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Distribution of Cadmium in Human Blood Cultured in Low Levels of $CdCl_2$: Accumulation of Cd in Lymphocytes and Preferential Binding to Metallothionein (40569)[1, 2]

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Cadmium (Cd) has been implicated as a causative agent in several human pathological conditions including renal dysfunction (1–3), essential hypertension (4), Itai-Itai disease (2, 3), and renal cancer (5). Recent studies also showed that Cd has marked effects upon the immune response in experimental animals (6–9). To delineate the mechanisms of these toxicological actions of Cd, a complete description of its metabolism in exposed animals is essential. Since exposure of blood is a primary event in any mode of administration of Cd to experimental animals (2, 3), the question arises as to how Cd is metabolized in the blood, specifically in blood cells. For example, do blood cells accumulate Cd during exposure, do the different classes of blood cells accumulate Cd differentially, and do the different classes of blood cells display different responses?

In this report, we present studies of Cd metabolism in human blood exposed in culture to Cd levels comparable to blood Cd levels found in cases of occupational human exposure to Cd (10). Experiments were performed to determine (i) whether Cd accumulates in blood cells in culture and, if so, whether erythrocytes and lymphocytes accumulate Cd differentially, and (ii) whether in these cell populations Cd is bound specifically to the low-molecular-weight, cysteine-rich metal-binding protein(s), metallothionein(s), previously shown to have a specificity for binding Cd and to be synthesized both in animal tissues and in cultured mammalian cells in response to Cd exposure ((11–17) and reviewed in (18)). A preliminary account of this work has been presented (19).

Materials and methods. Collection and culturing of blood. Venous blood (10 ml) was collected from each of two adult male individuals into a sterile syringe containing 1 ml of anticoagulant citrate dextrose solution (U.S.P.) obtained from a blood collection bag (Cutter Laboratories, Inc.). The whole blood was allowed to stand at room temperature for 16 hr prior to establishing the cultures. The desired amount of blood was removed and mixed with nine parts of RPMI-1640 (Gibco) containing freshly added glutamine, penicillin, and streptomycin. After dilution of the blood into culture medium, 1/100 vol of 2×10^{-5} *M* $CdCl_2$ (Amersham, carrier-free $^{109}CdCl_2$) at 2 μCi/ml was added to the blood culture. The concentrations of $CdCl_2$ solutions were verified by atomic absorption spectroscopy, and the sp act of $^{109}CdCl_2$ was corroborated using a calibrated gamma spectrometer (20). Cultures (10 ml/culture) were incubated without agitation at 37° in a CO_2 incubator. During culture, the blood cells settled to the bottom of the culture tubes, forming a loosely packed pellet.

Harvest and separation of blood cells. After 72 hr in culture, approximately three-fourths of the top of the culture medium overlaying the cell pellet was removed and saved. The blood cells were resuspended gently in the remaining culture medium and diluted with an equal volume of physiological saline at room temperature (~22°). The diluted blood cell suspension (4 ml) was carefully layered over 3 ml of lymphocyte separation medium (LSM solution, Bionetics Laboratories, Inc.) with a specific gravity of 1.078. After centrifugation at 400*g* for 30 min at room temperature, the band of lymphocytes at the interface between the supernatant-diluted culture medium and the LSM solution was withdrawn carefully with a Pasteur pipet. The

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supernatant-diluted culture medium and the LSM solution were removed separately and saved for further analysis. The pellet containing predominantly red blood cells (or erythrocytes) was resuspended in 4.5 ml of buffer A (0.01 *M* Tris–Cl, pH 7.4, 0.01 *M* KCl, 0.0015 *M* $MgCl_2$, 0.1 m*M* dithiothreitol) at 0–4°. The lymphocyte suspension withdrawn from the step gradient was diluted with Hanks' balanced salt solution (Gibco) and centrifuged at 500*g* for 6 min. The lymphocyte pellet was resuspended in 1 ml of buffer A at 0–4°.

