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Research Article

Colony-stimulating activity (CSA) is essential for in vitro differentiation of bone marrow cells into colonies of granulocytes and mononuclear cells. While blood monocytes and macrophages are a major source of CSA, recent studies have indicated that CSA may be produced by lymphocytes responding to immunologic stimulation. Lymphocytes, purified from spleens and thymuses of mice by glass wool columns, were incubated in CMRL-1066 medium with fetal calf serum in vitro. Lymphocytes from the thumus and spleen released CSA when cultured in vitro, with peak levels of CSA observed after 7 days of incubation. Stimulation of cultures with phytohemagglutinin, concanavalin A, or pokeweed mitogen resulted in a 2-5-fold increase in CSA release, with peak levels of CSA released after 4 days of incubation. Thymus-dependent lymphocytes were responsible for the release of CSA from unstimulated and mitogen-stimulated cultures, since the incubation of these cultures with rabbit anti-mouse T cell sera abolished their ability to release CSA. Anti-mouse B cell sera had no effect on the ability of lymphocyte cultures to release CSA. These studies suggest that thymocytes and thymus-derived lymphocytes can release CSA in vitro and may be responsible for the increase in CSA observed in certain immunologic reactions.

Release of Colony-Stimulating Activity from Thymus-Derived Lymphocytes

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ABSTRACT Colony-stimulating activity (CSA) is essential for in vitro differentiation of bone marrow cells into colonies of granulocytes and mononuclear cells. While blood monocytes and macrophages are a major source of CSA, recent studies have indicated that CSA may be produced by lymphocytes responding to immunologic stimulation. Lymphocytes, purified from spleens and thymuses of mice by glass wool columns, were incubated in CMRL-1066 medium with fetal calf serum in vitro. Lymphocytes from the thymus and spleen released CSA when cultured in vitro, with peak levels of CSA observed after 7 days of incubation. Stimulation of cultures with phytohemagglutinin, concanavalin A, or pokeweed mitogen resulted in a 2–5-fold increase in CSA release, with peak levels of CSA released after 4 days of incubation. Thymus-dependent lymphocytes were responsible for the release of CSA from unstimulated and mitogen-stimulated cultures, since the incubation of these cultures with rabbit anti-mouse T cell sera abolished their ability to release CSA. Anti-mouse B cell sera had no effect on the ability of lymphocyte cultures to release CSA. These studies suggest that thymocytes and thymus-derived lymphocytes can release CSA in vitro and may be responsible for the increase in CSA observed in certain immunologic reactions.

INTRODUCTION

The differentiation of marrow cells into granulocytes and mononuclear cells in vitro (1–3) requires the addition of a source of colony stimulating activity (CSA) [1] (2, 4). Previous studies in our laboratory and others have indicated that the monocyte/macrophage system in the blood (5–7) and tissues (8–10) of animals and man is a major source of CSA. Recently, increased levels of CSA have been found in certain immunologic reactions such as graft versus host reactions (11), mixed leukocyte cultures (11, 12), and mitogenic stimulation of lymphocytes (12, 13), suggesting that the lymphocyte may be a source of CSA in certain situations. The role of lymphocytes and, more specifically, the type of lymphocytes involved in the release of CSA was further investigated and forms the basis of this report.

METHODS

Animals

Inbred strains of 2–3-mo-old (C75BL × DBA) F_1 mice bred in our laboratory from stock purchased from Jackson Laboratories (Bar Harbor, Maine) were used for most experiments. In certain experiments, C57BL and AKR mice, purchased from Jackson Laboratories, were also used. Rabbits were purchased from local breeders.

