ON THE RELATION OF PRODUCTS OF ACTIVATED LYMPHOCYTES TO CELL-MEDIATED CYTOLYSIS*, ‡

By CHRISTOPHER S. HENNEY,§ JUDITH GAFFNEY, and BARRY R. BLOOM

(From the Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, the O'Neill Memorial Research Laboratories, Good Samaritan Hospital, Baltimore, Maryland 21239, and the Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461)

(Received for publication 4 June 1974)

Thymus-derived lymphocytes have been shown to carry out three major immunological functions—helper function in antibody formation, production of soluble mediators, and cell-mediated cytotoxicity. The relationships between the cells involved in these three phenomena and their mechanisms of action remain unknown. This study was undertaken to explore whether a relationship exists between mediator production and T-cell-mediated cytolysis, both manifestations of cell-mediated immunity. Experiments were specifically designed to test the hypothesis that T-cell-mediated cytolysis is effected by a soluble mediator, produced by the sensitized lymphocyte upon interaction with an antigen-bearing ("target") cell (1, 2). The experimental basis for this hypothesis derives principally from demonstrations that both antigen and T-cell mitogens can stimulate lymphocyte populations to produce soluble factors which are toxic for certain cell types in vitro (3, 4). Under these same conditions, direct cell-mediated cytotoxicity may also be observed (5). The total lack of specificity demonstrated in such cytotoxic systems stands in marked contrast to the exquisite specificity observed in T-cell-mediated lysis (6, 7). Nevertheless, it is conceivable that the specificity of T-cell killing resides in the interaction between effector and target cell; i.e. that lymphocytes, activated by membrane associated antigens, "inject" nonspecific mediators only into the membrane of the "triggering" target cell (8, 9). Thus, the mechanism of lysis in all cases could be similar.

A number of experimental observations are consistent with the concept that mediator production may be related to the mechanism of T-cell-mediated lysis. For example, production of migration inhibitory factor (MIF)[1] (10) and lymphotoxin (LT) (1) is suppressed by inhibitors of protein synthesis, and T-cell-mediated cytolysis is sub-

* This work is supported by grants AI-10280, AI-07118 and AI-10702 from the National Institute of Allergy and Infectious Diseases and a grant from the National Science Foundation.

‡ This is communication no. 127 from the O'Neill Memorial Research Laboratories.

§ Recipient of Research Career Development Award AI-70393 from the National Institute of Allergy and Infectious Diseases.

[1] cAMP, cyclic AMP; Con A, concavalin A; CT, cholera enterotoxin; LT, lymphotoxin; MIF, migration inhibitory factor; PPD, purified protein derivative (tuberculin).

stantially diminished by cycloheximide and actinomycin D (11). Further, inhibition of DNA synthesis affects neither the production of mediators by sensitized lymphocytes (12, 13), nor the lytic activity of effector T cells (14).

Recent observations made in this laboratory (9, 15, 16) and in others (17), suggest that the expression of lytic activity is dependent on functional cellular secretion. While the evidence for this conclusion is circumstantial, when considered collectively, it seems compelling. Cytolysis is inhibited by augmented cyclic AMP (cAMP) (15), by colchicine (16, 17), and by both EDTA and EGTA (14, 18). These agents have consistently been observed to modulate secretory phenomena in a wide variety of cell types (19). The striking inverse relationship between cAMP levels and cytolysis is perhaps the most insistent argument that T-cell-mediated cytolysis may involve secretory processes.

Materials and Methods

Reagents.—Pactamycin was a generous gift from. Dr. William Wechter (Upjohn Co., Kalamzaoo, Mich.). Cholera enterotoxin (CT) was a gift from Dr. Richard A. Finkelstein (The University of Texas, Dallas, Texas). Emetine and colchicine were purchased from the Sigma Chemical Co., St. Louis, Mo. Vinblastine was obtained from Eli Lilly & Co., Indianapolis, Ind.

Lymphocyte Populations.—Mouse splenic lymphocyte suspensions were prepared from normal C57BL/6 mice and from mice immunized 10 days previously with P815 mastocytoma cells of the DBA/2 strain by methods previously described (11, 15). Guinea pig lymph node cells from tuberculin-sensitive animals were obtained 3–6 wk after immunization with 1.5 mg of killed H37Ra (Difco Laboratories, Detroit, Mich.) emulsified in Freund's incomplete adjuvant as previously described (20).

Target Cells.—Mouse mastocytoma cells (P815 of the DBA/2 strain) were maintained in ascitic fluid in adult DBA/2 female mice, and were used both as immunizing antigens and as target cells in cytolytic assays. The cells were passaged at weekly intervals by intraperitoneal administration of 0.2 ml ascitic fluid (approximately 10^7 cells). Target cells containing ^{51}Cr were prepared as described previously (11, 21).

Cytolytic Assays.—The cytolytic assay employed throughout this study was that previously described (11, 21). Lymphocytes (10^7) and target cells (10^5) were incubated for 4 h at 37°C in Eagle's medium containing 10% fetal calf serum.

At the end of incubation the cells were centrifuged and the ^{51}Cr content of an aliquot of cell-free supernate counted. The percentage of the ^{51}Cr originally cell associated which was released into the supernate on incubation was equated with the percent target cell lysis (11). The percent specific cytolysis was evaluated by subtracting from the lysis obtained with immune C57BL/6 lymphocytes that amount of ^{51}Cr released in the presence of the same number of normal C57BL/6 spleen cells (9). In assessment of the inhibitory activity of various reagents employed in these studies, the specific cytolysis recorded in the presence of inhibitor was evaluated as a percentage of that specific lysis observed in the absence of inhibitor. As previously described (21), cytolysis as measured in this manner was highly reproducible, and populations of lymphocytes harvested 10 days after similar immunization schedules exhibited a cytolytic activity that varied only of the order of 10%. Replicate analyses performed at the same time on an individual lymphocyte population showed standard deviations of $< \pm 2.5\%$.