Cell fractionation procedures. All of the following operations were performed at 0–4°. Red blood cell and lymphocyte suspensions in buffer A were treated with 1/10 vol of 10% NP-40 (a nonionic detergent, Particle Data Laboratories, Ltd., Elmhurst, Ill.) and were mixed using a vortex mixer for 30 sec. After 15 min, another 30-sec vortex mixing was performed. Nuclei were removed from the lysed cell suspensions by centrifugation at 750*g* for 5 min. The supernatants (cytoplasms) were decanted carefully, frozen in a dry ice–ethanol bath, and stored at −20°. The pellets were resuspended in buffer A.

Cytoplasmic fractions from the red blood cell and lymphocyte lysates were fractionated further by column chromatography (1.2 × 75-cm column) using Sephadex G-75 (Pharmacia) equilibrated with 0.05 *M* Tris–Cl (pH 8.4). Samples were eluted at 0.3–0.5 ml/min with 0.05 *M* Tris–Cl (pH 8.4). Column chromatography was performed in a constant-temperature room at 20°. Calibration of the columns was performed with serum albumin (Technicon, standard), DNase I, and cytochrome *C* (both obtained from Worthington). The elution profile for hemoglobin was obtained by measuring for each fraction the absorption of 545 nm light as described elsewhere (21).

Monitoring of Cd in cells and in subcellular fractions. Samples (0.05–0.2 ml) in scintillation vials were adjusted to 1 ml with distilled water, and 0.1 ml 2% sodium lauroyl sarcosine (Schwarz/Mann) was added to solubilize the samples containing intact cells. Either PCS (Nuclear-Chicago Corp.) or Aquasol II (New England Nuclear Corp.) scintillation solvent was added to each sample, and the ^{109}Cd activity was determined using an automatic liquid scintillation spectrometer (17). Hemoglobin-containing samples were analyzed in an automatic gamma spectrometer (Packard).

Results. Exposure of human blood cells to $CdCl_2$ in tissue culture results in uptake of Cd by the blood cells (Tables I and II). During the 72-hr exposure period used in this study, there was no evidence of cell death (e.g., hemolysis), and the blood cells did not form clumps. Table I shows that a significant portion of Cd in the growth medium was incorporated into blood cells during a 72-hr exposure to 2×10^{-7} *M* $CdCl_2$. Separation of blood cells using lymphocyte separation medium provided two cellular populations: (i) a population comprised predominantly (>95%) of lymphocytes, and (ii) a red blood cell or erythrocyte population containing <0.02% of other cell types. In this separation, >80% of the total blood lymphocytes are found in the isolated lymphocyte population. As shown in Table II, lymphocytes accumulate approximately 800-fold more Cd than red blood cells on a per cell basis. Using the data in Table II and a volume of 87×10^{-12} cm^3/cell for both red blood cells and lymphocytes (22), the Cd concentration in an average red blood cell is approximately 1 μM, while the concentration in an average lymphocyte is approximately 620 μM. The Cd concentration in the culture medium was 0.2 μM so that the relative Cd accumulation in red blood cells and lymphocytes is 5- and 3000-fold, respectively. It should be noted that the measurements reported here for Cd incorporation by cells very likely include Cd bound to the cell surface so

TABLE I. Cd DISTRIBUTION IN 3-DAY BLOOD CULTURES (%)[a]

Culture medium	Red blood cells	Lymphocytes
80 ± 4	15 ± 1	5 ± 2

[a] The ± figure gives the full range of variation among the different cultures and the different individuals.

TABLE II. Cd INCORPORATION INTO RED BLOOD CELL AND LYMPHOCYTE POPULATIONS

Cell population	μg Cd/10^9 cells
Red blood cells	0.010 ± 0.001
Lymphocytes	6 ± 3

[a] The ± figure gives the full range of variation among the different cultures and between the different individuals.

that the Cd concentrations calculated above could overestimate the intracellular Cd concentration.

The distribution of Cd in cell lysates is given in Table III. In the lymphocyte lysate, approximately 4% of the total cellular Cd sedimented with the nuclear fraction. Only trace amounts of Cd were detected in the pellet of the red blood cell lysate, as would be expected, since erythrocytes are anucleated.

