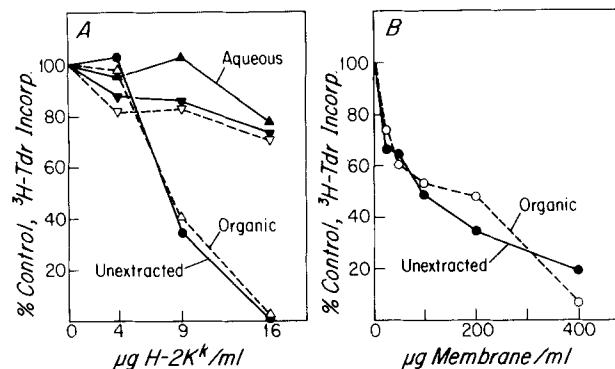
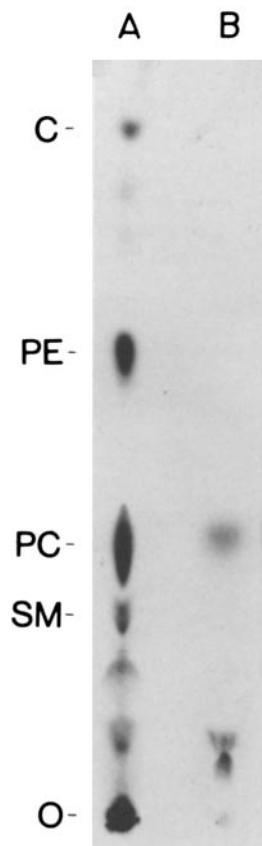


FIGURE 5 Inhibitory activity is soluble in organic solvents. Affinity-purified H-2K^k, an equal volume of buffer (buffer control), and membranes were extracted with butanol/6 M pyridinium acetate, pH 4.2. The separated aqueous and organic phases were dried and dissolved in 0.5% DOC, TBS. Lipid was added and liposomes were prepared as described in Materials and Methods. Liposomes were then tested for inhibitory activity in an LPS-induced mitogenic response. Concentrations of H-2 antigen and membrane are expressed as protein content of the sample before extraction. (A) Extraction of purified H-2K^k. Incorporation of [³H]Tdr in the absence of liposomes (100% control) was 87,000 \pm 3,000 cpm and the stimulation index was 34. Unextracted H-2K^k (●); H-2K^k, organic phase (Δ); H-2K^k, aqueous phase (\blacktriangle); buffer control, organic phase (∇); buffer control, aqueous phase (\blacktriangledown). (B) Extraction of membranes from RDM-4 cells. Incorporation of [³H]thymidine in the absence of liposomes (100% of control) was 153,000 \pm 10,000 cpm and the stimulation index was 7.9. Unextracted membranes (●); membranes, organic phase (○).

FIGURE 6 Lipid composition of RDM-4 membranes and affinity purified H-2K^k. Amounts of RDM-4 membrane (78 $\mu\text{g}/\text{ml}$; lane A) and H-2K^k (7 μg ; lane B) that had equal growth-inhibitory activity were examined by thin-layer chromatography. Unextracted membrane and H-2K^k (dialyzed to remove DOC) were applied to a silica gel G plate, dried, and chromatographed as described in Materials and Methods. The positions of lipid standards run in parallel are indicated and include C, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; and SM, sphingomyelin. O designates the origin. Lipids were visualized by exposing the plate to iodine vapor.



Figs. 1–5 must have contained inhibitor. However, the small amounts of lipid used relative to H-2 antigen did not contribute to the observed inhibition. $\sim 100 \mu\text{g}/\text{ml}$ of membrane is needed for 50% inhibition. Based on a ratio of 0.6 nmol of phospholipid per microgram of membrane protein, this would correspond to about 60 nmol of lipid. In contrast, H-2 antigen-containing liposomes were prepared using 10 nmol or less lipid per microgram of H-2 antigen. Thus, over the dose range needed to observe inhibition by the H-2 antigen preparations (0.5–2 μg), the added lipid was present in too low an amount to be inhibitory, as shown by the absence of activity in the control liposomes lacking the H-2 antigen.

Thin-layer chromatography was done to assess the lipid composition of inhibitor preparations. Affinity-purified H-2K^k (dialyzed to remove DOC) and membranes were applied to a silica gel G plate, developed with chloroform/methanol/water (60:35:8), and lipids were visualized by exposure to iodine vapor. At least 10 lipid species, including the major phospholipids, were detected in the membrane sample (Fig. 6, lane A). In comparison, only three iodine-staining spots were detected in the H-2 antigen sample (Fig. 6, lane B). The amounts of membrane and H-2 antigen run in lanes A and B (Fig. 6) had equal growth-inhibitory activity, as measured in the B cell mitogenic response assay, and comparison of these samples indicates that the growth inhibitor is a minor lipid or lipid-like component of the membrane. Large-scale purification and characterization of the inhibitor is in progress.