MIF Assays.—Guinea pig lymph node cells were cultured in 1.5 ml Eagle's MEM in the absence of serum for 18 h at 22.5×10^6 viable cells/Leighton tube at 37°C under an atmosphere of 7.5% CO_2 in balanced air. For studies utilizing pactamycin, emetine, vinblastine, and col-

chicine, supernates were dialyzed against saline and then water to remove drug, lyophilized, and reconstituted with MEM containing 15% heated guinea pig serum. For experiments employing CT, which is partially nondialyzable, supernates were frozen at −80°C, thawed, and reconstituted with 15% heated guinea pig serum.

In all experiments to be described, several types of controls were included: (*a*) lymphocytes cultured in the absence of antigen, to which antigen was restored upon harvesting the cells; and (*b*) lymphocytes cultured in the absence of antigen but in the presence of the drug (to which antigen was restored upon harvesting the supernates).

Experimental samples included: (*a*) cultures stimulated with purified protein derivative (tuberculin) (PPD) (25 μg/ml); (*b*) cultures stimulated with PPD in the presence of the drug; and (*c*) cultures stimulated with PPD (to which the drug was added upon harvesting the supernate). This latter control was particularly important in the studies on CT, in which the CT was found to exert some effect on the indicator macrophages. For calculation of the indices of migration, two formulae were used: (*a*) without drug, (migration of PPD supernate)/(migration of MEM supernate [+PPD]) × 100; and (*b*) with drug, (migration of PPD supernate with drug)/(migration of MEM supernate with drug [+PPD]) × 100. Brackets denote materials added to supernates after culture and removal of cells).

In other experiments, lymphocytes were "pulsed" for 1 h with drug, washed three times, and then cultured with or without antigen. Formula (*b*) was then used to calculate migration inhibition. In general, it should be noted that the drugs exerted no inhibitory activity on the migration of normal guinea pig macrophages.

LT.—The same supernates assayed for MIF activity were assayed for the presence of LT. The culture fluids were millipore filtered (0.44 μ) and placed on monolayers of 10^5 L cells grown on Linbro plates (16-mm tissue culture, Linbro Chemical Co., New Haven, Conn.) in Eagle's medium containing 10% inactivated fetal calf serum. After incubation for 72 h the cell monolayers were washed, and the cells cultured for an additional 4 h in the presence of 1 μCi of [^{3}H]leucine. The incorporation of [^{3}H]leucine into material insoluble in 5% TCA was then measured.

Assays for Protein Synthesis.—Lymphocytes (10^7/ml) were suspended in leucine-free Eagle's medium supplemented with nonessential amino acids and incubated at 37°C in a humid atmosphere of 10% CO_2 in air. For short-term cultures (less than 6 h) 1 μCi of [^{3}H]leucine (>40 Ci/mmol sp act: Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was included in the culture. For long term cultures (6 h or longer) 2 μCi of [^{3}H]leucine (2 Ci/mmol sp act: Schwarz/Mann Div., Becton, Dickinson & Co.) was added. At the end of the incubation period, 4 ml of 5% TCA was added to the culture and the tubes were centrifuged at 1,000 × *g* for 10 min. The supernate was discarded and the pellets were resuspended in 5 ml of TCA and recentrifuged. The final pellets were resuspended in 1 ml of tissue solubilizer (NCS, Amersham/Searle Corp., Arlington Heights, Ill.) which was then incorporated into a toluene scintillation cocktail (Liquifluor, New England Nuclear, Boston, Mass.) before counting.

cAMP.—The cAMP content of spleen cell suspensions was kindly measured by Dr. Elizabeth Gillespie of Johns Hopkins University by the method of Brown et al. (22). The adaptation of this method to lymphoid cell suspensions has previously been described in detail (15, 23).

RESULTS

Effect of Inhibitors of Protein Synthesis on T-Cell-Mediated Cytolysis and on MIF Production.—Spleen cell suspensions from normal C57BL/6 mice were cultured in vitro in the presence of [^{3}H]leucine and the incorporation of the isotope into cell-associated protein assayed 4 and 18 h later. The effect of adding various concentrations of the protein inhibitors pactamycin or emetine

to the culture medium was assessed. Data for the 18-h cultures are shown in Fig. 1. Both pactamycin (10^{-7}–10^{-5} M) and emetine (10^{-6}–10^{-5} M) were found to totally inhibit [^{3}H]leucine incorporation by the spleen cell suspensions. Vital dye exclusion tests performed at the end of culture showed that in the concentrations employed, the drugs did not affect cell viability. The inhibition of amino acid incorporation in 4-h cultures was similar (results not shown). Additionally, protein synthesis by a human lymphoblastoid line (8866) was similarly suppressed by pactamycin over the same range, 10^{-8}–10^{-5} M (results not shown). The drug effects, in all cases, were largely irreversible; cells treated with inhibitor at 37°C for 30–60 min and then washed, showed a suppression of protein synthesis over an 18-h period comparable to that observed in cells cultured continuously in the presence of drug.

Pactamycin and emetine both markedly suppressed the T-cell-mediated destruction of DBA/2 mastocytoma cells by spleen cell suspensions from alloimmunized C57BL/6 mice. Effector spleen cell populations were incubated at 37°C for 1 h in Eagle's medium containing various concentrations of emetine or pactamycin. The spleen cell suspensions were then washed three times, and lymphoid cells added to ^{51}Cr-labeled DBA/2 mastocytoma cells at a 100:1 ratio.

