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Effect of Penicillamine on the Proliferative Response of Human Lymphocytes (40011)

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D-Penicillamine (D-pen), β,β-dimethyl cysteine, is a drug which has been shown to have various biological functions. Among these are copper chelation (1), interaction with cystine to form a mixed disulfide (2), inhibition of collagen fibril formation (3), and biochemical antagonism of vitamin B-6 (4). *In vitro*, it inhibits poliovirus replication (5), and has been reported to suppress transformation of antigen- and mitogen-stimulated lymphocytes (6).

D-Pen has been shown to be an effective therapeutic agent in the treatment of rheumatoid arthritis. The clinical response to the drug is accompanied by a fall in titer of rheumatoid factor and a decrease in immune complexes in serum and synovial fluid (7). It is possible that the clinical response to D-pen is the consequence of an effect of this agent on the immune system. This study was undertaken to investigate the interaction of D-pen with human lymphocytes *in vitro*.

Materials and methods. Cell cultures. Lymphocytes were isolated from normal heparinized blood (20 units/ml) on a Ficoll-Hypaque gradient. The cells were washed twice with Hank's balanced salt solution (HBSS) and cultured at a density of 1×10^6 cells/ml in RPMI 1640 supplemented with 2 m*M* glutamine and 20% fetal calf serum. The purity of the isolated mononuclear cells was determined by differential counts of the stained cells, and was more than 90%. Cell viability was tested by the exclusion of trypan blue, and 97% of the cells were viable.

A continuous culture of normal human lymphocytes (Associated Biomedic Systems) was maintained in RPMI 1640 supplemented with glutamine and 5-7% fetal calf serum.

Mitogen stimulation. Cell cultures were stimulated with phytohemagglutinin (PHA) (Burroughs-Wellcome Co.), 5 μg/ml, concanavalin A (Con A) (Difco Laboratories), 20 μg/ml, and pokeweed mitogen (Difco Laboratories), 30 μg/ml. All cultures were run in triplicate and were incubated for a total of 72 hr, unless otherwise indicated, at 37° in 5% CO_2. D-Pen was dissolved in HBSS and added to cell cultures at varying concentrations at indicated times. Unstimulated lymphocytes, treated as above, were used as controls.

Mixed lymphocyte cultures. Mononuclear cells were isolated and cultured at a density of 1.5×10^6 cells/ml, as above. The stimulator cells were incubated with mitomycin C, 25 μg/ml, for 25 min at 37°. The treated cultures were centrifuged, washed twice with HBSS, and resuspended in complete medium. Aliquots (1 ml) of stimulator and responder cells were mixed and incubated with various concentrations of D-pen for 144 hr at 37° and 5% CO_2.

DNA, RNA, and protein synthesis. DNA synthesis was measured by the addition of 0.5-1.5 μCi/ml of [^{3}H]thymidine (22 Ci/mmole, Schwartz/Mann) to cell cultures 18 hr before harvesting, unless otherwise indicated. The cultures were centrifuged, washed with cold HBSS, and placed in an ice bath. Cells were lysed with 1 ml of cold H_2O and lysates were precipitated by the addition of 2 ml of 10% trichloroacetic acid (TCA). The precipitates were collected on Whatman GF/C glass-fiber filters and washed with 5% TCA. The incorporated radioactivity was measured in a Beckman scintillation spectrometer.

RNA and protein synthesis was measured by incubation of 0.1-0.5 μCi/ml of [^{14}C]uridine (488 mCi/mmole, Schwartz/Mann) or [^{14}C]leucine (340 mCi/mmole, Schwartz/Mann) with the cell cultures for 3 hr at 37° in 5% CO_2, and incorporated radioactivity was measured as above. The rate of RNA and protein synthesis was measured as describe previously (5).

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E rosette formation. The ability of D-pen-treated and untreated lymphocytes to form rosettes with sheep red blood cells (SRBC) was determined by the method of Bentwich *et al.* (8). Mononuclear cells at a density of 5×10^6 cells/ml were incubated in HBSS with varying concentrations of D-pen for 2 hr at 37°. The drug was removed by centrifugation and washing. The washed cells were resuspended in HBSS at the above density. One-tenth milliliter of a 0.5% suspension of sheep erythrocytes in HBSS was added to 0.1 ml of lymphocyte suspension, followed by the addition of 0.02 ml of heat-inactivated AB serum which had been absorbed with SRBC. The cell suspensions were incubated at 37° for 5 min, centrifuged at 200*g* for 5 min, and counted in a hemacytometer. Lymphocytes with three or more erythrocytes attached were considered rosettes.

Results. Effect of D-pen on DNA, RNA, and protein synthesis. D-Pen at concentrations ranging from 10 to 500 μg/ml was incubated for 3 and 6 hr with resting human lymphocytes and with human lymphocytes maintained in continuous culture. The drug had no effect on DNA, RNA, or protein synthesis.

