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Potassium Cyanate, An *in Vitro* Inhibitor of Lymphocyte Blast Transformation without *in Vivo* Activity[1] (39346)

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The ability of cyanate to react with proteins has been extensively studied in recent years following reports of its capacity to inhibit erythrocyte sickling *in vitro* (1). Studies of the cyanate reaction with hemoglobin have shown that the N-terminal amino groups of the B-chain are carbamylated first, but at higher concentrations of cyanate many other amino groups react as well (2). Since protein carbamylation is a general and nonspecific reaction, cyanate has been studied for its effects on other cell constituents, as well as for its toxicity to various organ systems *in vivo*. Alter *et al.* (3) found definite evidence of depressed hemoglobin synthesis in red cell precursors treated with cyanate, and long term studies have demonstrated severe toxicity in both humans and animals treated chronically with cyanate doses over 35 mg/kg/day. In humans, peripheral neuropathy and cataract formation (4) have been the most prominent symptoms of toxicity.

These findings have led some workers to develop cyanate for the extracorporeal treatment of blood to avoid exposing other body organs. In one ongoing study Harwood and Diederich (5) exposed blood for 4 hr at 37° to 50 m*M* cyanate in a dialysis machine which resulted in the carbamylation of hemoglobin to the extent of 2.3 mole of cyanate per mole of hemoglobin tetramer. These methods expose all blood constituents to cyanate and carbamylate many types of proteins in cells and in the plasma.

Alkylating agents, of which cyanate is one, are well known for selective toxicity toward lymphocytes while the prototype of such agents, nitrogen mustard, has been shown to inhibit the sickling of red blood cells *in vitro* (6). Protein synthesis is an early, essential component of the lymphocyte response to mitogens. It was of interest, therefore, to examine the effect of exposure to cyanate on the cellular arm of the immune response.

Methods and materials. Isolation of lymphocytes and PHA stimulation. Blood was obtained from laboratory personnel or from apparently healthy blood bank donors. In a typical experiment 40 ml of blood was collected in sterile plastic centrifuge tubes containing 10,000 USP units of heparin in isotonic saline (Panheprin, Abbot Laboratories, North Chicago, Ill.). Lymphocyte-enriched plasma was obtained by adding 5 ml of 5% dextran in saline with an average molecular weight of 200,000 (Poviet Prodeuctin, Amsterdam, Holland) and allowing the erythrocytes to sediment for 30 min at 37°. Nylon fiber columns were made by filling a 30-ml glass syringe with nylon wool (Scrub nylon fiber ST-242, Fenwall Laboratories, Morton Grove, Ill.) washed with 1 liter of distilled water and sterilized. The columns were then washed with a small amount of tissue culture medium and the leukocyte-enriched plasma mixed with an equal volume of prewarmed medium, was passed over the column at a flow rate of 1 ml per min or less at 37°. The column was washed with additional tissue culture medium and the combined eluate containing lymphocytes and a variable but small number of erythrocytes and platelets was centrifuged at 400 rpm for 10 min. The loosely packed cells were diluted in tissue culture medium and counted in a hemocytometer.

The medium used in all experiments was Dulbecco-Vogt modified Eagle's medium containing 10% calf serum, and all incubations were carried out in a total volume of 1 ml containing 10^6 lymphocytes. Phytohemagglutinin P (Difco Laboratory, Detroit, Mich., lot number 575119) was put into

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solution prior to each experiment and used in a final concentration of 250 μg/ml. Lymphocytes were pretreated with potassium cyanate (Eastmen Kodak, Rochester, N.Y., and recrystallization from ethanol) at a concentration of 400 μg/ml, unless otherwise specified, and incubated for 30 min at 37°. They were then washed twice with tissue culture medium and resuspended to a final concentration of 10^6 per ml. Control cells were initially treated with an equivalent amount of potassium chloride. Phytohemagglutinin was then added and the culture tubes were loosely capped and incubated under an atmosphere of 5% CO_2 in room air for 3 or 7 days. At the end of the incubation period, lymphocyte viability and blast transformation were assayed by mixing a drop from each culture tube with a drop of freshly dissolved acridine orange 2.5 μg/ml in isotonic saline and by examining the cells under ultraviolet illumination (7). The nuclei of viable cells appeared bright green and blasts were identified by their size and orange cytoplasm. On Day 3, 0.125 μCi of [^{3}H]thymidine, 16 Ci/mmol (Schwarz Bioresearch, Orangeburg, N.Y.) was added for a period of 18 hr. The following day, the contents of each tube were collected on Whatman GF-C glass fiber filters (8). The tubes were rinsed several times with saline which was added to the filter. The filters were then washed with 50 ml of cold isotonic saline followed by 25 ml of cold 5% trichlorocetic acid (TCA) and 25 ml of methyl alcohol. The filters were placed in glass liquid scintillation vials and 0.5 ml of hyamine was added. The vials were stoppered and incubated at 37° overnight. The following morning 10 ml of toluene scintillator was added and the vials counted.