Lymphocyte cultures

Cell suspensions from the spleens and thymuses of mice were prepared by gentle dispersion through stainless steel sieves into cold CMRL-1066 media. Care was taken to remove the evident perithymic lymph nodes from the thymuses by the method described by Leckland and Boyse (14). Mononuclear cell suspensions were obtained by Ficoll-Hypaque gradient centrifugation (15) (Sigma Chemical Co., St. Louis, Mo., and Winthrop Laboratories, New York, respectively). Nonadherent cell fractions were obtained by

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[1] *Abbreviations used in this paper:* B cell, bone marrow-derived lymphocyte; CM, conditioned media; Con A, concanavalin A; CSA, colony-stimulating activity; [^{3}H]TdR, tritiated thymidine; PHA, phytohemagglutinin; PKW, pokeweed mitogen; T cell, thymus-derived lymphocyte.

filtering mononuclear cells through Pyrex wool columns (16). In certain experiments, other procedures, including multiple glass adherence and iron particle phagocytosis (17), were used to remove adherent cells. Cell suspensions contained at least 98% lymphocytes as judged by morphology on smears stained with Wright's stain and by in vitro phagocytosis of carbon particles (1/300). Conditioned medium (CM) was prepared by incubating these lymphocytes in various concentrations in CMRL-1066 medium in loosely capped plastic tubes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) in a humidified atmosphere of 10% CO_2 for varying periods of time. At the end of the incubation, cells were removed by centrifugation (2,000 *g*) for 10 min and the cell-free supernate (the CM) was passed through a Millipore filter and stored at −20°C, before assaying for CSA.

Adherent spleen cell cultures

Mononuclear spleen cell suspensions obtained by Ficoll-Hypaque centrifugation were incubated in 35-mm plastic petric dishes in 1 ml of CMRL-1066 supplemented with 15% fetal calf serum. After 2 h of incubation at 37°C, the nonadherent cells were removed by vigorous washing with Seligmann's basic salt solution, and adherent cells were resuspended in culture media. The process was repeated 48 h later at which time greater than 95% of the adherent cells were macrophages as judged by morphology and phagocytosis of India ink. Phagocytosis was measured by adding India ink at a concentration of 1/300 to the cells and shaking in a 37°C water bath for 30 min. After washing the cells three times, they were stained and counted for ingestion of carbon particles.

Assay for CSA

CSA of CM from various cell fractions was assayed by its ability to stimulate colony formation from murine marrow in the soft-gel system described by Pluznik and Sachs (1) and Bradley and Metcalf (2). Methylcellulose was used in place of agar (18). Briefly, normal marrow cells were suspended in 1.6% methylcellulose, 10% horse serum, and CMRL-1066 medium. To 0.9 ml of the mixture of cells, horse serum, and medium, 0.1 ml of test CM was added for stimulation. 1 ml of the combined mixture was plated in 35 × 10-mm Falcon culture dishes. After incubation for 7 days, the number of colonies (more than 50 cells) was counted with the aid of an inverted microscope. For morphologic identification, individual colonies were removed with a Pasteur pipette, smeared between glass cover slips, and stained with Wright's stain.

Mitogen stimulation

One to two million lymphoid cells were cultured in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% heat-inactivated fetal calf serum (Flow Laboratories, Inc., Bethesda, Md.). Purified phythohemagglutinin (PHA) at 1 μg/ml (Wellcome Reagents Ltd., Beckenham, Kent, England), concanavalin A (Con A) at 10 μg/ml (2 × crystallized, ICN Nutritional Biochemicals Div., Cleveland, Ohio), or pokeweed mitogen (PKW) at 7 μl/ml (Grand Island Biological Co.) was added to lymphocyte cultures as indicated.

([^{3}H]TdR) incorporation

8 h before the termination of the mitogen-stimulated lymphocyte cultures, 1 μCi of [^{3}H]TdR (2 Ci/mm sp act, Amersham/Searle Corp., Arlington Heights, Ill.) was added. Cells were collected on Millipore filters as described by Davie and Paul (19). Trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation spectrometer (model 3375 Tri Carb, Packard Instrument Co., Inc., Downer Grove, Ill.).

Preparation of antisera

Rabbit anti-T cell sera. Mouse thymocytes (1×10^9) were emulsified in complete Freund's adjuvant and rabbits were injected intramuscularly on two occasions, 1 mo apart, and exsanguinated 1 wk after the final injection (20). All sera were heat inactivated for 30 min at 56°C and then stored at −20°C. The following sequential absorptions (packed absorbant/serum =1/10 in volume; 30 min at 0°C) were performed according to Raff (21): (*a*) homogenized mouse liver (once); (*b*) mouse red blood cells (twice); and (*c*) absorbtion with bone marrow mononuclear cells until sera had no cytotoxicity for bone marrow cells. Commercial antisera, purchased from Microbiological Associates, Inc. (Rockville, Md.) and prepared according to the method of Davis, Cooperband, and Masanich (22), were also used.