As can be seen (Fig. 1), both pactamycin and emetine at concentrations of 10^{-5} M totally inhibited cytolysis, apparently demonstrating the dependence of the lytic event on continuous protein synthesis. The concentration of inhibitors employed did not affect ^{51}Cr release from target cells in the presence of normal C57BL/6 spleen cells. It will be noted (Fig. 1) that emetine and pactamycin both totally inhibited amino acid incorporation at concentrations as low as 10^{-7} M; cytolysis at these concentrations was only minimally suppressed. A detailed analysis of this discordance will be the subject of a separate communication.

Guinea pig lymph node cells treated with pactamycin or with emetine also showed a diminished capacity to produce MIF when stimulated with specific antigen (Fig. 1). Lymph node cells obtained from animals exhibiting strong delayed hypersensitivity skin reactions to PPD, were treated for 1 h at 37°C with pactamycin (10^{-5} M) or emetine (10^{-5} M) and were then washed and incubated with 25 μg/ml PPD. Under these conditions MIF production was abolished. Treatment of sensitized lymphocytes for 24 h with lower concentrations of drug was associated with a dose-dependent inhibition of MIF production, which, from a quantitative standpoint, paralleled closely the effects of inhibitor dilution of [^{3}H]leucine incorporation (Fig. 1), particularly seen in the case of emetine (Fig. 1 *b*).

The Effects of In Vitro Treatment of Lymphoid Cells with CT on T-Cell-Mediated Cytolysis and on MIF and LT Production.—Because pharmacologic agents which increase intracellular cAMP have been shown to suppress T-cell-mediated cytotoxicity (15, 24), several such drugs were tested for their effect on LT and MIF production. In initial studies, dibutyryl cAMP and theophyl-

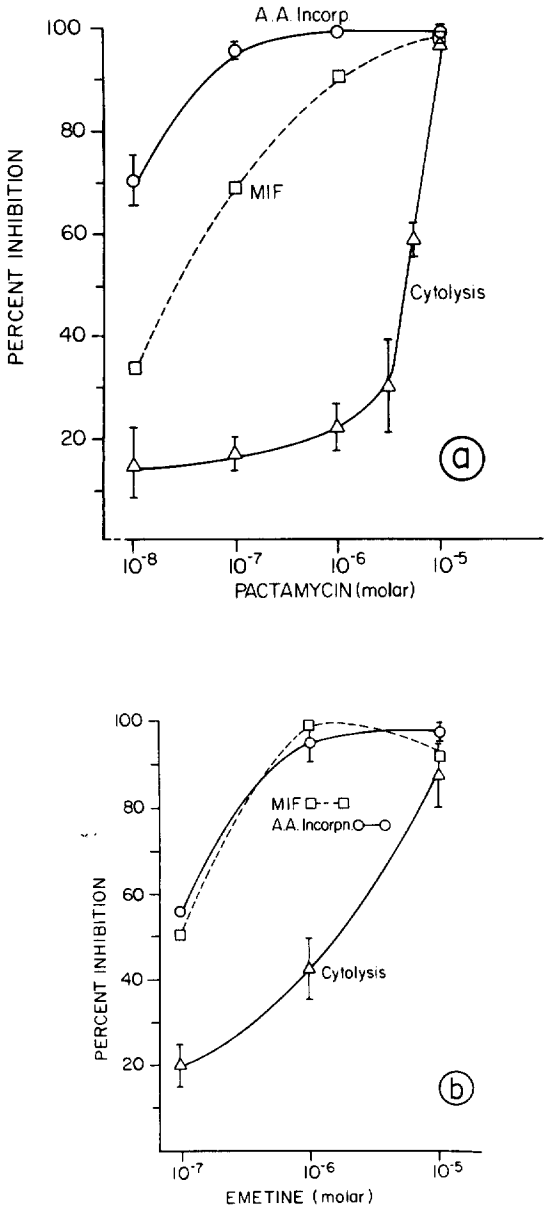

FIG. 1. Effect of the protein synthesis inhibitors (*a*) pactamycin and (*b*) emetine on the amino acid incorporation and the specific (anti-DBA/2) cytolytic activity of C57BL/6 mouse spleen cells and on PPD-induced MIF production by guinea pig lymph node cells. For details see text.

line were found to be without effect, while prostaglandin E_1 was occasionally inhibitory. Because the cytotoxicity assay requires less time than the 16 h necessary to produce MIF and LT, we chose to study the effects of CT, a potent and protracted adenylate cyclase-stimulating agent. We have previously shown that cholera toxin can suppress the cytolytic activity of effector T cells both in vivo and in vitro; a suppression presumably mediated via elevation of lymphocyte cAMP levels (23, 25).

Lymph node cells from PPD-sensitized guinea pigs were treated with 100 ng/ml cholera toxin for 24 h in the presence or absence of 25 μg/ml PPD. Three sets of controls were used: (*a*) cells incubated without antigen (to which PPD was restored after harvesting the supernate); (*b*) cells cultured with PPD alone; and (*c*) cells cultured with PPD (to which cholera toxin was added after harvesting the supernates). The supernatant fluids were then assayed for MIF and LT; the cell pellets for cAMP content. Results from 4 representative animals out of over 20 studied are presented in Table I. These animals were selected because they were sensitized and tested on the same day, and tests for LT, MIF, and cAMP were performed in parallel. It should be noted that in other experiments not shown, lymphocytes were cultured for 1 h with CT, washed three times, and then cultured in the presence or absence of antigen, with essentially no effect on mediator production.

Treatment of mouse and guinea pig lymphocytes with CT caused marked elevation in cAMP levels which persisted through a 24-h incubation period (Table I and references 23 and 25). Elevation of cAMP levels were first demonstrable 1–2 h after treatment with CT, and in earlier studies we have shown that cAMP remains at an augmented level for 24–48 h (25).

