

INHIBITION OF MATURATION OF HUMAN PRECURSOR  
LYMPHOCYTES BY COFORMYCIN, AN  
INHIBITOR OF THE ENZYME ADENOSINE DEAMINASE\*

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High concentrations of adenosine are known to be toxic to fibroblasts and lymphocytes under conditions of in vitro culture (1, 2). Normally, accumulation of adenosine nucleotides in all mammalian cells is prevented by the presence of adenosine deaminase, an aminohydrolase which converts adenosine to inosine (3). A genetically determined deficiency of adenosine deaminase has been associated with the autosomal recessive form of severe combined immunodeficiency, a syndrome in which precursor lymphocytes fail to mature into T cells and B cells (4-7). Erythrocytes of affected infants convert exogenous adenosine to AMP and ATP at an abnormally increased rate as a consequence of the enzyme defect, and fail to form inosine from the exogenous adenosine (8). These metabolic disturbances can be mimicked in normal erythrocytes by coformycin (8), a potent competitive inhibitor of adenosine deaminase (9, 10). In this study, the effects of coformycin were examined on the in vitro function of normal lymphocytes.

Materials and Methods

*Isolation of Lymphocytes.* Lymphocytes were obtained from tonsils, thymuses, and peripheral blood. Tonsils from children (4- to 10-yr old) undergoing elective tonsillectomy and thymus biopsies (obtained with informed parental consent from children undergoing thoracic surgery) were teased into single cell suspensions. The cells were filtered through sterile glass wool and washed before fractionation in RPMI-1640 medium containing antibiotics as previously described (11). Blood from normal adult donors was collected in heparin (100 U/ml), and sedimented with dextran (1 ml of 6% Macrodex 70000/10 ml blood). The leukocytes, in medium TC-199 containing 10% heat-inactivated serum (from an AB+ donor), were passed over a column of glass beads which was prewarmed at 37°C and nonadhering cells were collected and washed three times in TC-199.

*Fractionation of Lymphocytes.* Lymphocytes were suspended at a concentration of no more than  $10^6$  cells/ml in 17% bovine serum albumin. The cell suspension was added to the top of the gradient, centrifuged, and cells from each layer harvested (11). Purified B cells were prepared from layers seven and eight which were rosetted with sheep red cells (E), and nonrosetted cells were retained at the gradient interface in Ficoll-Triosil (density 1.080). After fractionation procedures, cells were washed three times in medium TC-199 before use.

*Rosettes with E.* Washed E were adjusted to 1% vol/vol concentration in magnesium-free phosphate-buffered saline. Suspensions of lymphocytes ( $2 \times 10^6$  cells/ml) containing 10% absorbed fetal calf serum were added to an equal volume of a 1% suspension of E. The suspensions were incubated at 37°C (20 min), centrifuged at 200 g (5 min), and stored at 4°C (at least 1-2 h). Rosettes

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were counted in a Neubauer hemocytometer. At least 200 lymphocytes were counted, and results are expressed as the nearest percent.

**Cellular Culture.** Lymphocytes were suspended immediately after fractionation in medium RPMI-1640 supplemented with 15% heat-inactivated human AB+ serum at  $1$  or  $2 \times 10^6$  lymphocytes/ml. 1 ml cultures in  $16 \times 125$  mm plastic culture tubes were maintained at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in air.

**Mitogens and Antigens.** Bacto-phytohemagglutinin P (PHA) (Difco Laboratories, Detroit, Mich.) and pokeweed mitogen (PWM) (Grand Island Biological Co., Grand Island, N. Y.) were used at a final dilution of 1/100 of the stock solution. Concanavalin A (Con A) (Pharmacia Fine Chemicals, Uppsala, Sweden) was used at  $5 \mu\text{g/ml}$  of culture. Tetanus toxoid (T.T.) (Massachusetts Biological Laboratory, Boston, Mass.) was used at a concentration of  $10 \mu\text{g/ml}$  culture. Lymphocyte mitogenic factor (LMF) was produced and used as previously described (12).

**Measurement of [ $^3\text{H}$ ]Thymidine Incorporation.** Cellular proliferation was measured by counting TCA-precipitable [ $^3\text{H}$ ]thymidine on filter paper disks which were assayed for radioactivity in duplicate (11).

**Immunoglobulin G Synthesis.** B-cell or precursor lymphocyte populations were cultured at  $2 \times 10^6$  cells/ml in RPMI-1640 containing 15% human heat-inactivated serum from a AB+ donor. On the 6th day of culture, cells were washed and resuspended in medium TC-199 (deficient in valine, leucine, and isoleucine) to which  $2 \mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]leucine, and  $1 \mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]valine and [ $^{14}\text{C}$ ]isoleucine were added. 2 days later supernates were harvested and newly formed IgG was measured (13). Results are expressed as the difference in counts per minute of the goat antirabbit serum and the normal rabbit serum precipitates.

