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ALTERATION OF LYMPHOCYTE RESPONSE BY SULFHYDRYL AND DISULFIDE COMPOUNDS

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Abstract—The effects on the proliferative response of human lymphocytes of the sulfhydryl agents D-penicillamine and 5-thiopyridoxine, some analogues of these agents, and their respective disulfides were investigated. The sulfhydryl agents inhibited the proliferation of mitogen-stimulated lymphocytes and suppressed the mixed lymphocyte reaction (MLR) in one-way mixed lymphocyte cultures (MLC). The inhibition in both systems was partial and dose dependent. Prolonged preincubation of responder cells with these sulfhydryl agents, followed by their removal prior to MLC, resulted in enhancement of DNA synthesis. Pretreatment of the stimulator cells was without effect. Analysis of the incubation mixtures of the sulfhydryl compounds showed that they had undergone disulfide formation. Disulfide compounds enhanced DNA synthesis in both mitogen-stimulated cultures and in MLC. The enhancement was dose dependent and limited in concentration range. Preincubation of lymphocytes with disulfides also resulted in enhancement of DNA synthesis when these cells were stimulated with PHA or used as responder cells in one-way MLC. Similar treatment of stimulator cells was without effect. The ability of lymphocytes to form rosettes with sheep erythrocytes was not altered by sulfhydryl agents or their disulfide derivatives.

D-Penicillamine (D-pen), a sulfhydryl-containing agent effective in the treatment of rheumatoid arthritis [1], has been shown to inhibit the proliferative response of human lymphocytes [2]. 5-Thiopyridoxine (5-TP), a sulfhydryl-containing analogue of vitamin B_6, and its disulfide derivative, pyridoxine-5-disulfide (5-TP-SS), are also effective in treatment of rheumatoid arthritis, producing clinical and immunological changes similar to those found after treatment with D-pen [3*]. This study was undertaken to investigate the effects of these and related sulfhydryl compounds and their disulfide derivatives on the proliferative response of normal human lymphocytes. The additional compounds studied were: cysteine, 5-vinyl-4-thiopyridoxine (VTP), penicillamine disulfide (Pen-SS), cystine and 5-vinyl-pyridoxine-4-disulfide (VTP-SS).

METHODS

Cell cultures. Peripheral blood lymphocytes (PBL) 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 mM glutamine and 20% fetal calf serum (FCS). The purity of the isolated mononuclear cells was determined by differential counts of the stained cells, and was more than 90 per cent. Cell viability was tested by the exclusion of trypan blue, and 97 per cent of the cells were viable.

Mitogen stimulation. Cell cultures were stimulated with phytohemagglutinin (PHA) (Burroughs-Wellcome Co., Triangle Park, NC), 5 μg/ml, concanavalin A (Con A) (Difco Laboratories, Detroit, MI), 20 μg/ml, and pokeweed mitogen (PWM) (Difco Laboratories), 20 μg/ml. All cultures were run in triplicate and were incubated for a total of 72 hr, at 37° in 5% CO_2. Stock solutions of the sulfhydryl-containing agents were made in 0.05 M Tris–HCl buffer, pH 7.4, and the disulfides in 0.002 M HCl, and were kept frozen at −20°. Working solutions were made by dilution of the above in RPMI 1640, and these were added to the cell cultures at the concentrations indicated. Unstimulated lymphocytes similarly treated were used as controls.

Mixed lymphocyte cultures. Mononuclear cells were isolated and cultured at a density of 2×10^6 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 of stimulator and responder cells were mixed at a ratio of 2 to 1 and incubated for 144 hr at 37° in 5% CO_2.

DNA. DNA synthesis was measured by the addition of 0.5 to 1.5 μCi/ml of [^{3}H]thymidine (22 Ci/m-mole, Schwartz/Mann, Orangeburg, NY.) to cell cultures 18 hr before harvesting. 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% trichloro acetic 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.

E-rosette formation. The ability of untreated lymphocytes and lymphocytes treated with sulfhydryl- and disulfide-containing agents to form rosettes with sheep erythrocytes (SE) was determined by a procedure described by Moretta *et al.* [4]. SE were washed three times with HBSS and a 5% suspension of the cells was prepared in HBSS. Neuraminidase (*Vibrio cholorae*, B grade, CalBiochem, La Jolla, CA.) was diluted 1:10 in

* E. C. Huskisson, I. A. Jaffe, P. S. Scott and P. A. Dieppe, *Arthritis Rheum.*, submitted.

