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COMPARISON OF COBALT AND CHROMIUM BINDING TO BLOOD ELEMENTS

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SUMMARY

Macromolecular binding of metals is thought to be a prerequisite for induction of metal sensitivity. In this study, the binding of cobalt(II) to blood components was investigated. Incubation of ^{60}Co with blood yielded a mean erythrocyte binding of 10.3×10^7 Co atoms/cell. Incubation of the metal with serum resulted in binding of 8.3×10^{-9} mol Co/mg protein. A comparison was made with analogous binding of chromium(III), a metal recognized for its sensitizing potency. Binding of chromium to proteins and blood cells was 20-fold higher than that obtained with cobalt. With both metals, binding to serum proteins was non-specific. The greater binding by chromium, when compared with cobalt, is consistent with the greater sensitizing ability of this metal.

Key words: Macromolecular-metal binding; Metal sensitivity; Cobalt sensitivity; Protein-metal interactions; Metal-erythrocyte binding

INTRODUCTION

Case histories of hypersensitivity to cobalt have been well documented. Reports of contact sensitivity have come from workers in the pottery industry [1], cement industry [2,3] and in the hard metal industry where cobalt is used as a cementing agent in metal carbides [4]. Respiratory sensitivity has also been noted following industrial exposure to cobalt. Four cases

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of allergic alveolitis in a hard metal plant were attributed to exposure to grinding coolant which contained dissolved cobalt [5].

Cobalt hypersensitivity has been induced experimentally in animals [6,7]. Using either the guinea pig maximization test [8], the split adjuvant technique [9], or the injection protocols of both Polak et al. [10] and Gross et al. [11], guinea pigs developed sensitivity following exposure to cobalt chloride.

It is widely accepted that low molecular weight compounds first conjugate in vivo with biological materials before initiating sensitization. The specificity of the resulting sensitivity is uncertain. Whether the response is directed toward the metal hapten or toward new antigenic determinants [12] formed by interaction of the metal with carrier molecules, is not known. In either case, the sensitizing potency of a metal ion is expected to depend in part on its ability to conjugate with macromolecules.

Blood components have received attention as likely in vivo carriers in metal sensitization. Cohen [13] found that all of 44 chromium sensitive patients showed positive reactions when tested by intradermal injection with a chromium-human serum albumin complex. A smaller number of patients (8/20) had positive reactions when challenged with a chromium-gamma globulin complex. Patients with chromium sensitivity exhibited a tuberculin-type reaction when injected intradermally with $CrCl_3$ and $CoCl_2$ bound to human serum albumin but not when injected with cobalt bound to rabbit liver glycogen [14]. These results suggested that metal sensitivity is carrier dependent and that serum proteins may be in vivo carriers. Jandl and Simmons [15] studied agglutination of red cells by metallic cations. They observed that many multivalent metallic cations, including cobalt, readily attached to the surface of washed red blood cells leading to agglutination.

The purpose of the present study was to examine the binding of cobalt to serum proteins and red blood cells. In order to determine if binding to macromolecules correlated with sensitizing potency, similar binding studies were performed with chromium, a metal generally recognized to be one of the more potent metal sensitizers.

MATERIALS AND METHODS

Cobalt, chromium binding to erythrocytes

Blood was obtained by clipping the nailbed of English smooth-haired quinea pigs (Hilltop Lab, Scottdale, PA). For binding studies, the typical protocol involved mixing 100 μl heparinized blood with 2.5 μl of 0.15 M $^{60}CoCl_2$ (6.9×10^9 cpm/mol) or 0.15 M $^{51}CrCl_3$ (8.8×10^{10} mCi/ml, obtained from the University of Pittsburgh Radiation Department). ^{51}Chromium, 2 mCi/ml as $CrCl_3$ was purchased from New England Nuclear. Because of the high specific activities, solutions of lower specific activity were prepared by adding non-radioactive cobalt chloride, $CoCl_2 \cdot 6H_2O$ (J.T. Baker), or chromic chloride, $CrCl_3 \cdot 6H_2O$ (Mallinkrodt) as follows. Solutions of labelled and unlabelled metal chlorides were mixed, then evaporated to dryness under

nitrogen using a heat lamp. The crystals were redissolved in an appropriate quantity of distilled water (25—50 μl). The evaporation step was necessary to remove HCl since the radioisotopes were supplied in 0.1 M or 0.5 M HCl solutions. Absorbance spectra before and after evaporation and redissolution indicated that this procedure caused no loss or change of chemical species.

The mixture of $CoCl_2$ (or $CrCl_3$) and blood was incubated at 37°C for 1 h, then centrifuged at 9000 *g* for 15 s. Plasma was removed and analyzed for metal-bound proteins as described in the following section. The erythrocytes were washed repeatedly with 0.85% saline then radioactivity was measured using a Packard 5110 Auto-Gamma Scintillation Spectrometer. Erythrocyte numbers were determined by direct counting using a hemocytometer.