**Coformycin.** Coformycin (9, 10) was a gift from Dr. H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan). Dilutions were made with saline and 0.1 ml of diluted solution was added to 1-ml cultures. Lymphocytes in culture were either exposed for  $1/2$  h to coformycin or during the entire course of the experiment.

## Results

**Effect of Coformycin on T- and B-Cell Functions.** When peripheral blood lymphocytes were rosetted with E, the number of E rosettes formed in the presence of coformycin (37%) did not differ significantly from the number of E rosettes formed in the absence of coformycin (44%). Similarly, coformycin had no effect on the rosetting of thymus cells.

Coformycin in concentrations higher than  $1 \times 10^{-5}$  M inhibited the rate of [ $^3\text{H}$ ]thymidine incorporation into unfractionated blood lymphocytes, stimulated with PHA but had no effect when used in ion concentrations lower than  $1 \times 10^{-5}$  M (Table I). A concentration of  $1 \times 10^{-6}$  M was chosen for all subsequent experiments. Coformycin did not inhibit responses to Con A, PWM, or T.T.

TABLE I  
Responses of T Cells and B Cells

Coformycin	0	$10^{-5}$ M	$10^{-6}$ M	$10^{-7}$ M	$10^{-8}$ M
T-cell mitogens					
—	2,075 $\pm$ 492				
PHA*	106,720 $\pm$ 5,176	69,670 $\pm$ 4,792	107,373 $\pm$ 6,140	99,834 $\pm$ 3,678	92,816 $\pm$ 8,166
Con A*	34,365 $\pm$ 1,002	31,236 $\pm$ 526	25,834 $\pm$ 212	32,746 $\pm$ 3,166	26,846 $\pm$ 6,658
PWM†	88,352 $\pm$ 4,700	79,812 $\pm$ 3,911	77,983 $\pm$ 3,397	—	—
T.T.*	119,214 $\pm$ 1,097	113,221 $\pm$ 5,095	126,254 $\pm$ 4,524	—	—
B-cell mitogen					
LMF‡	29,386 $\pm$ 526	—	30,272 $\pm$ 120	—	—

Results expressed in net counts per minute and per  $10^6$  cells plus one standard deviation. [ $^3\text{H}$ ]thymidine incorporation measured on the 3rd (for PHA, Con A, and PWM) and the 7th days of culture (for T.T. and LMF plus T.T.). Controls = (average  $\pm$  1 SD) = 2,075  $\pm$  492.

\* Unfractionated lymphocytes.

† T-cell enriched (albumin layers 4-5).

‡ B cells (albumin layers 6-8 depleted of E-reactive cells).

When purified B cells were cultured with T-cell LMF, the presence of coformycin did not affect the proliferative response of B cells (Table I). IgG synthesis by LMF-stimulated B cells was similar in the presence of coformycin (1,324 cpm) or in its absence (1,160 cpm).

*Effects of Coformycin on Precursor Cells.* Cells with precursor characteristics were present in albumin gradient layers 1-3 of tonsil lymphocytes (7). Inability of these precursor cells to react with E, high spontaneous [ $^3\text{H}$ ]thymidine incorporation, and inability to react with PHA were some of their characteristics. All lymphoid cells in layer one of albumin gradients exhibited these characteristics, while cells from layers two and three were contaminated with T cells.

Precursor cells differentiated during 10 days of culture into T cells (14) (Fig. 1). The presence of coformycin inhibited the appearance of E-reactive cells (Fig. 1). This inhibition did not correlate with the number of cells in culture nor with the number of dead cells. In a typical example, on the 7th day, in the control culture, there were  $5 \times 10^5$  cells, 55% of which excluded trypan blue and 34% of which were E positive. In the coformycin-treated culture, there were  $5 \times 10^5$  cells, 44% of which excluded trypan blue and only 1% of which were E positive.

The spontaneous incorporation of [ $^3\text{H}$ ]thymidine in precursor cells was decreased by coformycin (Table II). Coformycin also inhibited PHA-induced prolif-