Rabbit anti-B cell sera. B cells were obtained from normal DBA mice after treatment with anti-θ-C3H serum and guinea pig complement (C′) according to Lamelin, Lishowka-Bernstein, Matte, Ripes, and Vassalli (20). Rabbit were injected with 1×10^9 B cells as described for T cells. The following sequential absorptions were performed as described above: (*a*) homogenized mouse liver (once); (*b*) mouse red blood cells (twice); and (*c*) mouse thymocytes (10–15 times) until the serum was no longer cytotoxic for mouse thymocytes.

Measurement of cytotoxicity

Cytotoxicity was assayed by incubating $0.5–2.5 \times 10^6$ target cell/cm^3 CMRL-1066 with 1/4 dilution of antisera for 1 h at 37°C. Agarose-absorbed guinea pig complement (23) was added in a volume of 0.1 ml and the cultures were incubated for 2 h. An equal volume of 0.2% trypan blue was added to an equal volume of the cultures and 200 cells were counted for viability. Experiments in which complement alone killed more than 10% of the cells were excluded. The cytotoxic index (CI), which represents the specific killing for each antisera, was calculated by using the following equation: CI = 100 × (% killed with antisera − % killed with normal rabbit serum)/(100 − % killed with normal rabbit serum).

Gel filtration

CM from lymphocyte cultures was concentrated tenfold with polyethylene glycol (Fisher Scientific Co., Pittsburgh, Pa.) and then dialyzed overnight with 0.015 M tris-HCl buffer, pH 7.4. A Sephadex G-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden), 60 × 2.6 cm, was used for gel filtration. A 5-ml sample was placed on the column and ascending elution was performed with a pressure head of 25 cm. 5-ml fractions were collected and Millipore filtered before assaying for CSA. Void volume was delivered with blue dextran 2000 and the column was standardized with bovine serum albumin and chymotrypsinogen.

Immunofluorescence

Lymphocytes obtained by Pyrex wool columns were freed of dead cells by brief osmotic shock (10-s suspension in distilled water) and freed of erythrocytes by incubation in

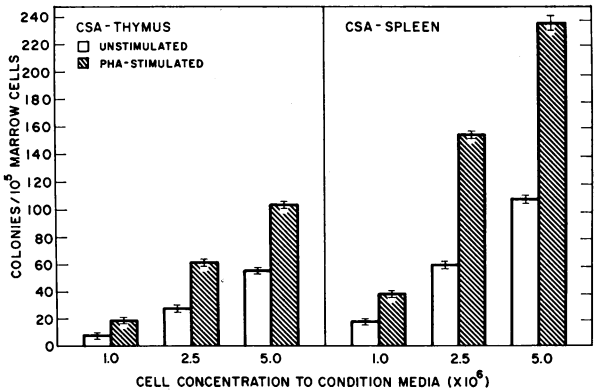

FIGURE 1 Effect of cell concentration and PHA stimulation on the ability of lymphocytes to release CSA in vitro. Values represent the mean±SE of the number of colonies/10^5 marrow cells stimulated by 0.1 ml CM.

ammonium chloride (0.75% in 0.02 M Tris buffer, pH 7.4). To 5×10^6 lymphoid cells suspended in 0.5 ml of RPMI 1640 media containing 1% bovine serum albumin, 0.1 ml of specific antiserum or normal rabbit serum was added. The cells were then washed twice with RPMI 1640 media containing 5% albumin. Fluoresceinated goat anti-rabbit immunoglobulin (0.1 ml) was added to the cell pellets and a further 30-min incubation was carried out. After three final washings, the cells were examined in the living state under dark field illumination using a Zeiss photomicroscope II (Carl Zeiss, Inc., New York). 200 cells were counted in each experiment.

