damaged or blocked, perhaps by autoimmune mechanisms (*18*), must be elucidated by future investigation. However, our model provides support for the concept that the receptor abnormality may be of fundamental importance in myasthenia gravis.

S. SATYAMURTI
DANIEL B. DRACHMAN, FRED SLONE
Department of Neurology,
Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

References and Notes

1. W. Fields, Ed., "Myasthenia gravis," *Annu. N.Y. Acad. Sci.* **183**, 1–386 (1971).
2. D. Elmqvist, W. W. Hofmann, J. Kugelberg, D. M. J. Quastel, *J. Physiol.* **174**, 417 (1964).
3. J. E. Desmedt, in *New Developments in EMG and Clinical Neurophysiology*, J. E. Desmedt, Ed. (Karger, Basel, 1973).
4. D. Grob and R. J. Johns, in *Myasthenia Gravis*, H. R. Viets, Ed. (Thomas, Springfield, Ill., 1961), p. 127.
5. J. A. Simpson, *Ann. N.Y. Acad. Sci.* **183**, 241 (1971).
6. D. M. Fambrough, D. B. Drachman, S. Satyamurti, *Science* **182**, 293 (1973).
7. C. C. Chang and C. Y. Lee, *Br. J. Pharmacol. Chemother.* **28**, 172 (1966).
8. G. Biesecker, *Biochemistry* **12**, 4403 (1973).
9. C. Y. Lee, C. C. Chang, T. H. Chiu, P. J. S. Chiu, T. C. Tseng, S. Y. Lee, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.* **259**, 260 (1968).
10. C. Y. Lee, *Annu. Rev. Pharmacol.* **12**, 265 (1972).
11. C. Ozdemir and R. R. Young, *Ann. N.Y. Acad. Sci.* **183**, 287 (1971). The decrement in percent is equal to

$$\frac{\text{amp. 1st pot.} - \text{amp. 4th pot.}}{\text{amp. 1st pot.}} \times 100$$

12. L. P. Rowland, H. Aranow, Jr., P. F. A. Hoefer, in *Myasthenia Gravis*, H. R. Viets, Ed. (Thomas, Springfield, Ill., 1961), p. 411.
13. The percent change is equal to

$$\frac{\Delta \text{ after treatment} - \Delta \text{ before treatment}}{\Delta \text{ before treatment}} \times 100$$

where Δ is the decrement.
14. J. Goodgold and A. Eberstein, *Electrodiagnosis of Neuromuscular Disease* (Williams & Wilkins, Baltimore, 1972), pp. 146–149.
15. A. Slomic, A. Rosenfalck, F. Buchthal, *Brain Res.* **10**, 1 (1968).
16. H. C. Fertuck and M. M. Salpeter, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1376 (1974).
17. Desmedt has postulated a presynaptic defect, based chiefly on the presence of the post-activation exhaustion phenomenon in myasthenic muscles, and its absence in partially curarized muscles of cat or man (*3*, pp. 241 and 305). We are unable to explain why curare does not produce this phenomenon and α-cobratoxin does, although the marked differences in their pharmacokinetic properties may be significant in this regard. Since post-activation exhaustion is not well understood, no firm conclusions about the site of the underlying disorder should be based on its presence or absence. In our study we have merely concluded that specific blockade of ACh receptors is capable of producing the characteristic physiological phenomena of myasthenia gravis. The site of the lesion is inferred from present knowledge about the location of ACh receptors.
18. J. A. Simpson, *Scott. Med. J.* **5**, 419 (1960); J. Patrick and J. Lindstrom, *Science* **180**, 871 (1973).
19. We thank Dr. Douglas Fambrough for helpful discussion during the course of this study. Supported by NIH grants HD04817-04 and 5-PO1-NS1090-02 and a fellowship grant (to S.S.) from the Muscular Dystrophy Association of America and a Henry R. Viets Student Research Fellowship award (to F.S.) from the Myasthenia Gravis Foundation, Inc. Address reprint requests to D.B.D.