Effect of D-pen on mitogen-stimulated lymphocytes. Addition of D-pen to lymphocyte cultures simultaneously with PHA, Con A, or pokeweed mitogen resulted in inhibition of transformation. This inhibition was partial, dose dependent, and maximal at 100 μg/ml (Table I). At this concentration, DNA synthesis was inhibited by 50% in PHA- and Con A-stimulated cultures, and by 40% when pokeweed was used as the mitogen. It had no effect on the number of surviving cells in the unstimulated control cultures.

TABLE I. EFFECT OF D-PEN ON DNA SYNTHESIS OF MITOGEN-STIMULATED LYMPHOCYTES.

D-Pen (μg/ml)	Counts per minute of [^{3}H]thymidine/10^6 cells at 72 hr		
	PHA	Con A	Pokeweed
0	14,000	18,000	10,000
25	13,000	15,500	9,200
50	9,500	13,000	6,200
100	6,800	8,700	6,000
200	7,000	8,500	5,800
400	7,400	9,000	6,200

Incubation of the cells with D-pen for 3 hr, followed by removal of the drug by centrifugation and washing prior to mitogen stimulation, resulted in a 20% inhibition of DNA synthesis.

Effect of D-pen on mixed lymphocyte cultures. Addition of D-pen to one-way mixed lymphocyte cultures resulted in inhibition of DNA synthesis (Fig. 1), which was maximal (60%) at a concentration of 100 μg/ml. Incubation of the stimulator cells with D-pen for 3 hr, followed by removal of the drug before addition of the responder cells, had little effect on DNA synthesis. Similar incubation of the responder cells resulted in a 40% inhibition of DNA synthesis (Fig. 2). The inhibitory effect was not augmented when *both* stimulator and responder cells were pretreated with the drug.

Effect of D-pen on E rosette-forming cells. Incubation of lymphocytes with D-pen for 2 hr and removal of the drug by centrifugation and washing before the addition of sheep red blood cells had no effect on the number of E rosettes.

Kinetics of D-pen inhibition of transformation. Since the proliferative response of lymphocytes to mitogen stimulation has been most extensively studied with PHA, the following experiments were performed with this mitogen.

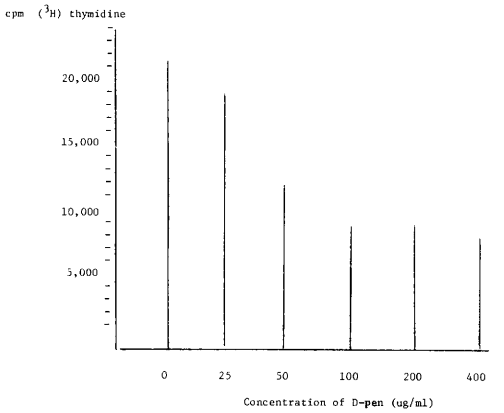

FIG. 1. Effect of D-pen on DNA synthesis in mixed lymphocyte cultures. Lymphocytes were isolated and cultured as described in the text. D-Pen was added at the concentrations shown to 2-ml aliquots of a mixture of stimulator and responder cells. Cultures were incubated for 144 hr at 37° in 5% CO_2. [^{3}H]Thymidine was added 18 hr before harvesting and DNA synthesis was determined.

The effects of 100 and 300 μg/ml of D-pen on protein, RNA, and DNA synthesis of PHA-stimulated cells were determined when the drug and mitogen were added to lymphocyte cultures simultaneously. Protein, RNA, and DNA synthesis was measured in triplicate cultures at 3, 20, 48, and 72 hr poststimulation. D-Pen had no effect on DNA, RNA, and protein synthesis of the stimulated cells in the first 20 hr of incubation (Table II). At 48 and 72 hr, inhibition of DNA synthesis was about 50% and there was a reduction in RNA and protein synthesis in parallel with the DNA inhibition (Table II).

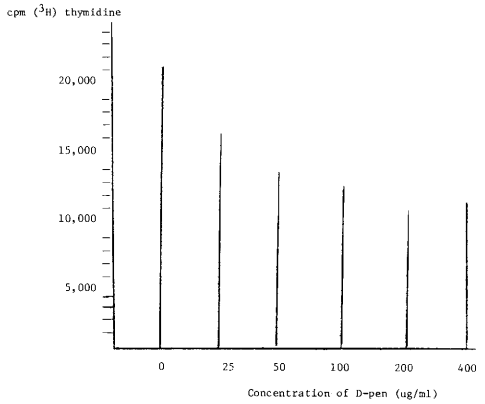

FIG. 2. Effect of incubation and removal of D-pen on responder cells in mixed lymphocyte cultures. Lymphocytes were isolated and cultured as described in the text. The responder cells were treated with concentrations of D-pen as shown, and incubated for 3 hr at 37° in 5% CO_2. D-Pen was removed by centrifugation and washing. Cultures (1 ml) of stimulator cells were added to cultures (1 ml) of the D-pen-treated and washed responder cells. Cultures were incubated for 144 hr at 37° in 5% CO_2. [^{3}H]Thymidine was added 18 hr before harvesting and DNA synthesis was determined.