HBSS and added to the SE suspension at a ratio of 1:5 (v/v). The mixture was incubated for 45 min at 37°. Cells were washed three times with HBSS and suspended at a density of 5×10^6/ml in RPMI 1640 supplemented with 20% FCS (absorbed with SE). Equal volumes of neuraminidase-treated SE and PBL cultures were mixed, incubated at 37° for 10 min, centrifuged at 1400 rev/min for 3 min and incubated for 1 hr at 4°. The pellet was gently dispersed and rosettes were counted. Lymphocytes with three or more SE attached were considered rosettes.

Disulfide formation. Disulfide formation in solutions of sulfhydryl-containing agents was determined by ascending paper chromatography with phenol–water as the solvent. The agents were dissolved in distilled water at a concentration of 4 mg/ml and incubated at 37° for 18 hr and applied to Whatman No. 1 paper. Chromatograms were air dried and sprayed either with iodine (3% solution) or with ninhydrin, and developed at 60°.

Analysis of data. Student's *t*-test was employed to assess the significance of the effect of sulfhydryl- and disulfide-containing agents on lymphocyte response.

RESULTS

Effects of sulfhydryl-containing agents on mixed lymphocyte reaction and on mitogen-stimulated lymphocytes. Addition of the sulfhydryl-containing agents D-pen, cysteine, 5-TP and VTP to one-way mixed lymphocyte cultures (MLC) resulted in inhibition of mixed lymphocyte reaction (MLR). The inhibition was partial and dose dependent. The maximum effective dose and the degree of inhibition varied for each agent (Fig. 1a). The inhibitory effect was reproducible in eight sets of experiments and was statistically significant for all agents ($P < 0.01$).

In order to determine whether the inhibition of MLR by sulfhydryl agents was due to an effect on the stimulator or responder cell populations, the following experiments were performed. The stimulator or responder cells were incubated with each agent for 18 hr at 37° and 5% CO_2. The drugs were removed by centrifugation and washing prior to MLC. The results indicated that the pretreatment of stimulator cells with the above agents had no effect on MLR, while pretreatment of the responder cells unexpectedly produced enhancement of MLR (Fig. 1b). This stimulatory effect resulting from prolonged incubation of the responder cells with sulfhydryl agents was reproducible in eight sets of experiments, and was statistically significant for all agents tested ($P < 0.05$).

A similar pattern of response was obtained in mitogen-stimulated cultures. Addition of sulfhydryl-containing agents to PHA-stimulated lymphocytes resulted in inhibition of transformation (Table 1, column A). With D-pen and cysteine, the degree of inhibition was the same regardless of the mitogen; however, 5-TP and VTP produced a greater inhibition with Con A (50 per

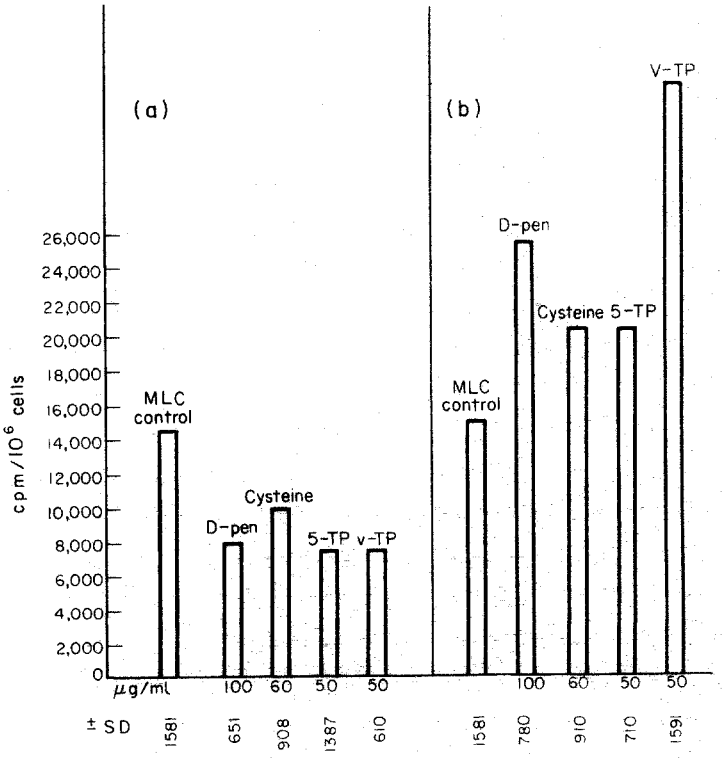

Fig. 1. Effects of sulfhydryl agents on DNA synthesis in one-way lymphocyte cultures. Lymphocytes were isolated and cultured as described in the text. The drugs were either added at the initiation of MLC (a) or were incubated with the responder cells for 18 hr (b) and removed by centrifugation and washing before addition of the stimulator cells. All cultures were incubated for 144 hr at 37° in 5% CO_2. Eighteen hr before harvesting, [^{3}H]thymidine was added. The concentrations shown on the abscissa were selected for optimum effect without cytotoxicity. Each data point is the average of eight different sets of experiments. $P < 0.01$ was obtained for treated cultures as compared to untreated controls (panel a). $P < 0.05$ was obtained for pretreated cultures (panel b).