20 September 1974; revised 26 November 1974

Adenosine Inhibition of Lymphocyte-Mediated Cytolysis: Possible Role of Cyclic Adenosine Monophosphate

Abstract. *The in vitro destruction of tumor cells by specifically sensitized mouse lymphocytes was inhibited by adenosine; this inhibition was markedly potentiated by the presence of an inhibitor of adenosine deaminase. The inhibition of cytolysis by adenosine was accompanied by a rapid elevation in lymphocytic adenosine 3′,5′-monophosphate (cyclic AMP) concentrations. Both the inhibition of cytolysis and the elevation of cyclic AMP were reversed by prolonged incubation of the lymphocytes in the presence of adenosine or, more rapidly, by removal of the adenosine. Low concentrations of adenosine also caused an elevation of cyclic AMP in human lymphocytes, and this effect of adenosine may contribute to the lack of immune response associated with adenosine deaminase deficiency.*

Recently there have been reports (*1*) of a number of children with combined immunodeficiency disease associated with a deficiency of adenosine deaminase (E.C. 3.5.4.4). A chance association of these two rare defects would appear to be highly improbable. Evidence against the possibility of the deletion of a single genetic locus involving both adenosine deaminase production and the immune response has been presented (*2*). Green and Chan (*3*) have reported that established fibroblasts or lymphoid cells die in the presence of 10^{-4} to $10^{-5}M$ adenosine. They found that pyrimidine nucleotide concentrations were markedly depressed and that the toxic effects of adenosine were prevented by pyrimidine sources, such as uridine. This led them to speculate that the association of combined immunodeficiency disease and adenosine deaminase deficiency was the result of adenosine-induced pyrimidine starvation of lymphoid cells. Adenosine has been reported to cause an increase in cyclic AMP in a variety of cell types (*4*), and elevated concentrations of cyclic AMP in the effector lymphocytes are known to be inhibitory to lymphocyte-mediated cytotoxicity (LMC) (*5*). Our studies with cytotoxic lymphocytes have shown that adenosine is an inhibitor of LMC and that its inhibitory activity is correlated with elevated lymphocytic cyclic AMP.

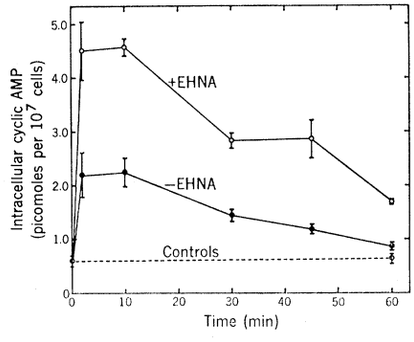

Fig. 1. Cyclic AMP concentrations in peritoneal exudate lymphocytes as a function of time after addition of adenosine (37.5 μM) ± EHNA (7.9 μM) to the cell suspensions. The cells (1×10^7 in 5.0 ml of buffer solution) were incubated for 30 minutes at 37°C, at which time ($t = 0$) adenosine or EHNA plus adenosine were added to the incubation mixtures. Duplicate control incubations were prepared with and without EHNA (7.9 μM); these two types of controls yielded identical cyclic AMP values. Each point represents the mean ± standard error of the mean (S.E.M.) for four determinations.

The in vitro model for lymphocyte-mediated immunity we have used has been described (*6*) and is based on the model reported by Berke *et al.* (*7*). Briefly, the target cell is a mouse ascites leukemia, EL4, which multiplies rapidly and kills C57BL mice after intraperitoneal injection. However, intraperitoneal injection of these cells into an allogeneic strain of mice, such as CD-1, results in the production of sensitized lymphocytes and ultimate rejection of the tumor by an unknown mechanism.

Cytotoxic peritoneal exudate cells were obtained from CD-1 mice after rejection of the EL4 cells, by repeated lavages of the peritoneal cavity. Nonadherent peritoneal exudate lymphocytes were obtained by passing the crude population of cells over a glass wool column and used in both the in vitro cytolysis and the cyclic AMP experiments. These cell populations contained over 90 percent small to medium-size lymphocytes. While the exact proportion of cytolytic lymphocytes is not known, Sullivan *et al.* (*8*) have reported that more than 80 percent of the peritoneal exudate lymphocytes obtained from a number of strains of mice after intraperitoneal injection of

Table 1. Inhibition of lymphocyte-mediated cytolysis by adenosine, alone and in the presence of 7.9 μM EHNA. Lysis was measured by the ^{51}Cr released after 70-minute incubations of ^{51}Cr-labeled EL4 cells with peritoneal exudate lymphocytes and drugs, compared to the ^{51}Cr released after control incubations lacking drugs.

Adenosine (μM)	Percentage inhibition of lysis of EL4 cells with	
	Adenosine	Adenosine + EHNA
150	51	87
75	41	82
37.5	44	80
18.8	33	73
9.4	28	67
4.7	16	53

EL4 cells contained a unique antigen which may reflect the acquisition of cytolytic capacity. The lymphocytes were washed immediately before use to separate them from the relatively high concentrations of extracellular cyclic AMP. Phosphate-buffered saline (*9*) supplemented with 10 percent heat-inactivated fetal calf serum was used for all washes and incubations.