Further resolution of the intracellular Cd distribution was obtained by molecular sieve chromatography with Sephadex G-75. Column chromatography was performed on the cytoplasm from the lysates of (i) total blood cells prior to separation, (ii) red blood cells, and (iii) lymphocytes. The results from these measurements are shown in Figs. 1A–C. Figure 1A indicates that, in the cytoplasm from total blood cell lysate, there are at least four distinguishable peaks of Cd-binding activity. Peak I (Fig. 1, fraction 20) corresponds to the material that is excluded from the column (indicating $M_r > 70{,}000$). Peak II (fraction 23) elutes near the hemoglobin peak and appears as a shoulder on peak I. Peak III (fraction 30) precedes the elution of cytochrome *C* (cochromatographed with the lysate in separate experiments), and peak IV (fraction 40) elutes as a species smaller than cytochrome *C*. Chromatographic profiles of the cytoplasmic fractions from separate red blood cell and lymphocyte populations are shown in Figs. 1B and C, respectively. In the red blood cell lysate, only three distinct Cd-binding peaks corresponding to peaks I, II, and III are observed (Fig. 1B). It is clear that the Cd-binding component(s) associated with peak IV is absent. In contrast to the elution profile of the red blood cell cytoplasm, the lymphocyte cytoplasm contains only Cd-binding species corresponding to peaks I and IV in Fig. 1A, with most of the activity appearing with peak IV. Figure 1D serves as a check for appearance of cellular Cd-binding components in the growth medium as a result of cell death and lysis during Cd exposure. This profile shows no indication of cellular components in the growth medium after 72 hr of exposure to Cd. Further, absorbance measurements (data not shown) indicate no

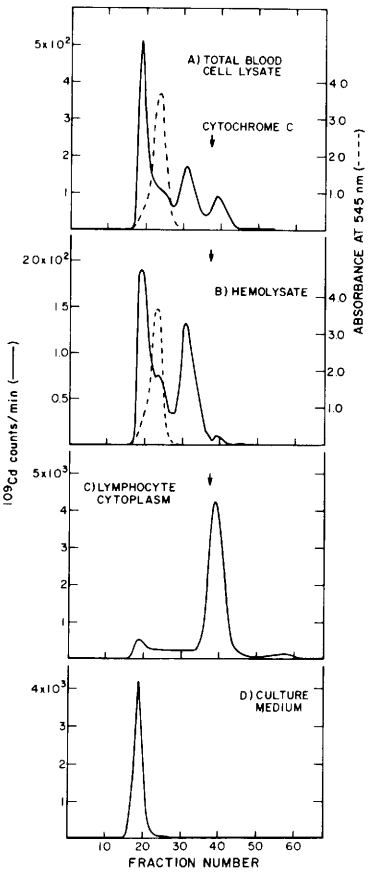

FIG. 1. Sephadex G-75 column chromatographs of (A) total blood cell lysate, (B) red blood cell lysate (hemolysate), (C) lymphocyte cytoplasm, and (D) culture medium. Four peaks of ^{109}Cd activity are distinguishable: peak I eluting at fraction 20, peak II coeluting with hemoglobin in fraction 23, peak III eluting at fraction 30, and peak IV eluting at fraction 40. The dashed line in frames A and B indicates the elution profile of hemoglobin obtained by measuring for each fraction the absorption of 545-nm light (21).

TABLE III. DISTRIBUTION OF Cd IN LYSATES OF RED BLOOD CELL AND LYMPHOCYTE POPULATIONS

Cell population	Incoporated Cd (%)	
	Cytoplasm	Nuclei
Red blood cells	>99	<1[a]
Lymphocytes	96	4

[a] Since red blood cells contain no nuclei, this figure includes nuclei of nonred blood cell types coisolating with the red blood cells. Also, this estimate was based on measurements of ^{109}Cd only two- to threefold > background so that a significant portion of this label could be due to trace amounts of ^{109}Cd left on tube walls after removal of the cytoplasm.

545-nm absorbing material eluting from the column, demonstrating the absence of detectable hemolysis during culturing.