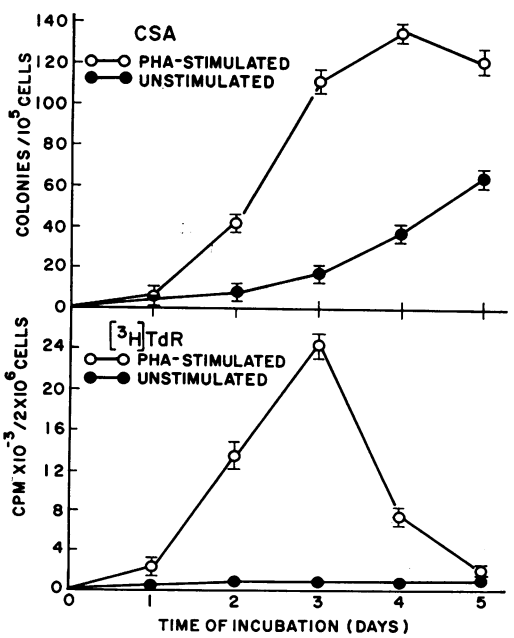

FIGURE 2 Effect of PHA on the time-course of [^{3}H]TdR incorporation and the release of CSA. Values for [^{3}H]TdR incorporation (bottom) and CSA release (top) are expressed as the mean±SE.

RESULTS

Lymphocytes isolated from the spleens and thymuses of mice were able to release CSA when incubated in vitro (Fig. 1). Activity was observed after 2–3 days of incubation, reaching peak levels after 7 days. A good correlation existed between the number of cells incubated and the amount of CSA released. While 10^6 thymocytes or spleen lymphocytes released small amounts of CSA, increased levels of CSA were observed with incubation of higher cell concentrations. CSA from 5×10^6 spleen lymphocytes stimulated 108±2.00 colonies, while CSA released from the same concentration of thymocytes stimulated 56±1.67 colonies. Lymphocytes isolated from the spleen released significantly ($P < 0.001$) more CSA than comparable concentrations of thymocytes. These results did not appear to be strain dependent, for lymphocytes obtained from C57Bl and AKR mice were also able to release CSA in vitro.

Stimulation of lymphocytes with PHA resulted in a 2–4-fold increase in CSA levels from various cell concentrations after 7 days of incubation (Fig. 1). As with the unstimulated lymphocytes, there was a two-fold greater release of CSA from PHA-stimulated spleen cells than from thymus cells. CSA obtained by incubating 5×10^6 spleen lymphocytes with PHA stimulated 228±4.67 colonies/0.1 ml compared to 108±2.0 colonies from unstimulated cells. CSA from the same number of PHA-stimulated thymocytes 102±1.67 colonies compared to 56±1.67 colonies from unstimulated cells.

The effect of PHA stimulation on the time-course of release of CSA from lymphocytes is seen in Fig. 2. PHA stimulation of [^{3}H]TdR incorporation increased to a maximum at day 3 (24,098±3,191 cpm/2×10^6 cells) and fell to control levels by day 5 (1,638±428 cpm). A 2–5-fold increase in CSA release occurred during this same period from PHA-stimulated lymphocytes as compared to unstimulated cells with maximum difference on day 3. The increase in CSA release closely followed the increase in [^{3}H]TdR incorporation. After 3 days of incubation, CSA from PHA-stimulated spleen lymphocytes stimulated 116± 4.00 colonies as compared to 19±1.67 colonies from unstimulated cells. In PHA-stimulated cultures, CSA release occurred earlier than in unstimulated controls and reached maximal level by day 4. On the other hand, unstimulated cultures of spleen lymphocytes had a lag of 3 days before significant amounts of CSA were released and reached maximal amounts by day 7. Further incubation to 10 days resulted in virtually no change in CSA levels with 142±4.7 colonies/0.1 ml for PHA-stimulated cultures and 63±3.3 colonies for unstimulated cultures.

In addition to PHA, other mitogens were capable of releasing increased amounts of CSA from thymus and spleen lymphocytes (Table I). Spleen lymphocytes stimulated with Con A incorporated a greater amount of [^{3}H]TdR (42,622±8,128 cpm) than cells stimulated by either PHA or PKW which incorporated 24,723± 3,926 and 13,472±1,520 cpm, respectively. Increased amounts of CSA were released by all mitogens with the increase being 2–5 times greater than from unstimulated cells. While the greatest amount of CSA was obtained from PKW-stimulated cells (182±5.67 colonies), this was not significantly different than that observed with Con A and PHA which stimulated 166 ±4.67 and 156±4.33 colonies, respectively.