Cobalt, chromium binding to plasma proteins

Binding was evaluated by assessing labelled proteins eluted from molecular exclusion chromatographic columns. Following incubation of $^{60}CoCl_2$ or $^{51}CrCl_3$ with blood, the plasma fraction was applied to Sephadex G-200 and/or Sepharose 4B columns (1 cm × 30 cm). Proteins were eluted with 0.0027 M borate—0.1 M NaCl buffer (pH 7.4). One milliliter fractions were collected at a flow rate of 0.1 ml/min. The radioactivity of each fraction was measured and, whenever possible, the protein concentration was determined from absorbance at 280 nm. All columns were pretreated with bovine serum albumin (BSA) prior to first use.

Identification of serum proteins binding cobalt or chromium

To assess binding capacity of metals to serum proteins in the absence of blood cells, radioactive metal chlorides (10 μmol) were incubated with 100 μl guinea pig serum for 1 h at 37°C. Following incubation, the protein-bound metals were separated from unbound metals by molecular exclusion chromatography using Sephadex G-25 (1 cm × 21.5 cm; FR = 0.4 ml/min), Sephadex G-200 (1 cm × 30 cm; FR = 0.1 ml/min), Sepharose 4B (1 cm × 30 cm; FR = 0.2 ml/min) or a column (1 cm × 30 cm) containing 15 cm of Sephadex G-200 above 15 cm of Sepharose 4B (FR = 0.1 ml/min). All columns were operated at room temperature. The radioactivity of each fraction was measured, and the protein concentration determined from absorbance at 280 nm or from colorimetric determination (Bio-rad protein reagent, Bio-Rad Laboratories). Columns were calibrated by determining elution volumes of standard purified proteins: bovine gamma globulin (150 000 daltons) BSA (70 000 daltons) and ovalbumin (45 000 daltons).

EXPERIMENTAL

Cobalt binding to erythrocytes

Ten μmoles of $CoCl_2$ (6.9×10^9 cpm/mol Co) were added to 200 μl freshly drawn guinea pig blood. The amount of cobalt bound to erythrocytes

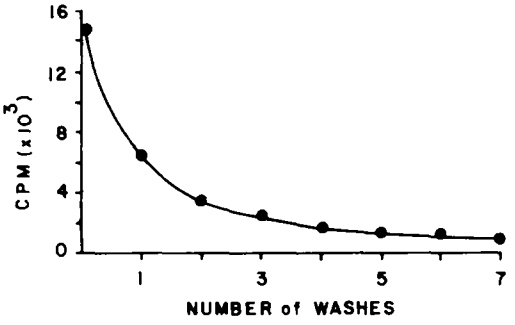

Fig. 1. Binding of cobalt(II) to blood cells. 200 μl blood (4.8×10^9 cells/ml) were incubated with 10 μmol $^{60}CoCl_2$ (6.9×10^9 cpm/mol). Cells were washed repeatedly with 100 μl saline until cpm bound remained constant (background count = 136 cpm).

after thorough washing is shown in Fig. 1. After 5—7 washes, counts remained at 1,050 cpm above background (136 cpm). This value represents binding of 1.4% of the added radioactivity and indicated 1.6×10^{-16} mol (9.7×10^7 Co atoms) bound per red blood cell. A replicate cell binding experiment using 5 μmol $CoCl_2$ (3.8×10^{10} cpm/mol) and 100 μl blood yielded 1.8×10^{-16} mol. (1.1×10^8 Co atoms) bound per red blood cell (Table I).

TABLE I

COBALT AND CHROMIUM BINDING TO BLOOD COMPONENTS[a]

	Cobalt		Chromium	
	Amount bound	% of total Co added	Amount bound	% of total Cr added
Blood cells	9.7×10^7 atoms/cell	1.4	2.0×10^9 atoms/cell	36
	11.0×10^7 atoms/cell	0.76	2.2×10^9 atoms/cell	49
Mean ± S.D.	10.3×10^7 (± 0.91) atoms/cell		2.1×10^9 (± 0.14) atoms/cell	
Plasma proteins in presence of cells	12×10^{-9} mol/mg	0.59	not ascertained due to severe hemolysis of red cells	
Serum proteins in absence of cells	8.2×10^{-9} mol/mg	0.32	1.6×10^{-7} mol/mg	8.3
	8.4×10^{-9} mol/mg	0.37	2.1×10^{-7} mol/mg	10.7
			2.7×10^{-7} mol/mg	8.0
Mean ± S.D.	8.3×10^{-9} (± 14) mol/mg		2.1×10^{-7} (± 0.55) mol/mg	

[a] 10 μmoles $CoCl_2$ or $CrCl_3$ were incubated with 200 μl blood or 100 μl serum. Blood cells were removed by centrifugation and washed thoroughly. Unbound metals were separated from protein-bound metals by molecular exclusion chromatography.