DISCUSSION

Plasma membranes isolated from lymphoid cells inhibit the *in vitro* growth of both normal lymphocytes and lymphoid

tumor cells (15). The inhibitory component(s) of the membrane was found to co-purify with H-2K and D antigens when these cell surface glycoproteins were purified by monoclonal antibody-affinity chromatography. Insensitivity of the inhibitory activity to protease and heat treatment indicated that the H-2 glycoprotein was not the inhibitor, and this was confirmed by demonstration that plasma membranes from a cell line lacking surface H-2 antigens had inhibitory activity. Furthermore, the inhibitor could be separated from the H-2 glycoprotein by gel filtration in DOC-containing buffer. The properties of the inhibitor suggested that it might be a lipid or lipid-like membrane component, and this was confirmed by the finding that inhibitory activity could be extracted from H-2 preparations and from membranes by organic solvents. Comparison of the lipid content of H-2K^k and membrane extracts having equal inhibitory activity indicated that the inhibitor is a minor lipid component of the membrane.

Although the growth inhibitor is not H-2K or D, nor dependent on them for activity, it co-purified with the glycoproteins during affinity chromatography on MAb-Sepharose columns specific for the H-2 antigens. Plasma membranes cause 50% growth inhibition at 20–80 $\mu\text{g}/\text{ml}$ (15) whereas H-2 antigen preparations have comparable activity at 0.5–6 $\mu\text{g}/\text{ml}$. Thus, the inhibitor was purified at least 10–40-fold with respect to the membrane protein. It was also highly purified with respect to membrane lipids, as shown by comparison of the lipid content of membrane and H-2 preparations having equal inhibitory activity (Fig. 6). The basis for the co-purification of inhibitor and H-2 antigens has not been determined, but these findings suggest the possibility that H-2 glycoproteins and inhibitor may be associated. If so, this association is retained in TX-100 during solubilization and affinity chromatography but is not seen in DOC, in that the glycoprotein and inhibitor are separated by gel filtration in this detergent. These results might be explained by the observation that H-2 antigens undergo a partial and reversible denaturation in DOC, as evidenced by lower affinity binding to MAbs and increased susceptibility to proteolytic cleavage in this detergent in comparison to TX-100 (33). A detailed examination of the possibility of H-2 antigen association with the inhibitor, and the basis for their co-purification, will be possible once the inhibitor has been characterized and obtained in radiolabeled form.

The small size of the inhibitor-bearing liposomes made it possible to directly examine the reversibility of growth inhibition. Even after several days in the presence of profoundly inhibitory concentrations of liposomes, cells rapidly resumed growth upon removal or dilution of the liposomes (Fig. 2). This confirms and extends the findings made using membranes (15), which indicated that the inhibitor was acting to slow the rate of cell growth without increasing the rate of cell death. The reversibility of membrane-mediated growth inhibition suggests possible parallels with the observation that density-arrested lymphoid cells rapidly resume growth upon dilution to lower density (14).

Several properties of the growth inhibition suggest that it may occur under normal conditions via cell–cell contact when cells are at high density, possibly by surface receptor recognition of the inhibitor. The inhibitory activity is localized to the plasma membrane and membranes are active at concentrations of 20–80 $\mu\text{g}/\text{ml}$ (15). Based on yields obtained upon membrane purification (15, 16), it can be estimated that this is the amount of plasma membrane present on $\sim 1\text{--}4 \times 10^7$

cells. This compares favorably with the maximal cell density of $2-6 \times 10^6$ cells/ml reached by lymphoid cells in vitro. The agreement is better if one considers the fact that some fraction of the membrane vesicles are probably present in an inside-out configuration (with the outside surface thus inaccessible) (34). Although accurate quantitation of the inhibitory concentration of purified inhibitor will not be possible until it is better characterized, it appears that it is active at very low (probably $<1 \mu\text{g/ml}$) concentrations. Furthermore, cells show a very sharp (often sigmoidal) dose response to the inhibitor, with inhibition going from 0 to almost 100% over a two- to fourfold concentration range (Figs. 1, 3, and 5). This is in contrast to many drugs which inhibit cell growth with a dose response curve over a one log or more concentration range. If inhibition by the membrane lipid is mediated via surface receptor recognition, its ready reversibility would indicate that continued interaction of the receptors with the inhibitor is necessary to maintain inhibition.

It is also possible that inhibition by the membrane component involves a mechanism other than recognition by a surface receptor. The lipid-like nature of the component raises the possibility that inhibition could occur via a lipid exchange process to result in transfer of inhibitor from membranes or liposomes to cells. However, because membranes inhibit growth of the same type of cells that they are obtained from, it is unclear that an exchange process could result in a net change in the composition of the cell surface membranes. Understanding of the mechanism of action of the inhibitor will clearly require further study.

Membranes from 3T3 cells inhibit the in vitro growth of 3T3 cells (9, 10) and the available evidence indicates that the inhibitory component(s) is a membrane protein(s) (11-13). In contrast, the inhibitory component of lymphocyte membranes is a lipid or lipid-like molecule whose activity does not depend on membrane proteins. A variety of evidence has suggested potential roles for cell surface lipids in cell-cell interactions which control growth or differentiation (reviewed in references 35, 36). To our knowledge, the results described here are the first demonstration of the occurrence of a membrane lipid that has profound but readily reversible growth inhibitory activity in both the native plasma membrane and in highly purified form, and that is active at concentrations consistent with its having a role in mediating density-dependent growth arrest. Structural characterization of the inhibitor (in progress) will provide the basis for further investigating the mechanism of inhibition and its physiological role in growth control. The fact that this membrane component inhibits lymphoid tumor cell growth also raises the possibility of its potential use in control of in vivo tumor growth.

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