While all mitogens tested resulted in a significant increase in the incorporation of [^{3}H]TdR into spleen lymphocytes, a somewhat different response was observed with thymocytes. While stimulation of thymocytes with Con A and PKW resulted in significant increases in [^{3}H]TdR incorporation (14,071±1,286 and 4,171±896, respectively), this was not observed with PHA stimulation, where the [^{3}H]TdR incorporation was similar to that observed in unstimulated controls (755±236 versus 455±108 cpm). The increase in CSA released by mitogen-stimulated thymocytes followed a similar pattern. There was no significant difference between the amount of CSA released from Con A (114±3.67)- or PKW (120±4.00)-stimulated cells, but both mitogens released significantly more than PHA-stimulated cells (42±1.33) ($P < 0.001$). In all instances mitogen-stimulated spleen lymphocytes released significantly ($P < 0.001$) greater amounts of CSA than did mitogen-stimulated thymocytes.

The addition of mitogen directly to cultures of bone marrow cells unstimulated and stimulated with conditioned media from mouse L cells neither stimulated nor inhibited colony formation at the concentrations used.

The morphology of cells growing in the colonies stimulated by CSA obtained from both mitogen-stimulated and unstimulated lymphocytes was granulocytic for the first 4 days of culture and by day 7 the cells were entirely macrophages, similar to that observed with stimulation by other CSAs (24).

Since mature lymphocytes can be divided both physically and functionally (21) into T (thymus-dependent) lymphocytes and B (thymus-independent or bone marrow) lymphocytes, the question was raised as to which cell type was responsible for CSA release. Heteroantisera can be obtained by immunization with mouse thymocytes, which after appropriate absorption react only with mouse T lymphocytes (20). Heteroantisera to B cells were prepared according to Lamelin et al. (20). With these antisera, it is possible to specifically kill either cell population in the presence of complement and thus eliminate it from the cell suspension. The different specificities of these antisera, as determined both by cytotoxicity and fluorescent antibody assays, are seen in Table II. Rabbit anti-T cell serum was cytotoxic for thymic lymphocytes while the anti-B cell serum was not. The results obtained by using both cytotoxicity and fluorescence are in close agreement. Additional evidence that these two antisera have different specificities rests on the following observations: (*a*) the two antisera give additive killing of lymphocytes when used together; (*b*) the activity of the anti-T cell serum can be eliminated by absorption with thymocytes but not with bone marrow lymphocytes, while the opposite is true of anti-B cell sera; (*c*) the values for

TABLE I

Effect of Mitogen Stimulation on the Release of CSA from Lymphocytes

Source of lymphocytes	Mitogen*	[^{3}H]TdR‡	Colonies§
		cpm/2 × 10⁶ cells	*per 10⁵ marrow cells*
Spleen	—	1,578±308	48±1.33
Spleen	PHA	24,723±3,926	156±4.33
Spleen	Con A	42,622±8,128	166±4.67
Spleen	PKW	13,472±1,520	182±5.67
Thymus	—	455±108	6±0.67
Thymus	PHA	752±236	42±1.33
Thymus	Con A	14,071±1,286	114±3.67
Thymus	PKW	4,171±896	120±4.00

* PHA at 1 μg/ml, Con A at 10 μg/ml, and PKW at 7 μl/ml were used.

‡ [^{3}H]TdR incorporation was measured after 3 days of incubation. Values are expressed as the mean±SE.

§ Number stimulated by 0.1 ml conditioned media prepared by incubating 2 × 10^6 lymphocytes/ml. Values are expressed as the mean±SE.

TABLE II

Frequency of Different Lymphocytes in Spleen and Thymus Cells Determined by Specific Antisera

Cell source	T cells*		B cells*		Others‡	
	Cyto	Fl	Cyto	Fl	Cyto	Fl
	%		%		%	
Thymus	98±2	97±1	1±1	5±2	0	0
Spleen	39±3	35±4	54±3	56±3	6±2	11±3

* Percentage of cells bound by each antisera (anti-T cell or anti-B cell) was determined by trypan blue cytotoxicity (Cyto) and indirect immunofluorescence (Fl) as described in Methods. Values represent the mean±SE of three experiments.