The in vitro cytolytic assay involved duplicate incubations of 2.5×10^5 lymphocytes with an equal number of ^{51}Cr-labeled EL4 cells in a total volume of 1.0 ml. These cells were incubated with gentle rocking (five cycles per minute) at 37°C in plastic tissue culture plates (35 by 10 mm) in an atmosphere of moist air. Adenosine was added to achieve the specified concentrations. The contents of the plates were assayed for released radioactivity after the specified incubation time. *erythro*-9-(2-Hydroxy-3-nonyl)adenine (EHNA), a potent inhibitor of adenosine deaminase (*10*), was employed in the indicated experiments at a concentration of 7.9 μM; this concentration of EHNA was without effect either on cytolysis or on the cyclic AMP content of the lymphocytes.

For the cyclic AMP determinations, 1×10^7 lymphocytes were incubated, in duplicate, in 5.0 ml of the buffer solution with the agents under study. After the specified incubation period, cells were harvested by centrifugation at 200*g* for 5 minutes, resuspended in 5 ml of fresh buffer solution, and rapidly extracted with an equal volume of cold 1.0*M* perchloric acid containing approximately 0.2 pmole (12,000 disintegrations per minute) of [8-^{3}H]-cyclic AMP. The extracts were clarified by centrifugation, neutralized with KOH, centrifuged to remove the precipitated $KClO_4$, evaporated to dryness, and dissolved in 1.0 ml of 0.1*M* HCl. These acidified extracts were chromatographed on columns (0.5 by 4.5 cm) of Bio-Rad AG 50W-X8 (200 to 400 mesh), which were equilibrated and eluted with 50 m*M* HCl. The cyclic AMP fractions (6 to 10 ml) were evaporated to dryness and dissolved in 1.0 ml of 50 m*M* sodium acetate (*p*H 6.2). The amount of [8-^{3}H]cyclic AMP present in 200 μl of sample was measured to determine the percent of recovery (usually 30 to 50 percent). Total cyclic AMP concentrations in 100-μl portions of the samples were determined by cyclic AMP radioimmunoassay (*11*) after prior succinylation of the samples to render the assay 100-fold more sensitive (*12*). Each sample was assayed in duplicate, and a third 100-μl portion of each sample was assayed after treatment with beef heart 3′,5′-nucleotide phosphodiesterase. This enzymatic treatment routinely destroyed more than 97 percent of the measured cyclic AMP. Reagents for the cyclic AMP radioimmunoassay were purchased from Collaborative Research Inc., Waltham, Massachusetts.

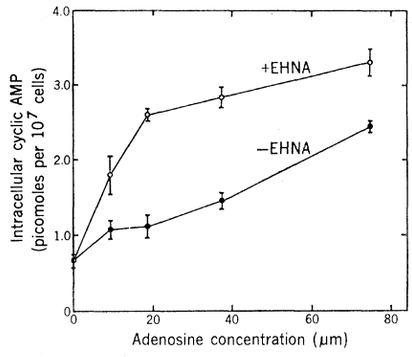

Fig. 2. Effect of adenosine concentration on lymphocyte cyclic AMP concentrations 30 minutes after addition of adenosine ± EHNA (7.9 μM) to the cell suspensions. Experimental conditions were identical to those described in the legend to Fig. 1. Each point represents the mean ± S.E.M. for four determinations.

The inhibition of LMC by adenosine is shown in Table 1. The 50 percent inhibitory dose (ID_{50}) of adenosine was approximately 150 μM in the absence of EHNA and 4.7 μM in its presence. The potentiative effect of the deaminase inhibitor was also apparent when horse serum (which does not contain adenosine deaminase) was substituted for fetal calf serum. The deamination product of adenosine, inosine, was not inhibitory to cytolysis even at 375 μM.

The inhibitory effect of adenosine (with or without EHNA) was lost after prolonged incubation (Table 2); recovery of cytolytic activity could be achieved without delay by washing the cells and resuspending them in fresh buffer solution. Lymphocyte-mediated cytotoxicity was inhibited by N^6,2′-*O*-dibutyryl cyclic AMP with an ID_{50} of 2.0 m*M*.