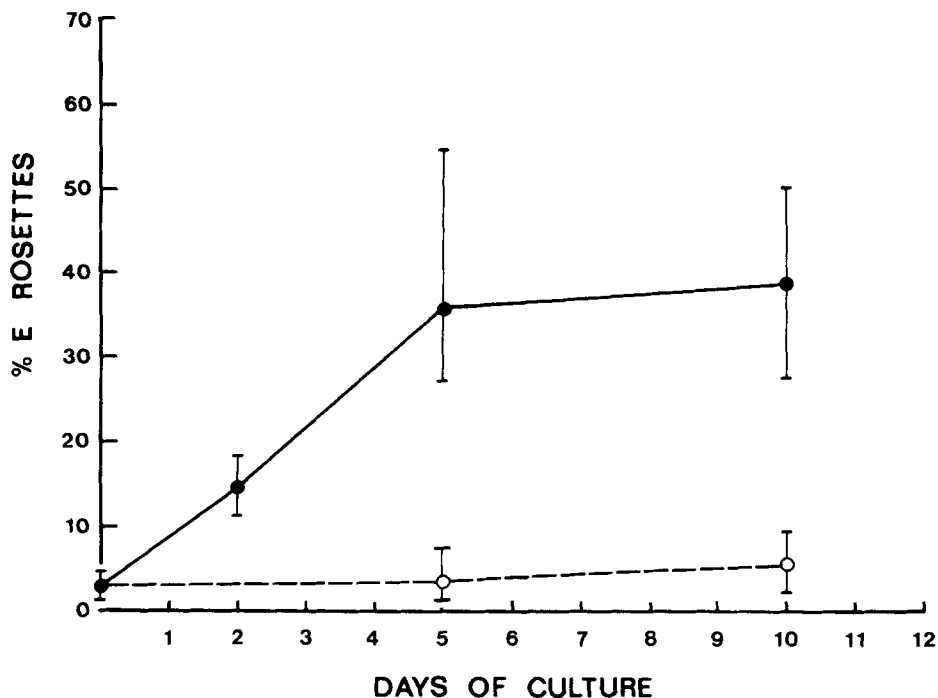


FIG. 1. Effect of coformycin on E reactivity of human precursor cells in culture. Results expressed in percents of E-rosetting lymphocytes at various time intervals of culture. (●) in the absence of, or (○) in the presence of coformycin ( $10^{-6}$  M). (Average results obtained from four different tonsil lymphocytes from albumin gradient layers one and two. Bars indicate two standard deviations.)

TABLE II  
*Effect of Coformycin on Spontaneous [<sup>3</sup>H]Thymidine Incorporation or Lectin Induced Proliferative Response*

	Exp. 1*		Exp. 2†		Exp. 3‡	
	Control	PHA	Control	PHA	Control	PHA
In the absence of coformycin	6,236 ± 457	5,185 ± 260	2,430 ± 164	37,943 ± 3,121	3,181 ± 60	48,930 ± 3,718
In the presence of coformycin	1,434 ± 26	374 ± 141	1,636 ± 59	1,189 ± 52	440 ± 47	55,496 ± 4,663

Results expressed in net counts per minute and per  $10^6$  cells ± one standard deviation.

\* PHA added on day 4, cultures harvested on day 7.

† PHA added on day 7, cultures harvested on day 10.

‡ PHA added on day 0, cultures harvested on day 7.

erative responses of the precursor cells (Table II) when PHA was added to the culture on day 4 or day 7. However, in experiments where PHA was added at the beginning of culture, coformycin did not alter the proliferative response of the cells harvested after 7 or 10 days of culture.

Precursor cells synthesized IgG at day 8 in culture in the presence of  $1 \times 10^{-6}$  M of coformycin, an amount that inhibited inception of E rosetting and PHA proliferation (28,024 cpm vs. 19,680 cpm in control culture).

#### Discussion and Summary

A limiting concentration of coformycin,  $1 \times 10^{-6}$  M, a potent inhibitor of adenosine deaminase, has no apparent effect on T- or B-cell function in vitro, although larger amounts of this drug inhibit proliferative responses to mitogens. On the other hand,  $1 \times 10^{-6}$  M of this inhibitor has a profound effect on precursor lymphocytes. Lymphoid cells, which have neither T- nor B-cell characteristics have been identified in blood, tonsils, bone marrow, and thymus and have been observed to mature under conditions of in vitro culture into typical T or B lymphocytes (14, 15). On the one hand, maturation of these precursor lymphocytes into T cells, as defined by E rosetting, and proliferation in the presence of PHA, is very sensitive to the presence of coformycin. On the other hand, maturation of precursor lymphocytes into immunoglobulin-secreting cells is not impaired by coformycin. The metabolic effects of coformycin which are ultimately responsible for the inhibition of T-cell maturation are not known at present.

Infants affected with severe combined immunodeficiency have lymphoid cells with precursor characteristics but no or very few mature T cells and B cells (7). The experiments reported here have mimicked in vitro the alterations observed in infants affected with this disease. From these findings it may be surmised that the B-cell defect in adenosine deaminase deficiency may be secondary to the failure of T-cell maturation. Indeed, the B-cell number is often normal in adenosine deaminase-deficient infants (5). A child with a genetically determined deficiency of nucleoside phosphorylase, the next enzyme in the salvage pathway which converts inosine to hypoxanthine, has a profound T-cell deficiency but normal numbers of B cells (16). Further studies of purine metabolism in lymphocytes are clearly needed to define the remarkable immunosuppressive

effects of these metabolic disturbances induced by chemical inhibitors or genetically determined enzyme deficiencies in the purine metabolic pathways.

In the present experiments, it was observed that the presence of PHA in cultures of precursor cells overcame the effects of coformycin. This was probably attributable to the recruitment effect of small numbers of cells (2-6%) which escaped the effect of coformycin and matured into T cells.

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