‡ Cells not bound by anti-T and anti-B serum when added to culture together.

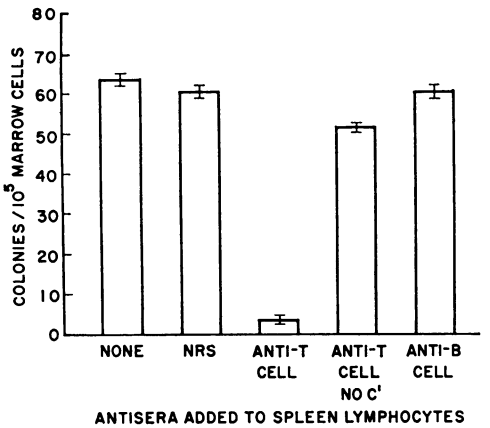

FIGURE 3 Effect of incubation with antisera on the ability of spleen lymphocytes to release CSA. Lymphocytes previously treated with antisera were put in fresh media and incubated for 7 days. NRS, normal rabbit serum; anti-T cell, antisera to thymus-derived lymphocytes; anti-B cell, antisera to bone marrow-derived lymphocytes; C', complement. Values represent the mean±SE of the number of colonies stimulated by 0.1 ml CM.

the relative proportions of B and T cells in the spleen were 38 and 54% and are in close agreement with results previously reported with heterologous and alloantisera (20, 21, 25).

The ability of T cell-depleted and B cell-depleted lymphocyte cultures to release CSA is seen in Fig. 3. There was no significant change in the amount of CSA released from spleen lymphocytes when incubated with anti-B cell sera. CSA from spleen lymphocytes incubated with anti-mouse B cell sera stimulated 61±3.33 colonies as compared to 64±4.33 colonies stimulated by CSA from control cultures not containing antisera. On the other hand, CSA from lymphocytes incubated with anti-mouse T cell sera resulted in almost complete inhibition of colony stimulation (4±0.67 colonies/0.1 ml), and was similar to that observed with unstimulated cultures. This failure to release CSA was dependent upon cell lysis, since cells bound by antisera in the absence of complement were still capable of releasing CSA (52±2.67 colonies/0.1 ml) in an amount which did not differ significantly from control values. Similar results were obtained with PHA-stimulated spleen lymphocytes and these are seen in Table III. Anti-T cell sera eliminated both the release of CSA

TABLE III

Effect of Lymphocyte Antisera on PHA-Stimulated Cultures

Serum added	Colonies*	[³H]TdR‡
	per 10⁵ marrow cells	*cpm/2 × 10⁶ cells*
None	111±4.3	19,906±2,153
Normal rabbit	106±5.0	14,486±1,592
Anti-T cell	4±1.3	451±113
Anti-B cell	108±4.3	14,986±1,791

* Number stimulated by 0.1 ml CM prepared by stimulating 2 × 10⁶ lymphocytes/ml with 1 μg/ml PHA for 4 days. Values are expressed as the mean±SE of three separate experiments.
‡ [³H]TdR incorporation was measured after 3 days of incubation. Values are expressed as the mean±SE of three separate experiments done in triplicate.

TABLE IV

Effect of Supernates from Mitogen-Stimulated Lymphocytes on CSA Release from Spleen Cells

Cell type	Cell number	Supernate added*	Colonies‡
	×10⁶		*per 10⁵ marrow cells*
Macrophages	0.13	None	0
Macrophages	0.13	48 h PHA	27±2.4
Macrophages	0.13	72 h PHA	70±4.3
Macrophages	0.28	None	0
Macrophages	0.28	48 h PHA	29±2.0
Macrophages	0.28	72 h PHA	68±3.7
Macrophages	1.30	None	16±1.7
Macrophages	1.30	48 h PHA	18±2.3
Macrophages	1.30	72 h PHA	43±4.0
—	—	48 h PHA	29±2.3
—	—	72 h PHA	77±3.7
Lymphocytes	2.00	48 h Con A +methylglucoside	89±5.0
Lymphocytes	2.00	None	63±4.7
—	—	48 h Con A +methylglucoside	32±2.5

* Cell-free supernates were added directly to freshly cultured cells.
‡ Number stimulated by 0.1 ml CM harvested 4 days after the addition of the supernate. Values are expressed as the mean±SE of three separate experiments.