Elevated cAMP levels in mouse effector cell populations were associated with an almost total inhibition of lytic activity (legend Table I). These data have already been described in detail (15, 23). On the other hand, guinea pig lymph node cells with augmented cAMP levels produced, on stimulation with homologous antigen, amounts of LT equivalent to that produced by untreated cells (Table I). CT-treated cells which were washed before antigenic stimulation, produced amounts of MIF comparable to untreated controls. When CT was included throughout the period of culture, however, some suppression of MIF activity was noted (Table I). It was apparent that most of this effect was at the level of MIF action, for the addition of CT to MIF containing PPD supernates diminished their inhibitory activity on the migration of normal macrophages, in some cases dramatically (animal 4, Table I). Such findings are in concordance with those of Koopman et al. (26).

Cholera toxin treatment of mouse spleen cells had no effect on LT production induced by concanavalin A (Con A) (Table II). In this study, 6×10^6 C57BL/6 mouse spleen cells were incubated with 100 ng of CT for 1 h, washed, and then cultured for 72 h in the presence of 10 μg/ml Con A. The cell-free supernates were harvested, millipore filtered (0.44 μ pore size), and then cul-

TABLE I

CT Activation of Lymphocyte cAMP Levels: Effect on MIF and LT Production

Guinea pig	Culture conditions*	Lymphocyte cAMP levels‡	MIF		LT		
			Inhibition of migration§	Inhibition of MIF‖ production	Inhibition of a.a. incorporation by LT¶		Inhibition of LT production**
					undild.	1:3	
		p mol/10⁷ cells	%	%	%		%
1	Medium alone	9.9; 4.6	0	—	0		
	+ PPD‡‡	4.6; 4.6	70	—	92.4	50.6	—
	+ PPD + CT§§	92.3; 168.3	54	23	94.7	78.9	0
	+ PPD (CT)‖‖	9.9; 4.0	63	10	94.8	66.0	0
2	Medium alone	12.5; 10.6	0	—	0		
	+ PPD	21.2; 27.1	77	—	93.3	69.7	—
	+ PPD + CT	130	55	29	95.8	78.6	0
	+ PPD (CT)	8.4; 11.2	65	16	94.6	81.8	0
3	Medium alone	17.1; 14.8	0	—	0		
	+ PPD	8.5	56	—	97.6	26.9	—
	+ PPD + CT	205.6; 266.0	41	27	93.5	56.5	0
	+ PPD (CT)	15.0	46	18	91.2	58.3	0
4	Medium alone	Not measured	0	—	Not measured		
	+ PPD		65	—			
	+ PPD + CT		19	70			
	+ PPD (CT)		18	72			

Treatment (1 h, 37°C) of effector T-cell populations with 100 ng/ml of CT suppressed cytolysis 78.5 ± 14.2%; 10 ng/ml inhibited cytolysis 61.2 ± 10.1%; and 1 ng/ml 39.5 ± 7.3%.

* 2.2×10^7 guinea pig lymph node cells obtained 22 days after immunization with complete Freund's adjuvant (see text) were cultured for 24 h with or without antigen. After culture, the cells were separated and suspended in 5% TCA and immediately frozen until cAMP measurements were made. The supernates were assayed for MIF and LT.

‡ Number given is mean of triplicate assays on two individual cell samples. Replicates of each sample were always $< \pm 20\%$ and usually $< \pm 10\%$.

§ Relative to inhibition of migration observed in cultures incubated with CT in the absence of PPD and reconstituted with PPD at time of subsequent harvest. Values given for inhibition of migration are means of duplicate estimations.

‖ Relative to migration inhibition observed in the presence of PPD alone.

¶ Relative to supernates from cultures containing lymphocytes but no antigen.

** Relative to cultures containing antigen but no drug.

‡‡ 25 μg/ml PPD.

§§ 100 ng/ml CT added to the cultures at the time of antigenic stimulation.

‖‖ Cholera toxin added at time of supernate harvest (after 24-h incubation of cells with PPD alone).

tured with 10^5 mouse L cells for 72 h. Destruction of the L-cell monolayer was assayed as described in the Materials and Methods. As can be seen (Table II), CT-treated cells produced, in the presence of Con A, amounts of LT comparable to those obtained from untreated cells (Table II). In other experiments, not shown, 100 ng/ml CT was kept in the cultures throughout the period of incubation with Con A. The results obtained were similar: the presence of CT had no effect on LT production. Thus, under conditions in which the specific cytolytic activity of T cells was almost totally abolished, LT and MIF production by both mouse and guinea pig lymphoid cells remained basically unaffected.

Effects of Colchicine and Vinblastine on T-Cell-Mediated Cytolysis and on

TABLE II

Effect of Cholera Toxin and Colchicine on PHA-Induced Mouse Lymphotoxin Production

Cells	Treatment	LT assay			
		Inhibition a.a incorporation		Inhibition LT production*	
		Exp. I	Exp. II	Exp. I	Exp. II
		%		%	
C57BL/6 Spleen	None	0	0		
	Con A‡	84.6	83.5	—	—
	Con A + CT§	95.2	77.0	0	8
	Con A (+CT)‖	76.5	78.2	10	6
	Con A + colchicine¶	83.3	72.3	2	13
	Con A (+ colchicine)**	87.9	84.9	0	0
	Con A + 10^{-5} M pactamycin‡‡	0	1.8	100	98

6×10^6 C57BL/6 spleen cells were incubated alone or in the presence of drugs for 1 h at 37°C. The cells were then washed three times and cultured with 10 μg/ml Con A for 72 h at 37°C. The supernates obtained were millipore filtered (0.44 μ) and then placed on top of 10^5 L cells and cultured for a further 72 h. During the last 4 h of this culture 1 μCi[^{3}H]leucine was added and the amino acid incorporation into the residual cells then assayed.