Mixed lymphocyte culture reactions were performed as one-way reactions. The stimulating cells were treated with 50 μg of mitomycin C (Schwarz Mann, Orangeburg, New York) for 30 min. The cells were then washed twice with tissue culture medium and resuspended. Both stimulating and responder cells were treated with mitomycin to determine base incorporation values. Responder cells were pretreated with potassium cyanate or potassium chloride as described above. The cultures were allowed to incubate for 5 days. At the end of this time, the percentage of blasts was counted and viability assessed by means of acridine orange staining, and the incorporation of tritiated thymidine was measured as described above.

Results. Cyanate was noted to inhibit blast transformation in response to PHA as examined by morphologic change and the incorporation of [^{3}H]thymidine. More importantly 400 μg/ml of cyanate virtually abolished the response of lymphocytes to allogeneic lymphocytes (Table I). The values for each experiment represent the mean of triplicate culture tubes.

The dose of cyanate, 400 μg/ml, was chosen because it is equivalent to the 5 m*M* (405 μg/ml) dose demonstrated to inhibit erythrocyte sickling *in vitro*. Cyanate alone did not reduce thymidine incorporation in nonstimulated lymphocytes by more than 5%. The addition of 400 μg/ml of KCl to stimulated or unstimulated lymphocytes had no effect on [^{3}H]thymidine incorporation. Sodium cyanate was equally effective on a weight basis in inhibiting the PHA response. In MLC experiments the reverse experiment, namely the exposure of the stimulating or mitomycin-treated cells to KCNO for 30 min, had no effect on the response of untreated lymphocytes.

In view of the effect of cyanate on the *in vitro* response of lymphocytes in mixed lymphocyte culture, we decided to see whether cyanate was immunosuppressive *in vivo*. Some data were available on the pharmacology of cyanate in mice. Using a treatment schedule shown to produce significant carbamylation of hemoglobin in mice (9) we studied the ability of cyanate-treated animals to reject a first set skin allograft.

Brown CBA/J female mice obtained from Jackson Laboratories, Bar Harbor, Maine, were grafted with the skin obtained from white A/J mice. Twenty recipient mice with a mean weight of 17.52 ± 1.74 g were each given 525 μg of sodium cyanate (Pfaltz & Bauer, Flushing, N.Y.) by intraperitoneal injection with an average dose of 29.96 μg per gram body weight. Injections were given every 5 out of 7 days for a total of 16 injections as described by Cerami (9). The control CBA/J mice were given normal sa-

TABLE I. INHIBITION OF PHA-INDUCED BLASTOGENESIS AND THE MLC REACTION BY CYANATE.[a]

	Percentage blasts		Counts per minute[b]					
Expt.	PHA	PHA-KCNO	PHA	PHA-KCNO	Control[c]	MLC Ax Bm	MLC-KCNO A_{KCNO} × Bm	Control Am × Bm
1	78	4	30,867	9,332	247	15,560	998	396
2	70	2	5,585	567	182	2,136	187	110
3	74	3	60,196	1,710	510	7,769	173	265
4	78	13	101,190	30,383	9,481	9,726	506	265
5	70	6	40,810	3,630	1,380	4,705	325	182
6	68	5	19,690	3,740	367	9,269	166	138
Mean	73	5.5	41,389	8,227	2,027	9,246	392	226
SE	±4.3	±4.9	±14,823	±4,694	±1,532	±2,106	±135	±44

[a] MLC: responder cells (A) versus mitomycin-treated stimulator cells (Bm).
[b] Each value represents the mean of triplicate incubation tubes.
[c] Untreated cells; cells treated with KCNO alone were included in each experiment but the values were not significantly different from untreated cells.

line. At the end of this time, skin grafts were performed and injections of cyanate or saline were given an additional seven times. Nineteen treated and nine untreated mice survived, and all mice, in both groups, rejected their grafts on Days 10 or 11.