TABLE V

Cell Populations Present in Cultures during Mitogen Stimulation

Incubation	Mitogen added	Antisera added	Lymphocytes	Blasts	Phagocytes*
days			%	%	%
0	None	None	98	0	2
3	None	None	97	0	3
3	PHA	None	11	84	5
3	PHA	Anti-T cell	91	3	6
3	PHA	Anti-B cell	18	79	3
4	None	None	95	0	5
4	PHA	None	9	83	8
4	PHA	Anti-T cell	91	3	6
4	PHA	Anti-B cell	11	80	9

* Phagocytes were those cells ingesting India ink particles as determined in Wright's stained smears.

and [^{3}H]TdR incorporation, while lymphocytes treated with anti-B cell sera released CSA which stimulated 108 colonies/0.1 ml and incorporated 13,986 cpm of [^{3}H]TdR. The addition of either antisera to cultures of marrow cells stimulated with mouse L cell CM did not affect colony formation.

Since it is possible that stimulated T cells release a soluble factor which may stimulate other cells types, such as macrophages, to release CSA, an attempt to demonstrate such a factor was made. Spleen lymphocytes stimulated with mitogen were cultured for 48 and 72 h and the cell-free supernates were added to freshly cultured spleen macrophages and lymphocytes. The results as seen in Table IV indicate that no increase in CSA was found in any of the cultures to which the supernates were added. In the lymphocyte experiments, methylglucoside was added to the Con A-treated supernate so that the remaining mitogen could not directly stimulate the lymphocytes to produce CSA.

Analysis of the mitogen-stimulated cultures at days 3 and 4 showed that 5 and 9%, respectively, of the viable cells were phagocytic (Table V), indicating that there were less than 2×10^5 macrophages present in these cultures. Since appreciable CSA formation after 4 days requires at least 1×10^6 macrophages/ml (Table IV), it is unlikely that the macrophage is the source of CSA in the mitogen-stimulated cultures.

Studies with CSA from other sources have demonstrated that it is a heat-stable glycoprotein with a molecular weight of approximately 40–60,000 daltons (26, 27). The molecular weight of CSA obtained from both unstimulated and mitogen-stimulated lymphocytes was chromatographed on Sephadex G-200 columns (Fig. 4). CSA from PHA-stimulated cultures was present in a single sharp peak corresponding to a molecular weight of approximately 36,000 daltons as determined according to Whitaker (28). CSA from unstimulated lymphocyte cultures gave the same elution pattern.

DISCUSSION

The results of these studies indicate that lymphocytes from the thymus and spleen can release CSA when cultured in vitro and the amount of CSA released can be increased 2–5-fold by stimulation with PHA, Con A, or PKW. Stimulation by mitogens shortens the incubation period before CSA is released as well as the time required to obtain maximal release. Maximal release of CSA occurred by day 7 and incubation for longer periods up to 10 days did not result in a greater amount of CSA being released. At no time did unstimulated cultures release more than one-half the amount of CSA obtained from stimulated cultures, suggesting that cell stimulation was responsible for the increased production.

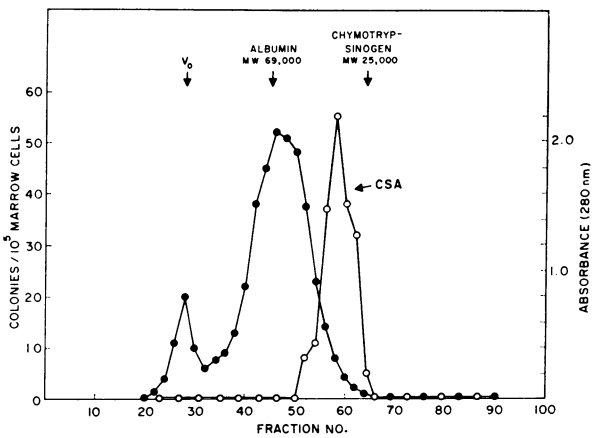

FIGURE 4 G-200 Sephadex filtration patterns of cell-free media stimulated with PHA after 7 days incubation with spleen lymphocytes. A single peak of CSA (○) was found as determined by the number of colonies stimulated by 0.2 ml of each column fraction. Void volume (V_0) was determined with blue dextran 2000. Optical density (●) is also shown.