Cholera toxin and colchicine inhibit T-cell-mediated cytolysis in a dose-related manner; at the 100 ng/ml cholera toxin concentration employed above, we have observed 79 ± 12% inhibition of specific cytolysis (23); 3×10^{-4} M colchicine inhibited cytolysis 72%.

* Calculated relative to cultures containing Con A but no drug.

‡ 10 μg/ml.

§ Spleen cells treated with 100 ng/ml cholera toxin for 1 h at 37°C then washed and incubated with Con A.

‖ Cholera toxin (100 ng/ml) added to supernate before assay only.

¶ Spleen cells treated with 3×10^{-4} M colchicine for 1 h at 37°C and then washed and incubated with Con A.

** Colchicine (3×10^{-4} M) added to supernate before assay only.

‡‡ Cells cultured with 10^{-5} M pactamycin at 37°C for 30 min and then washed 4–5 times before culturing with Con A. The supernate was then assayed for LT activity.

MIF and LT Production.—As reported earlier (16), effector spleen cells treated for 1 h at 37°C with colchicine at concentrations varying from 10^{-5} to 3×10^{-4} M showed diminished lytic activity. The degree of suppression was dose related (Table III). Vinblastine, another antimitotic agent with a propensity for microtubule subunits similar to that shown by colchicine (27), inhibited cytolysis comparably. Spleen cells treated with vinblastine for 1 h at 37°C were washed and their lytic capacity then compared to untreated cells. The effects (Table III) were similar to those observed with colchicine, complete inhibition occurring after treatment of the effector cells with concentrations of 10^{-4} M. Vinblastine-treated cells showed no diminished viability by dye exclusion tests. Treatment of target cells with colchicine and vinblastine had no effect on their susceptibility to lysis, nor did the drugs affect the release of ^{51}Cr from target cells in the presence of normal C57BL/6 spleen cells.

TABLE III

Effect of Colchicine and Vinblastine on T-Cell-Mediated Cytolysis

Drug	Specific cytolysis	Inhibition of specific cytolysis
	%	%
None	57.1 ± 7.0	
Colchicine 10^{-5} M	27.4 ± 6.3	52
10^{-4} M	18.8 ± 5.3	67
3×10^{-4} M	16.0 ± 6.2	72
10^{-3} M	7.4 ± 4.8	87
None	38.5	
Vinblastine 3×10^{-7}	34.7	10
10^{-6}	19.9	48
3×10^{-6}	13.8	64
10^{-5}	8.8	77
3×10^{-5}	4.7	88
10^{-4}	0.5	99

10^7 C57BL/6 spleen cells taken 10 days after immunization with 10^7 DBA/2 mastocytoma cells were incubated with or without drug for 1 h at 37°C. The cells were then washed and their lytic activity against 10^5 ^{51}Cr-labeled DBA/2 mastocytoma cells assessed. Percent specific cytolysis was calculated after subtraction of the percent ^{51}Cr released in the presence of 10^7 C57BL/6 spleen cells from unimmunized animals. Percent inhibition of specific cytolysis was calculated relative to effector cells incubated in the absence of drug.

The values recorded for colchicine inhibition are the means ± standard deviation derived from two experiments, in each of which the assays were performed in triplicate. The values recorded for vinblastine are means of triplicate assays in a single experiment. Duplication of this experiment gave similar values.

We had previously observed that vinblastine did not diminish the production of MIF by viable PPD-stimulated guinea pig lymph node cells (13). Similarly, treatment with colchicine (3×10^{-4} M) or colcemid for 24 h had little or no effect on the concurrent antigen-induced production of MIF or LT (Table IV). MIF containing supernates to which vinblastine, colchicine, or colcemid were added fully maintained their migration inhibitory activity, e.g. PPD supernates, 33.4% and 23.2% inhibition; the same supernates containing colcemid 1 μg/ml, 37.9% and 26.9%. At the same time, the migration of normal macrophages was found to be markedly increased by the presence of both colchicine and vinblastine.

The production of LT from mouse spleen cells in the presence of Con A was unaffected by pretreatment with vinblastine (3×10^{-5} M) (results not shown) or colchicine (3×10^{-4} M) (Table II), concentrations which almost totally inhibited the lytic activity of immune T-cells. LT action was similarly unaffected by addition of vinblastine or colchicine to active supernates.

TABLE IV

Effect of Colchicine on LT and MIF Production from Guinea Pig Lymph Node Cells

Exp.	LT			MIF		
	Inhibition of a.a incorporation*		Diminution of mediator production by colchicine‡	Inhibition of macrophage migration*		Diminution of MIF production by colchicine‡
	PPD	PPD + Colchicine		PPD	PPD + colchicine	
	%		%	%		%
I	92.4	94.8	0	70	63	10
II	93.3	95.0	0	77	59	23
III	70.7	88.5	0	50	48	4
IV	87.6	83.2	5	56	46	18
V	Not tested			33	46	−14
VI	Not tested			23	37	−14

Immune guinea pig lymph node cells as described in Table I were cultured for 24 h with or without antigen (25 μg/ml PPD) in the presence or absence of 3×10^{-4} M colchicine. After culture the cells were separated and the supernates assayed for both MIF and LT as described in the Materials and Methods.

* Relative to supernates obtained from lymphocytes cultured in the absence of antigen.

‡ Relative to the mediator produced in cultures containing antigen but no drug.