Incubation of peritoneal exudate lymphocytes with 37.5 μM adenosine resulted in a rapid increase in the cellular cyclic AMP, both in the presence and absence of EHNA (Fig. 1). At all times, the elevation in cyclic AMP caused by adenosine was greater in the presence of the deaminase inhibitor. In the absence of EHNA, lymphocytic cyclic AMP concentrations fell to control levels within 60 minutes; the presence of EHNA slowed this fall significantly but did not prevent its occurrence. In another experiment, when lymphocytes were washed free of adenosine (37.5 μM) and EHNA (7.9 μM) after a 10-minute incubation, the elevated (tenfold) cyclic AMP level returned to the control value within 30 minutes.

Table 2. Effect of preincubation on inhibition of LMC by adenosine. Lymphocytes (2.5 ml with 5×10^6 cells per milliliter) were incubated at 37°C with adenosine, alone or with EHNA, for the indicated times. Samples (0.5 ml) of these lymphocytes were then mixed with equal numbers of ^{51}Cr-labeled EL4 cells (in 0.5-ml volumes) and assayed for cytolytic activity in the usual manner (70-minute assay). The EL4 cells added to the last two groups contained 7.9 μM EHNA. Cytolytic activity (^{51}Cr release) is compared to that measured in parallel control incubations lacking adenosine.

Adenosine (μM)	EHNA (μM)	Percentage inhibition of lysis after incubation for				
		0 min	60 min	90 min	120 min	150 min
75	0	40	36	25	14	0
37.5	0	40	27	20	14	0
37.5	7.9	70	70	63	53	33
18.8	7.9	65	55	53	37	17

Lymphocytic cyclic AMP levels were elevated in a dose-dependent manner as the concentration of adenosine was increased up to 75 μM (Fig. 2). At all concentrations of adenosine tested, the elevation of cyclic AMP was markedly higher in the presence of the deaminase inhibitor. Purified (*13*) human peripheral blood lymphocytes also exhibited a dose-dependent increase in cyclic AMP in response to exogenous adenosine; a concentration of 3.8 μM adenosine caused a 2.0-fold increase in cyclic AMP after 30 minutes of incubation.

Several lines of evidence indicate that the ability of adenosine to inhibit cytolysis is unrelated to the inhibition of pyrimidine nucleotide synthesis described by Green and Chan (*3*). First, the inhibitory effect of adenosine is not prevented by the presence of a tenfold molar excess of uridine; uridine itself has no effect on cytolysis. Second, the inhibitory effect of adenosine is spontaneously reversible during prolonged incubation of cytolytic lymphocytes and adenosine. Third, high-pressure liquid chromatographic analysis (*14*) of the nucleotides of the lymphocyte population after 60 minutes of incubation with adenosine showed no effect on the pyrimidine nucleoside triphosphate pools.

The mechanism by which adenosine inhibits LMC appears to involve an elevation of lymphocytic cyclic AMP; the concentrations of adenosine which inhibit LMC (Table 1) are also effective in causing an increase in lymphocytic cyclic AMP (Fig. 2). The time-dependent decay of adenosine-enhanced cyclic AMP to control levels (Fig. 1) precedes the gradual recovery of cytolytic activity observed when the cells were incubated with adenosine (with or without EHNA) before the cytolytic assay (Table 2). This observation [in addition to the observation that cyclic AMP concentrations rapidly decrease after removal (washout) of adenosine and EHNA] is consistent with the hypothesis that the inhibitory effect of adenosine on LMC is mediated via raised cyclic AMP concentrations in the effector lymphocytes and that this inhibitory activity is reversed once the cyclic AMP concentration falls below some critical value.

It is apparent from this in vitro model that the absence of adenosine deaminase in lymphoid tissue would render these cells particularly sensitive to inhibition of an immune effector mechanism by adenosine. Adenosine formation and its subsequent reutilization (via adenosine kinase) or catabolism (via adenosine deaminase) appear to occur as a normal feature of cellular purine nucleotide metabolism (*15*). Deficiency of the enzyme adenosine deaminase may allow an abnormal expansion of the lymphoid pool of adenosine and thus lead to immunosuppressive effects such as that demonstrated in this report.

GERALD WOLBERG
THOMAS P. ZIMMERMAN
KAY HIEMSTRA
MARVIN WINSTON
LI-CHIANG CHU

Department of Experimental Therapy, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