There appeared to be a correlation between the incorporation of [^{3}H]TdR and CSA release (Table I) and both responses were abolished by anti-T cell sera. However, the observation that PHA-stimulated thymocytes release CSA while not incorporating a significant amount of [^{3}H]TdR suggest that the two parameters may not be directly associated. This area is now being investigated in our laboratory.

Spleen lymphocytes consistently produced more CSA than the same concentration of thymocytes. A possible explanation for this finding is that the bulk of thymocytes are immature, and larger numbers of thymocytes as compared to spleen lymphocytes are required for comparable graft versus host reactions (29) and mitogen stimulation (30). In support of this possibility was the greater increase in CSA release from thymocytes by Con A than PHA which is in keeping with the greater responsiveness of immature thymocytes to stimulation with Con A (30, 31).

Previous reports have demonstrated that human and murine spleen cells (11, 32) can release CSA, although the exact cell type responsible for its release was not determined. Lymphocytes can be separated into two distinct classes by the use of specific antisera to T and B lymphocytes. Such antisera were produced in rabbits by injecting either mouse thymocytes or mouse B cells, the latter obtained after treating spleen lymphocytes with anti-θ serum. These antisera were shown to be specific by both trypan blue dye cytotoxicity and indirect immunofluorescence.

Incubation of lymphocyte cultures with these antisera demonstrated that anti-T sera eliminated the ability of

lymphocytes to release CSA, indicating that the T lymphocyte was responsible for the release of CSA. Anti-B cell sera had no effect on the ability of lymphocytes to release CSA. Similar results with specific antisera were also obtained in PHA-stimulated cultures. These results suggest that the cells stimulated by PHA to release CSA and the cells spontaneously releasing it are both killed by anti-T cell sera and are probably the same cell.

These findings are in keeping with the observations of Parker and Metcalf (12) who studied unfractionated spleen cells from nude mice. They observed that PKW, a stimulator of both T and B cells, did not stimulate release of CSA from spleen cells of nude mice (congenitally athymic mice possessing no functional T cells), suggesting that PKW acted to release CSA from T cells.

While it is possible that macrophages present in the cultures could contribute to some of the CSA release, their concentration was too small to produce the amount of CSA obtained (9, 12). CSA release was not seen with concentrations of less than 10^6 spleen macrophages/ml. Determination of the cell types present during the culture period indicated that at no time did the concentration of macrophages exceed 2×10^5/ml. In addition, the use of iron particle phagocytosis and multiple glass adherence steps after Pyrex wool filtration did not reduce the amount of CSA released, suggesting that macrophage contamination was not responsible for CSA release in these lymphocyte cultures.

Another possibility was that stimulated T cells might be producing a factor which stimulated other cell types such as macrophages to release CSA. An attempt to demonstrate such a soluble factor in the supernates of mitogen-stimulated cultures was unsuccessful. Neither macrophages nor lymphocytes could be stimulated to release more CSA by the addition of these supernates.

Studies on the CSA released by the unstimulated and mitogen-stimulated lymphocytes indicated that it is similar to CSAs obtained from other sources (26, 27). The molecular weight of this lymphocyte CSA was approximately 36,000 as determined by gel filtration. A low molecular weight CSA similar to that recently obtained from human blood leucocytes (33) and mouse lung (34) was not found in these cultures.

A number of soluble lymphocyte products (lymphokines) are known to be released from stimulated and unstimulated lymphocytes (35). These factors include macrophage migration inhibition factor, chemotatic factor, and lymphotoxin. It is generally thought that these soluble factors originate only or mainly in T cells (35). The results of these studies suggest that T lymphocytes are capable of releasing still another factor (CSA) which may be responsible for increased levels of granulocytes and mononuclear cells seen in certain immunologic reactions.

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