DISCUSSION

One of the central problems in cellular immunology at the present time concerns the heterogeneity of T cells. In addition to differences in longevity, response to mitogens and circulation patterns, T-cell heterogeneity is also reflected in terms of biological activity. It is not yet possible to determine whether the helper and suppressor activities of T cells are mediated by different subpopulations from those responsible for mediator production and direct cytolysis or whether a single T-cell line exhibits different capabilities during different stages of its differentiation or maturation.

Our attention in this paper has been directed at the two T-cell functions most directly concerned with the effector arm of cell-mediated immunity: soluble mediator production and T-cell-mediated cytotoxicity. The experiments presented were designed to test the hypothesis that mediator production and T-cell-mediated cytolysis were necessarily related cell-mediated immune phenomena, and that soluble mediators were involved in the lytic mechanism.

Initial suggestions that mediator production and cell-mediated cytolysis might be related came from observations that both phenomena were suppressed by inhibitors of protein synthesis. Production of MIF (10) and LT (1) were shown to be inhibited by puromycin, and T-cell-mediated cytolysis was shown to be substantially diminished by cycloheximide and actinomycin D (11). In the present studies, pactamycin and emetine, both known to be potent inhibitors of the initiation of protein synthesis (28, 29), were able to suppress production of MIF, LT, and T-cell-mediated cytolysis totally at concentrations of 10^{-5} M, confirming the previous observations. However, on analysis of the

dose curves (Fig. 1), it became clear that mediator production was considerably more sensitive to inhibition by these agents than was T-cell-mediated cytolysis, e.g. at 10^{-6} M pactamycin, cytolysis was inhibited only 20%, while MIF production and amino acid incorporation were inhibited approximately 90%. Thus, while both MIF production and T-cell-mediated lysis are ultimately affected by pactamycin and emetine, there was a marked dissociation in the sensitivities to these drugs. Furthermore, inhibition of cytolysis did not seem to parallel the effect of the drugs on amino acid incorporation.

Further experimental dissociation of mediator production and T-cell-mediated cytolysis was observed when the effects of adenylate cyclase-stimulating agents were studied. In the present study, CT was employed to ensure potentiation of cAMP levels throughout the 24- to 48-h period required for mediator production. The T-cell-mediated lytic activity of spleen cell populations was dramatically inhibited after treatment with CT, yet augmentation of cAMP levels was without effect on the production of guinea pig MIF and LT or murine LT. In earlier studies, Koopman et al. observed that cAMP active drugs inhibited the action of MIF on guinea pig macrophages, but reported that inhibition of MIF production was only irregularly seen in the presence of isoproterenol or theophylline (26). While cAMP levels in the lymphocyte populations were not assayed in their work, from our earlier studies it would appear unlikely that augmentation of cAMP would have persisted throughout the assay period. We observed only a transient (10–30 min) augmentation of lymphocyte cAMP levels by isoproterenol, and no demonstrable elevation with theophylline (15). In the present study, we were able to confirm their observation that cAMP-active drugs inhibit the action of preformed MIF on guinea pig macrophages. Interestingly, CT had no effect on either the production or action of LT. The results were unequivocal, and were true of antigen-induced LT from guinea pig lymph node cells as well as for LT produced by mouse spleen cells after Con A stimulation. These findings are at variance with those of Lies and Peter (30), who reported that dibutyryl cAMP and theophylline diminished the production of LT from human lymphocytes. In that study, cAMP levels were not measured, and the drugs chosen would not be expected to give prolonged increases in cAMP levels. Additionally, their assay for LT involved ^{51}Cr release from DBA/2 mastocytoma cells after a 16-h incubation period. In the present studies, such cells treated with both our guinea pig and mouse LT did not cause ^{51}Cr release over this time period. It is conceivable, though unlikely, that two different cytotoxic factors are being measured.

It previously had been reported that vinblastine, while inhibiting cell proliferation, had no effect on MIF production by viable antigen-stimulated guinea pig lymphocytes (13). This observation was confirmed in this study, and similar findings were also made with colchicine and colcemid. These vinca alkaloids were similarly without affect on the production of LT. On the other hand, both colchicine and vinblastine were potent inhibitors of T-cell-mediated

cytolysis. Colchicine and vinblastine are, of course, antimitotic agents, but this is almost certainly not the mode of inhibition of cytolysis, for the lytic process occurs independently of DNA synthesis (14). It is known that these drugs have a propensity for the dimeric subunits of microtubules, and by binding to them may inhibit microtubular aggregation and thus interfere with secretory processes (27, 31). In this regard, it is noteworthy that colchicine and vinblastine usually inhibit secretion of prepackaged "granules" but generally fail to affect continuous secretory processes. Thus, colchicine inhibits the secretion of lysosomal enzymes (32) but not the secretion of immunoglobulin from plasma cells (33).

In summary, we demonstrate here that T-cell-mediated lysis was inhibited under conditions in which synthesis of two lymphocyte-derived mediators (LT and MIF) remain unaffected. While extrapolation from these studies of two mediators to all possible products of activated lymphocytes would be unwarranted, these studies provide no ground for support of the hypothesis that soluble mediators play an essential role in T-cell-mediated cytolysis. Two possible objections to this conclusion deserve address. Firstly, it is possible that the cAMP-active drugs, and colchicine, prevent lymphocyte "triggering" by cell-associated antigens, but not by soluble antigens, thus accounting for the observation that cytolysis is inhibited without affect on mediator production. There is, however, experimental evidence to refute this point. In a previous study we have shown that the specific binding of effector T cells to specific target cell monolayers was not affected by colchicine or the adenylate cyclase stimulating hormone, PGE_1, but was inhibited by cytochalasin B (34). Thus, the inhibitory activity of colchicine or cAMP stimulating drugs is not attributable to an effect on binding.