References

E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara, H. J. Meuwissen, *Lancet* **1972-II**, 1067 (19
H. D. Ochs, J. E. Yount, E. R. Giblett, S.-H. Chen, C. R. Scott, R. J. Wedgwood, *ibid.* **1973-I**, 1393 (19
J. Yount, P. Nichols, H. D. Ochs, S. P. Hammar, C. R. Scott, S.-H. Chen, E. R. Giblett, R. J. Wedgwood, *J. Pediatr.* **84**, 173 (1974).
M. B. van der Weyden, R. H. Buckley, W. N. Kelley, *Biochem. Biophys. Res. Commun.* **57**, 590 (1974).
3. H. Green and T.-S. Chan, *Science* **182**, 836 (1973).
4. A. Sattin and T. W. Rall, *Mol. Pharmacol.* **6**, 13 (1970); D. C. B. Mills and J. B. Smith, *Biochem. J.* **121**, 185 (1971); W. A. Peck, J. Carpenter, K. Messinger, *Endocrinology* **94**, 148 (1974); R. B. Clark, R. Gross, Y.-F. Su, J. P. Perkins, *J. Biol. Chem.* **249**, 5296 (1974).
5. T. B. Strom, A. Deisseroth, J. Morganroth, C. B. Carpenter, J. P. Merrill, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2995 (1972); C. S. Henney, H. R. Bourne, L. M. Lichtenstein, *J. Immunol.* **108**, 1526 (1972); H. R. Bourne, L. M. Lichtenstein, K. L. Melmon, C. S. Henney, Y. Weinstein, G. M. Shearer, *Science* **184**, 19 (1974); C. W. Parker, T. L. Sullivan, H. J. Wedner, in *Advances in Cyclic Nucleotide Research*, P. Greengard and C. A. Robinson, Eds. (Raven, New York, 1974), vol. 4, p. 1.
6. G. Wolberg, K. Hiemstra, J. J. Burge, R. C. Singler, *J. Immunol.* **111**, 1435 (1973).
G. Berke, K. A. Sullivan, B. Amos, *J. Exp. Med.* **135**, 1334 (1972).
K. A. Sullivan, G. Berke, D. B. Amos, *Transplantation* **16**, 303 (1973).
R. Dulbecco and M. Vogt, *J. Exp. Med.* **99**, 167 (1954).
H. J. Schaeffer and C. F. Schwender, *J. Med. Chem.* **17**, 6 (1974).
11. A. L. Steiner, C. W. Parker, D. M. Kipnis, *J. Biol. Chem.* **247**, 1106 (1972).
H. L. Cailla, M. S. Racine-Weisbuch, M. A. Delaage, *Anal. Biochem.* **56**, 394 (1973).
13. J. W. Hadden, E. M. Hadden, M. K. Haddox, N. D. Goldberg, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3024 (1972).
D. J. Nelson, C. J. L. Bugge, H. C. Krasny, G. B. Elion, *Biochem. Pharmacol.* **22**, 2003 (1973).
15. C. A. Lomax and J. F. Henderson, *Cancer Res.* **33**, 2825 (1973); M. H. Lerner and B. A. Lowy, *J. Biol. Chem.* **249**, 959 (1974).

1 October 1974; revised 25 November 1974 ■

Cell-Mediated Immunity to Antigens Associated with Endogenous Murine C-Type Leukemia Viruses

Abstract. *Splenic lymphocytes from (BALB/c × A/J) F_1 mice are cytotoxic for BALB/c fibroblasts infected with an endogenous C-type leukemia virus, but are not cytotoxic for uninfected BALB/c fibroblasts. These results indicate that mice do not exhibit cell-mediated immune tolerance to antigens associated with endogenous C-type viruses.*

Most, if not all, mouse strains carry within their cells the necessary genetic information to code for the production of endogenous C-type leukemia viruses (*1*). Expression of such virus genetic information varies greatly among strains, and may be related to the subsequent development of lymphomas. Little is known of the host's natural immune responses to endogenous C-type viruses. Recent studies have indicated that inbred mice develop humoral antibodies to several endogenous C-type viral antigens (*2*). We report here studies that indicate that mice may also develop cell-mediated cytotoxic responses against antigens associated with endogenous C-type viruses.

Male (BALB/c × A/J) F_1 mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Two BALB/c embryo fibroblast cell lines of common origin were established. One cell line was infected with an endogenous mouse-tropic C-type virus activated during a graft-versus-host reaction in a (BALB/c × A/J) F_1 mouse injected with BALB/c splenocytes (*3*). After 35 passages, 90 to 100 percent of these cells were producing infectious virus as determined by an infectious center assay previously described (*4*). The other BALB/c cell line was not infected, but was passed in parallel with the virus-infected line; this line remained virus-negative for both mouse-tropic and xenotropic C-type viruses, as determined by RNA-dependent DNA polymerase assays performed according to techniques described by Lieber *et al.* (*5*). These two fibroblast cell lines, histocompatible with the hybrid responder cells, served as target cells for all cytotoxicity assays.