Table 1. Effects of sulfhydryl-containing agents on DNA synthesis of unstimulated and PHA-stimulated lymphocytes *

	Counts/minute/10^6 cells ± S.D.		
		PHA-stimulated	
Agents	Unstimulated	Agents present throughout (A)	Preincubated for 18 hr (B)
0	2,000 ± 210	16,500 ± 790	16,500 ± 790
D-Pen (100 μg/ml)	1,850 ± 251	9,000 ± 1,000	18,000 ± 961
Cysteine (60 μg/ml)	2,050 ± 400	9,500 ± 690	18,500 ± 612
5-TP (45 mg/ml)	2,100 ± 358	11,000 ± 1,036	17,000 ± 890
VTP (40 μg/ml)	1,900 ± 310	12,000 ± 1,457	20,000 ± 1,581

* Lymphocytes were isolated and cultured as described in the text. Sulfhydryl agents were either added simultaneously with PHA or preincubated with lymphocytes for 18 hr at 37° in 5% CO_2. Eighteen hr before harvesting, [^{3}H]thymidine was added, and DNA synthesis was measured. Cultures of unstimulated lymphocytes were used as controls. Each data point is the average of eight different experiments. $P < 0.01$ was obtained for all treated cultures in column A. $P < 0.05$ was found for cysteine and VTP, and $P > 0.05$ for D-pen and 5-TP in pretreated cultures (column B).

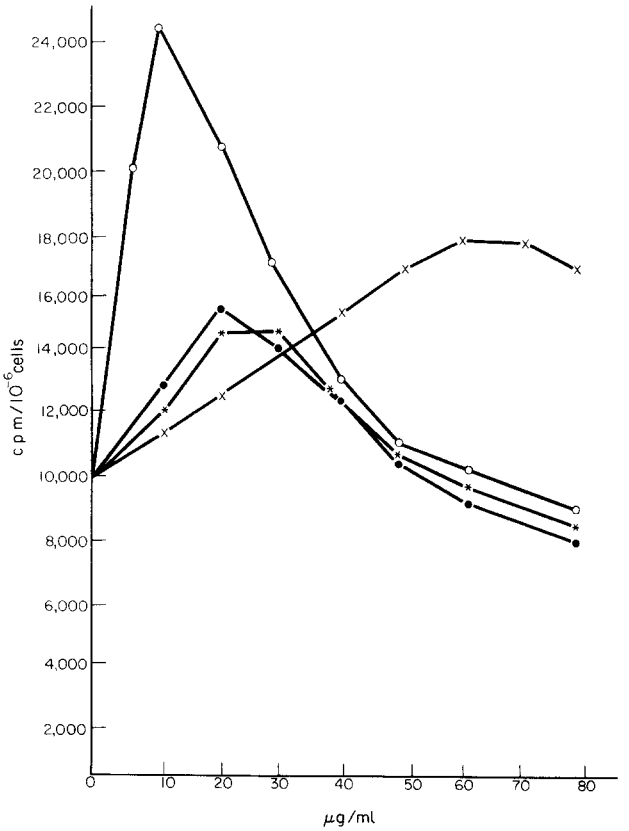

Fig. 2. Effect of increasing concentrations of disulfides on DNA synthesis in one-way lymphocyte cultures. Lymphocytes were isolated and cultured as described. Disulfides were added to cell cultures at the concentrations indicated, following mixing of the stimulator and responder cells. Cultures were incubated at 37° in 5% CO_2 for 144 hr. Eighteen hr before harvesting, [^{3}H]thymidine was added, and DNA synthesis was determined. Key: D-pen-SS (●—●); cystine (×—×), 5-TP-SS (*—*), and VTP-SS (○—○). Each data point is the average of eight different experiments.