Recent demonstrations that both B and T cells are able to produce mediators (references 35 and 36, and footnote 2) could present grounds for an alternative explanation of our observations. It could be argued that T-cell mediator production, along with T-cell-mediated cytolysis, could be inhibited by colchicine and the cAMP-active drugs, but that B-cell mediator synthesis could continue. Such arguments would demand either that B and T cells differ in the manner in which they synthesized and/or secreted products of activated lymphocytes, or, at the least, that they exhibited marked differences in susceptibility to several distinct classes of pharmacological agents. To date the effective dose-response relationships of the vinca alkaloids, pactamycin and emetine, and CT have been observed to be rather similar for a wide variety of cell types (19, 28, 29). While there may be differences in susceptibility of B and T cells to one of the drugs used in this study, it is improbable that differences would exist in susceptibility to all. It is perhaps worthy of note that the production of LT by mitogen-stimulated murine spleen cells was almost totally

[2] Bloom, B. R., E. Shevach, and I. Green. Manuscript in preparation.

inhibited by treatment with anti-thy.1 serum and complement (37), and that in the present experiments LT production was unaffected by treatment with CT, vinblastine, or colchicine (Table II). Nevertheless a definitive resolution of this problem must await results of experiments carried out on homogeneous lymphocyte subpopulations.

Our findings do not, of course, imply that soluble mediators lack other important biological functions in connection with delayed type hypersensitivity reactions or with tumor and allograft rejection. While a dissociation between cytolytic activity and mediator production under the conditions employed was clear, it cannot be argued on the basis of these experiments that these two functions are necessarily carried out by independent subpopulations of T cells. This may indeed be the case and a number of recent observations support this contention. Using TNP-conjugated mastocytoma cells and fixation with glutaraldehyde, Dennert was able to distinguish between T-helper and T-effector cells (38). These same T-cell activities have been selectively inhibited by treatment with the appropriate anti-Ly sera plus complement in mice (P. Kisielow, personal communication). The alternative possibility that mediator production and cytolytic activity are functions of the same T-cell line at different stages of maturation cannot be excluded by presently available evidence.

SUMMARY

Experiments have been designed to test the hypothesis that soluble mediator production and T-cell-mediated cytotoxicity are necessarily related phenomena, and that soluble mediators may be involved in the mechanism of cytolysis. To this end, agents known to inhibit T-cell-mediated lysis in vitro have been studied for their effects on the production of two lymphocyte-derived mediators, lymphotoxin (LT) and migration inhibitory factor (MIF).

A clear dissociation between mediator production and cell-mediated cytolysis was found using inhibitors of protein synthesis. Pactamycin and emetine, in doses of 10^{-7} M to 10^{-6} M, suppressed production of MIF and LT with only slight effect on killing of mastocytoma cells by immune T cells. On the other hand colchicine and vinblastine inhibited T-cell-mediated cytolysis in a dose-related manner but had no significant effect on either MIF or LT production. A striking dichotomy was also observed after augmentation of intracellular cyclic 3′5′ adenosine monophosphate (cAMP) levels with cholera enterotoxin. Increased cAMP levels were associated with abrogation of direct lytic activity, but were without significant effect on MIF or LT production in guinea pigs or mice. These findings indicate that mediator production and direct lymphocyte-mediated cytolysis can be experimentally dissociated and represent independent cell-mediated immune functions.

We wish to thank Dr. Elizabeth Gillespie, Department of Medicine, Johns Hopkins University, for kindly performing the cAMP assays and Mr. Kirk Ziegler for excellent technical

assistance. We are grateful to Dr. W. Wechter, Upjohn Company, Kalamazoo, Mich., and Dr. R. A. Finkelstein for generous gifts of pactamycin and cholera enterotoxin, respectively.

REFERENCES

1. Williams, T. W., and G. W. Granger. 1969. Lymphocyte *in vitro* cytotoxicity: correlation of derepression with release of lymphotoxin from human lymphocytes. *J. Immunol.* **103**:170.
2. Walker, S. M., and Z. J. Lucas. 1972. Cytotoxic activity of lymphocytes. II. Studies on the mechanism of lymphocyte mediated cytotoxicity. *J. Immunol.* **109**:1133.
3. Ruddle, N. H., and B. H. Waksman. 1968. Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. II. Analysis of mechanism. *J. Exp. Med.* **128**:1267.
4. Granger, G. A., and W. P. Kolb. 1968. Lymphocyte *in vitro* cytotoxicity: mechanisms of immune and non-immune small lymphocyte mediated target cell destruction. *J. Immunol.* **101**:111.
5. Perlmann, P., and G. Holm. 1969. Cytotoxic effects of lymphoid cells *in vitro*. *Adv. Immunol.* **11**:117.
6. Hayry, P., and V. Defendi. 1970. Mixed lymphocyte cultures produce effector cells: model *in vitro* for allograft rejection. *Science* (*Wash. D. C.*). **168**:133.
7. Brunner, K. T., and J.-C. Cerottini. 1971. Cytotoxic lymphocytes as effector cells of cell-mediated immunity. Prog. Immunol. (1st International Congress of Immunology) (B. Amos, editor), Academic Press, Inc. New York. pp. 386.
8. Bloom, B. R. 1971. *In vitro* approaches to the mechanism of cell-mediated immune reactions. *Adv. Immunol.* **13**:102.
9. Henney, C. S. 1973. On the mechanism of T-cell mediated cytolysis. *Transplant. Rev.* **17**:37.
10. David, J. R. 1965. Suppression of delayed hypersensitivity in vitro by inhibition of protein synthesis. *J. Exp. Med.* **122**:1125.
11. Brunner, K. T., J. Mauel, J.-C. Cerottini, and B. Chapuis. 1969. Quantitative assay for the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells *in vitro*: inhibition by isoantibody and by drugs. *Immunology.* **14**:181.
12. Bloom, B. R., J. Gaffney, and L. Jimenez. 1972. Dissociation of MIF production and cell proliferation. *J. Immunol.* **109**:1395.
13. Rocklin, R. E. 1973. Production of migration inhibitory factor by non-dividing lymphocytes. *J. Immunol.* **110**:674.
14. Mauel, J., H. Rudolf, B. Chapuis, and K. T. Brunner. 1970. Studies of allograft immunity in mice. II. Mechanism of target cell destruction *in vitro* by sensitized lymphocytes. *Immunology.* **18**:517.
15. Henney, C. S., H. R. Bourne, and L. M. Lichtenstein. 1971. The role of cyclic 3′-5′ adenosine monophosphate in the cytolytic activity of lymphocytes. *J. Immunol.* **108**:1526.
16. Plaut, M., L. M. Lichtenstein, and C. S. Henney. 1973. Studies on the mechanism of lymphocyte mediated cytolysis. III. The role of microfilaments and microtubules. *J. Immunol.* **110**:771.
17. Strom, T. B., M. R. Garovoy, C. B. Carpenter, and J. P. Merrill. 1973. Microtubule function in immune and nonimmune lymphocyte mediated cytotoxicity. *Science* (*Wash. D. C.*). **181**:171.