The effects of 100 and 300 μg/ml of D-pen on PHA-stimulated cells were determined at various time intervals. The drug was added simultaneously with PHA, and at 20 and 48 hr poststimulation. The addition of D-pen at zero time and 20 hr poststimulation resulted in a 50% inhibition of DNA synthesis; however, when the agent was added at 48 hr, it had no inhibitory effect (Table III).

Discussion. The above experiments show that D-pen inhibited lymphocyte responsiveness to nonspecific mitogen stimulation. In addition, the mixed lymphocyte reaction was also suppressed by the drug. The inhibition in both systems was partial and dose dependent (Table I, Fig. 1). These findings are in agreement with those reported by Schumacher *et al.* on the effect of D-pen on mitogen- and antigen-stimulated lymphocytes (6).

D-Pen had no effect on DNA, RNA, or protein synthesis in resting lymphocytes or lymphocytes maintained in continuous culture. We conclude, therefore, that the inhibitory effect of this agent is not due to

TABLE III. EFFECT OF D-PEN ADDED AT VARYING TIME PERIODS ON DNA SYNTHESIS IN PHA-STIMULATED LYMPHOCYTES.

Time of addition of D-pen (hr)	D-Pen (μg/ml)	Counts per minute of [^{3}H]thymidine/10^6 cells at 72 hr
0	0	24,000
	100	11,000
	300	12,000
20	0	25,000
	100	12,000
	300	11,500
48	0	23,500
	100	25,000
	300	24,000

TABLE II. EFFECT OF D-PEN ADDED SIMULTANEOUSLY WITH PHA ON MACROMOLECULAR SYNTHESIS MEASURED AT VARIOUS TIME PERIODS.

		Counts per minute/10^6 cells			
Macromolecule	D-Pen (μg/ml)	3 hr	20 hr	48 hr	72 hr
Protein	0	3,000	10,000	16,000	17,000
	100	3,400	9,500	10,000	11,000
	300	2,800	9,800	11,000	11,000
RNA	0	4,500	16,000	28,000	26,000
	100	4,200	17,000	15,000	14,000
	300	4,500	15,000	16,000	15,000
DNA	0	1,100	1,000	6,500	15,500
	100	1,000	1,200	3,200	8,000
	300	950	1,100	3,600	7,500

nonspecific inhibition of synthesis of those macromolecules.

The same degree of inhibition was produced when D-pen was added simultaneously with PHA or at 20 hr poststimulation, when the cells were fully committed to the proliferative state (Table III). [PHA stimulation of lymphocytes at 37° is not reversible after 6 hr (9).] The inhibitory effect, therefore, does not appear to be due to competition with or alteration of mitogen receptor sites on the cell surface.

The absence of effect of D-pen on lymphocyte surface antigens is indicated by the failure of the agent to inhibit rosette formation with sheep erythrocytes. In the mixed lymphocytes cultures, preincubation of the responder cells with D-pen resulted in inhibition of DNA synthesis (Fig. 2), while the preincubation of the stimulator cells had no effect. This also indicates that a nonspecific effect on lymphocyte surface antigens was not produced by the drug since that would have produced a similar degree of inhibition in both responder and stimulator cells.

In PHA-stimulated lymphocytes an increase in the rate of RNA and protein synthesis is evident shortly after stimulation (10, 11), and it becomes maximal in 24–48 hr. This increase in macromolecular synthesis is a prerequisite for the initiation of DNA replication, which commences approximately 20 hr poststimulation (12). D-Pen did not inhibit the induction of the increased rate of RNA and protein synthesis following PHA stimulation, as evidenced by the lack of effect of the drug on RNA and protein synthesis during the first 20 hr (Table II). The inhibitory effect of D-pen on lymphocyte transformation was evident only when the drug was present in the first 20 hr of culture. The inhibitory effect appeared to be related to some process(es) prior to the start of the replicative phase. DNA synthesis, which does not begin until about 20 hr poststimulation, is maximal in 48–72 hr (13). D-Pen inhibited DNA synthesis only when it was present during the first 20 hr after stimulation, even though it had no effect on the enhanced rate of RNA and protein synthesis during that period. When DNA synthesis was maximal, inhibition was not produced by the addition of D-pen (Table III). It is possible that D-pen may exert its inhibitory effect by blocking the signal for initiation of DNA replication or that it may partially inhibit the activation of enzymes required for this process. Further studies are in progress to more precisely define the mechanism of the inhibition.

Summary. Mitogen-stimulated normal human lymphocytes were exposed to D-penicillamine (D-pen) at various concentrations and different time intervals. D-Pen inhibited lymphocyte response to mitogen stimulation and was also inhibitory to the mixed lymphocyte reaction. The inhibition was found to be partial and dose dependent. The inhibition of lymphocyte transformation was not due to nonspecific inhibition of protein, RNA, or DNA synthesis. The drug was inhibitory only when it was present during the first 20 hr of culture. The inhibitory effect seemed to be related to some process prior to the initiation of the replicative phase. It is suggested that D-pen may block the signal for the initiation of DNA replication, or partially inhibit the activation of enzymes required for the above process.

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