It was considered likely that the lymphocyte population could respond to Cd exposure by inducing the synthesis of the Cd-binding metalloprotein, metallothionein (expected to elute similarly to peak IV in Figs. 1A and D), while the anucleated red blood cell population could not respond in this manner. The possibility that peak IV material is Cd-thionein was approached by comparing the chromatographic profile of the lymphocyte cytoplasm with the elution of Cd-thionein isolated from another source ((16, 17) and manuscript in preparation). This Cd-thionein is similar to Cd-thioneins from other sources (11–18) as judged by its cysteine-rich, leucine-deficient composition and its electrophoretic behavior (unpublished results). The result of cochromatography of the isolated Cd-thionein with lymphocyte cytoplasm is shown in Fig. 2. The Cd-thionein elutes exactly with peak IV material. The same result was observed for independent chromatography of lymphocyte cytoplasm and Cd-thionein, ruling out the possibility of aggregation of Cd-thionein and peak IV material. Separate experiments with isolated lymphocyte cultures exposed to Cd also indicate that peak IV material is Cd-thionein on the basis of the parameters mentioned above.

Discussion. In this study, human blood was exposed to low levels of $CdCl_2$ in tissue culture to investigate the metabolism of Cd in blood cells. The concentration of Cd used in these studies (2×10^{-7} *M* $CdCl_2$) is comparable to concentrations found in the blood of persons who have been exposed to Cd vapors in the soldering industry (10). The use of tissue culture has permitted Cd metabolism in human blood cells to be studied under controlled conditions of nutrient supply and Cd exposure. Tissue culture also permits one to separate the direct effects of Cd metabolism by blood cells from indirect effects arising from Cd metabolism in other tissues resulting in symptomatic changes in the blood. It is also important to determine whether Cd metabolism in other tissues is reflected in Cd metabolism in blood cells. Hence, to obtain a complete view of the indirect and direct effects arising in blood cells as a result of Cd

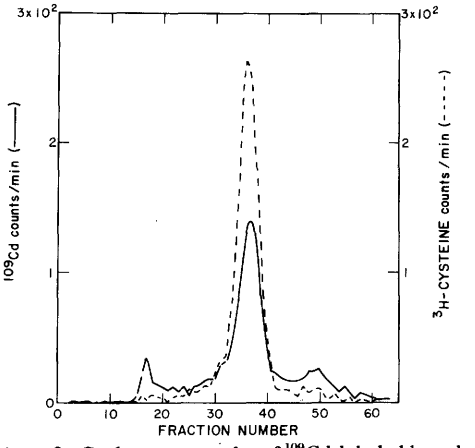

FIG. 2. Cochromatography of ^{109}Cd-labeled lymphocyte cytoplasm (solid line) with isolated [^{3}H]cysteine-labeled Cd-thionein (dashed line). [^{3}H]Cysteine-labeled Cd-thionein was isolated from cultured Chinese hamster cells grown in [^{3}H]cysteine (0.01 μCi/ml, New England Nuclear Corp.) during exposure to subtoxic metallothionein-inducing levels of Cd for 24 hr (17).

exposure, it will be necessary to compare Cd metabolism both in blood from animals exposed to Cd *in vivo* and in blood from the same animal species exposed *in vitro.*

During a 3-day exposure of cultured blood cells to the low level of Cd, a Cd accumulation in the cells was observed. This result is consistent with previous studies of Cd metabolism in blood of experimental animals treated with Cd salts at levels which produce some signs of toxicity (i.e., weight loss) during prolonged exposure (6 months) (2, 23–25). In the present study, separation of nucleated and anucleated blood cell types showed that the red blood cell population had an approximately 5-fold greater concentration of Cd than the culture medium, while the lymphocyte population accumulated Cd to a level ~3000-fold greater than the culture medium. This striking differential accumulation of Cd by lymphocytes suggests that different mechanisms for Cd uptake operate in the different types of blood cells. Attempts are in progress to determine how much of the cellular Cd is incorporated into cells (erythrocytes and lymphocytes) relative to how much is associated with exterior cell-surface components. These determinations could slightly modify our present estimates of Cd incorporation into blood cells, especially erythrocytes.