Discussion. The exact means by which cyanate inhibits lymphocyte activation *in vitro* is obscure, but one can speculate that it does so by carbamylating a protein involved in the process that results in blast transformation. The failure to demonstrate an effect *in vivo* may be dose related since 7.5% of a dose of ^{14}C-labeled cyanate given to a mouse was bound to the amino terminal valine of hemoglobin, and only 0.01% was found in the spleen, while lymph nodes and white blood cells were not specifically examined (2, 9). Furthermore, Nehlsen and Lalezari (10) have demonstrated prolongation of skin allografts in mice following the daily injection of sodium cyanate at approximately 60 μg/g of body weight, a dose they observed to cause moderate toxicity. It is also possible that a less stringent test of *in vivo* immunity than allograft rejection might be altered by cyanate administration.

Differences between the *in vitro* and *in vivo* toxicity of cyanate have been observed in a variety of biological processes. For example, 5 m*M* cyanate inhibits hemoglobin synthesis in isolated human reticulocytes but apparently does not inhibit erythroid cell maturation nor produce a lack of hemoglobin *in vivo* (11). In addition to these discordant results with cyanate apparent contradictions between *in vitro* and *in vivo* effects have been noted in studies on lymphocytes involving other agents. Aspirin, for example, has been reported to inhibit lymphocyte transformation *in vitro* but contradictory results have been obtained following its administration *in vivo* (12–16). A drug may be more toxic *in vitro* due to the absence of detoxifying or excretory mechanisms offered by the liver and kidney *in vivo*.

At the present time an extracorporeal method of cyanate treatment for sickle cell anemia is being considered in which the *in vitro* model of drug usage is more relevant. Blood will be exposed to high concentrations of cyanate for a short time, then cyanate will be removed by cell washing or dialysis and the blood reinfused. Our data suggest that lymphocytes exposed to this amount of cyanate may show a diminished response to both specific and nonspecific antigens. A large pool of lymphocytes residing outside the vascular compartment, in the lymph nodes, spleen and thymus, and bone marrow, will not be affected initially. With chronic, intermittent treatment, however, very many more lymphocytes will be exposed to cyanate. Our data do not bear directly on the immunologic status of such an individual, but they do suggest caution in the treatment of whole blood *in vitro* with cyanate until appropriate studies are done.

Summary. Potassium cyanate (400 μg/ml) inhibited the blastogenesis of lymphocytes in response to PHA and the response of lymphocytes in mixed lymphocyte cul-

tures. Cyanate administered to mice at a dose of 30 mg per kg per day did not delay allograft rejection.

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1. Cerami, A., and Manning, J. M., Proc. Nat. Acad. Sci. USA **68,** 1180 (1971).
2. Cerami, A., Manning, J. M., Gillette, P. N., deFuria, F., Miller, D., Graziano, J. H., and Peterson, C. M., Fed. Proc. **32,** 1669 (1973).
3. Alter, B. P., Kan, Y. W., and Nathan, D. G., Blood **43,** 57 (1974).
4. Smith, R. P., Proc. Soc. Exp. Biol. **142,** 1041 (1973).
5. Harwood, T., and Diederich, D., Abstract **235,** Amer. Soc. Hematol. (1974).
6. Roth, E. F., Jr., Nagel, R. L., Bookchin, R. M., and Grayzel, A. I., Biochem. Biophys. Res. Commun. **48,** 612 (1972).
7. Hathaway, W., Newly, L., and Githens, J., Blood **23,** 517 (1964).
8. Sorensen, S. F., Andersen, V., and Giese, J., Acta Path. Microbiol. Scand. **75,** 508 (1969).
9. Cerami, A., New Eng. J. Med. **287,** 807 (1972).
10. Nehlsen, S. L., and Lalezari, P., IRCS Med. Sci. **3,** 88 (1975).
11. deFuria, F. G., Miller, D. R., Cerami, A., and Manning, J. M., J. Clin. Invest. **51,** 566 (1972).
12. Opelz, G. T., Terasaki, P. I., and Hirata, A. A., Lancet **2,** 478 (1973).
13. Pachman, L. M., Esterly, N. B., and Peterson, R. D. A., J. Clin. Invest. **50,** 226 (1971).
14. Schneider, W., Pappas, A., and Scheurlen, P. G., Clin. Woch. **49,** 1187 (1971).
15. Crout, G. E., Hepburn, B., and Ritts, R. E., New Eng. J. Med. **292,** 221 (1975).
16. Smith, M. J., Hoth, M., and Davis, K., Ann. Int. Med. **83,** 509 (1975).

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