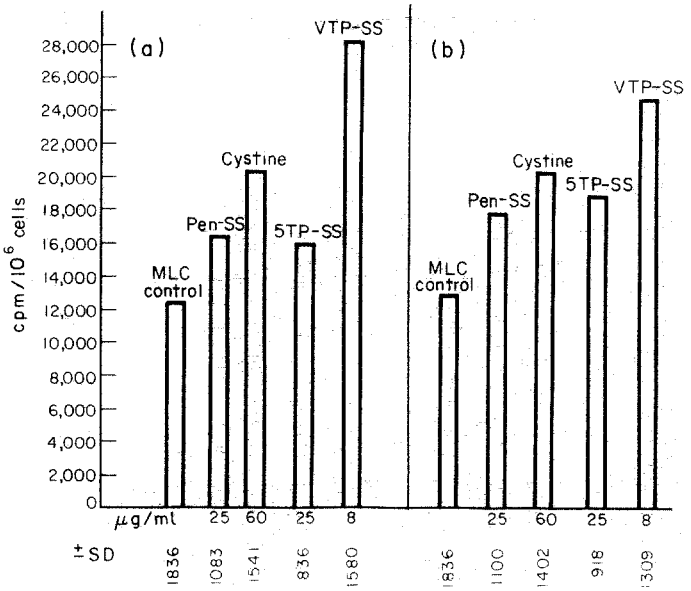

Fig. 3. Effects of disulfide compounds on DNA synthesis in one-way mixed lymphocyte cultures. Lymphocytes were isolated and cultured as described. Disulfides were either added at the initiation of MLC (a) or incubated with the responder cells for 18 hr (b) and removed by centrifugation and washing before addition of the stimulator cells. All cultures were incubated for 144 hr at 37° in 5% CO_2. Eighteen hr before harvesting, [^{3}H]thymidine was added, and DNA synthesis was determined. The concentrations shown on the abscissa were selected for optimum effect without cytoxicity. Each data point is the average of eight different experiments; $P < 0.01$ and $P < 0.05$ for panel a and panel b respectively.

cent) than with PHA or pokeweed (30 per cent). The inhibition was partial, dose dependent and characteristic for each agent. The dose at which maximum inhibition was produced is shown in Table 1. The inhibitory effect was consistent in eight separate experiments and was statistically significant ($P < 0.01$). At the concentrations studied, these compounds had no effect on DNA synthesis of resting cells; however, at high concentrations, they were all cytotoxic except D-pen.

Preincubation of lymphocytes with the agents for 18 hr, followed by their removal by centrifugation and washing, prior to mitogen stimulation, resulted in a consistent enhancement of DNA synthesis. This enhancement was statistically significant for cysteine and VTP ($P < 0.05$), but it was not significant for D-pen and 5-TP ($P > 0.05$). Paper chromatography of the sulfhydryl agents after incubation in HBSS for 18 hr at 37° showed formation of their respective disulfides. This suggested that the disulfides might have been responsible for the enhancement which was found following prolonged incubation of cells with sulfhydryl compounds. Therefore, we studied the effects of these disulfides on the proliferative response of lymphocytes.

Effects of disulfide-containing agents on mixed lymphocyte cultures and mitogen-stimulated lymphocytes. Addition of the disulfide derivatives, Pen-SS, cystine, 5-TP-SS and VTP-SS to one-way mixed lymphocyte cultures resulted in a reproducible enhancement of DNA synthesis which was statistically significant ($P < 0.01$) (Figs. 2 and 3a). The enhancing effect was concentration dependent, and it was evident only in a limited concentration range (Fig. 2). Enhancement was found to diminish with increasing concentration of the compounds. At concentrations higher than those shown, the disulfides were inhibitory. Preincubation of stimulator cells with the disulfides for 18 hr had no effect on the MLR. Similar treatment of the responder cells consistently produced enhancement of DNA synthesis (Fig. 3b). This stimulatory effect was reproducible and statistically significant ($P < 0.01$).

Addition of the disulfides to mitogen-stimulated cultures also resulted in enhancement of DNA synthesis (Table 2). The enhancement was reproducible and was statistically significant ($P < 0.01$). Preincubation of the cells with these disulfide compounds for 18 hr, followed by their removal prior to the addition of PHA, resulted in enhancement of DNA synthesis ($P < 0.05$) (Table 2).

Effect of sulfhydryl- and disulfide-containing agents on E-rosette formation. E-rosette formation was determined following incubation of lymphocytes with sulfhydryl- and disulfide-containing agents for 18 hr. The agents were removed by centrifugation and washing before addition of neuraminidase-treated sheep erythrocytes. There was no effect on E-rosette formation.

DISCUSSION

The above studies show that the sulfhydryl-containing agents, D-pen, cysteine, 5-TP and VTP, inhibited the proliferative response of lymphocytes in mixed lymphocyte cultures (Fig. 1a). Similar results were obtained with mitogen-stimulated lymphocytes (Table 1). Prolonged incubation of lymphocytes with sulfhydryl agents, prior to initiation of MLC or mitogen stimulation, resulted in enhancement of the proliferative response (Fig. 1b, Table 1). Chromatographic analysis of these agents following prolonged incubation showed the formation of their respective disulfides. This