18. Henney, C. S., and J. E. Bubbers. 1973. Studies on the mechanism of lymphocyte mediated cytolysis. I. The role of divalent cations in cytolysis by T-lymphocytes. *J. Immunol.* **110**:63.
19. Becker, E., and P. Henson. 1973. *In vitro* studies of immunologically induced secretion of mediators from cells and related phenomena. *Adv. Immunol.* **17**:94.
20. Bloom, B. R., and B. Bennett. 1971. The assay of inhibition of macrophage migration and the production of macrophage inhibitory factor (MIF) and skin reactive factor (SRF) in the guinea pig. *In In Vitro* Methods in Cell-Mediated Immunity. B. R. Bloom and P. R. Glade, editors., Academic Press, Inc., New York. 235.
21. Henney, C. S. 1971. Quantitation of the cell mediated immune response. I. The number of cytolytically active lymphoid cells induced by immunization with allogeneic mastocytoma cells. *J. Immunol.* **107**:1558.
22. Brown, B. L., J. D. M. Albano, R. P. Elkins, and A. M. Sgherzi. 1971. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′ cyclic monophosphate. *Biochem. J.* **121**:561.
23. Lichtenstein, L. M., C. S. Henney, H. R. Bourne, and W. B. Greenough. 1973. The effect of cholera toxin on in vitro models of immediate and delayed hypersensitivity: further evidence for the role of cAMP. *J. Clin. Invest.* **52**:691.
24. Strom, T., A. Deisseroth, J. Morganroth, C. B. Carpenter, and J. P. Merrill. 1972. Alteration of the cytotoxic action of sensitized lymphocytes by cholinergic agents and activators of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 2995.
25. Henney, C. S., L. M. Lichtenstein, E. Gillespie, and R. T. Rolley. 1973. In vivo suppression of the immune response to alloantigen by cholera enterotoxin. *J. Clin. Invest.* **52**:2853.
26. Koopman, W. J., M. H. Gillis, and J. R. David. 1973. Prevention of MIF activity by agents known to increase cellular cyclic AMP. *J. Immunol.* **110**:1609.
27. Borisy, C. G., and E. W. Taylor. 1967. The mechanism of action of colchicine. Binding of colchicine ^{3}H to cellular protein. *J. Cell. Biol.* **17**:19.
28. MacDonald, J. S., and I. H. Goldberg. 1970. An effect of pactamycin in the initiation of protein synthesis in reticulocytes. *Biochem. Biophys. Res. Commun.* **41**:1.
29. Grollman, A. P. 1972. Structural basis for inhibition of protein synthesis by emetine and cyclohexamide based on an analogy between alkaloids and glutarimide antibiotics. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3024.
30. Lies, R. B., and J. B. Peter. 1973. Cyclic AMP inhibition by cytotoxin ("lymphotoxin") elaboration by stimulated lymphocytes. *Cell. Immunol.* **8**:332.
31. White, J. G. 1969. Effect of colchicine and vinca alkaloids on human platlets. III. Influence on primary internal contraction and secondary aggregation. *Am. J. Pathol.* **54**:467.
32. Weissman, G., P. Dukor, and R. B. Zurier. 1971. Effects of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nat. New Biol.* **231**:131.
33. Parkhouse, R. M. E., and A. C. Allison. 1972. Failure of cytochalasin or colchicine to inhibit secretion of immunoglobulin. *Nat. New Biol.* **235**:220.
34. Henney, C. S., and J. E. Bubbers. 1973. Antigen-T lymphocyte interactions: inhibition by cytochalasin B. *J. Immunol.* **111**:85.
35. Yoshida, T., H. Sonozaki, and S. Cohen. 1973. The production of migration inhibitory factor by B-cells and T-cells of the guinea pig. *J. Exp. Med.* **138**:784.

36. MacDermott, R. P., R. E. Rocklin, L. Chess, J. R. David, and S. F. Schlossman. 1974. Reevaluation of in vitro cellular immunity using purified human T and B cells. *J. Clin. Invest.* **53**:49*a*. (Abstr.)
37. Shacks, S. J., J. Chiller, and G. A. Granger. 1973. Studies on in vitro models of cellular immunity: the role of T and B cells in the secretion of lymphotoxin. *Cell Immunol.* **7**:313.
38. Dennert, G. 1974. Evidence for non-identity of T killer and T helper cells sensitized to allogeneic cell antigens. *Nature (Lond.)*. **249**:358.