The observation that Cd is accumulated by lymphocytes in whole blood cultures provides a basis for speculating that exposure of animals to this toxic trace metal may affect the normal functioning of the immune response of which lymphocytes are essential components. This proposition is predicated upon the assumption that Cd metabolism by blood cells *in vivo* is analogous to Cd metabolism during exposure in culture. In the context of these considerations, it has been reported that Cd exposure affects immunocompetence in rabbits (7), rats (6), and mice (8, 9). In other studies, Cd has been shown to alter the mitogenic response of lymphocytes in culture (26). However, in the previous studies, Cd metabolism by lymphocytes was not followed during Cd exposure (6–9, 26). Hence, quantitation of Cd metabolism in blood cells (particularly lymphocytes) would be an interesting adjunct to studies of the effects of Cd exposure on the immune response in animals. Although the relevance of the present findings to the effects of Cd in immunocompetence will require further study, the observation that lymphocytes accumulate Cd in culture provides a preliminary basis for explaining why Cd-mediated effects on the immune response would be observed.

Examination of the subcellular distribution of Cd (i) in total blood cell lysates, (ii) in lysed erythrocytes, and (iii) in the lymphocyte cytoplasm has revealed several interesting features of Cd metabolism in these cell populations. The total blood cell lysate was found to contain at least four distinct macromolecular Cd-binding species. Note that the material eluting from the G-75 column in the excluded peak may contain several large Cd-binding species not resolved by this system. A peak of Cd-containing material appears to coelute with hemoglobin in the column profiles (Fig. 1B and unpublished results), although in the present study the apparent Cd-binding character of hemoglobin has not been ascertained. One of the Cd-binding species (Fig. 1A, peak IV) corresponds to Cd-thionein (see Fig. 2). With the exception of an additional Cd-binding species observed in the present studies (Fig. 1A, peak III), our findings are in agreement with previous observations by Nordberg *et al.* on the hemolysate of blood from mice chronically exposed to $CdCl_2$ (25). They found Cd bound mainly to a macromolecular species characteristic of Cd-thionein. However, in their studies, the blood cells were not separated for further study. In the present investigation, separation of the lymphocyte population from the erythrocyte population showed pronounced differences between the erythrocyte and lymphocyte Cd-binding macromolecules. Erythrocytes contained most of the peak I Cd-binding species and all of the peaks II and III material (see Figs. 1A and B). In contrast, the lymphocyte cytoplasm (containing 96% of the total cellular Cd) had very little peak I Cd-binding species, and the remainder was in Cd-thionein (peak IV, Fig. 1A). The accumulation of Cd specifically in metallothionein in lymphocytes might be expected, since it is known that cultured nucleated cells from several animal sources, as well as cells in tissues of exposed animals, respond to Cd exposure by inducing synthesis of a low-molecular-weight, cysteine-rich, Cd-binding metallothionein (13–18, 20). Since the induction of metallothionein synthesis is very likely regulated at the level of gene transcription ((27, 30) and unpublished results), it would be expected that anucleated human erythrocytes would lack the ability to synthesize metallothionein, while the nucleated blood cells (viz. lymphocytes) would have that capability.

Summary. Human blood was exposed to low levels of $CdCl_2$ (2×10^{-7} *M*) in tissue culture. During a 3-day exposure, Cd was accumulated by the blood cells. Erythrocytes incorporated Cd to a level ~5-fold greater than the concentration in the growth medium, while lymphocytes accumulated Cd to a cellular concentration ~3000-fold greater than that in the culture medium. In erythrocytes, Cd was bound to several macromolecular species, none of which corresponded to the specific, inducible, Cd-binding protein metallothionein. In contrast, most of the Cd in lymphocytes was found associated with a low-molecular-weight macromolecule characteristic of Cd-thionein. On the basis of these studies showing the accumulation of Cd by lymphocytes and the metabolic response of lymphocytes indicated by the sequestering of Cd in metallothionein, it is possible that Cd exposure may alter normal immunocompe-

tence associated with lymphocytes.

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