Cobalt binding to plasma proteins

Following incubation of $^{60}CoCl_2$ with whole blood the amount of cobalt bound to plasma proteins was determined using molecular exclusion chromatography. Binding of ^{60}Co to plasma proteins was evident from the plasma elution profile shown in Fig. 2. Fractions eluting at 9—17 ml displayed significant radioactivity (greater than background +2 S.D.). Of the total radioactivity applied to the column, 0.6% eluted with the proteins. The remaining radioactivity was eluted in the volume corresponding to cobalt salts (>26 ml). Results indicated 1.2×10^{-8} mol Co had bound/mg plasma protein when $CoCl_2$ was added to whole blood. Of the total cobalt added, approximately 1% bound to the red cells while 0.6% bound to proteins.

Incubation of cobalt with serum

Binding of cobalt to serum proteins is shown in Fig. 3. Following incubation of serum with $^{60}CoCl_2$, the mixture was applied to a Sephadex G-200/Sepharose 4B column. Radioactivity was detected in all protein fractions. The profile of cpm exactly paralled that for absorbance 280 nm. This result indicated non-specific binding of cobalt to serum proteins. The sharp rise in cpm starting at an elution volume of 25 ml represented 3.4×10^{-8} mol cobalt. Calculations indicated 8.2×10^{-9} mol of cobalt bound/mg protein. Results of replicate determinations are listed in Table I.

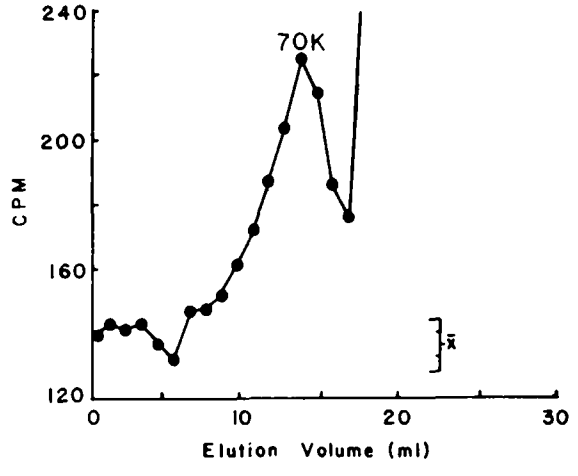

Fig. 2. Elution of Co(II)-plasma mixture from Sephadex G-200/Sepharose 4B column. $^{60}CoCl_2$ (10 μmol, 6.9×10^9 cpm/mol) was incubated with 200 μl blood. The plasma fraction was applied to a 1 × 30 cm column containing a bed of Sephadex G-200 atop an equal bed of Sepharose 4B. Elution was with 2.7 mM borate-0.1 M NaCl buffer, (pH 7.4). One milliliter fractions were collected using a flow rate of 0.1 ml/min. Bar indicates background cpm + 2 S.D. K = 1000 daltons.

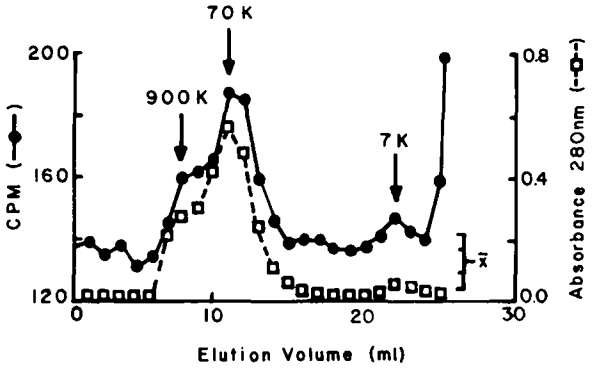

Fig. 3. Fractionation of Co(II)-serum mixture using Sephadex G-200/Sepharose 4B. $^{60}CoCl_2$ (10 μmol, 6.9 × 10^9 cpm/mol) was incubated with 100 μl serum. Elution was with 0.5 M borate buffer (pH 7.4); 1 ml fractions were collected. Bar indicates background cpm + 2 S.D.

Chromium binding to blood components

A study was made of the ability of chromium to bind with blood components. Ten μmoles chromium chloride (8.8 × 10^{10} cpm/mol Cr) were incubated with 200 μl blood for 1 h at 37°C. After repeated washing of cells with saline, 338 930 cpm remained bound (Table I). This value represented 2.0 × 10^9 chromium atoms bound per cell (3.3 × 10^{-15} mol/cell). A replicate experiment yielded 444 140 cpm or 2.2 × 10^9 Cr atoms/cell (3.7 × 10^{-15} moles/cell). The extent of chromium binding to blood proteins could not be evaluated because of erythrocyte hemolysis.

Chromium binding to serum proteins

^{51}Chromium was incubated with serum and the mixture applied to a Sephadex G-200 column. Results are shown in Fig. 4. Radioactivity eluted

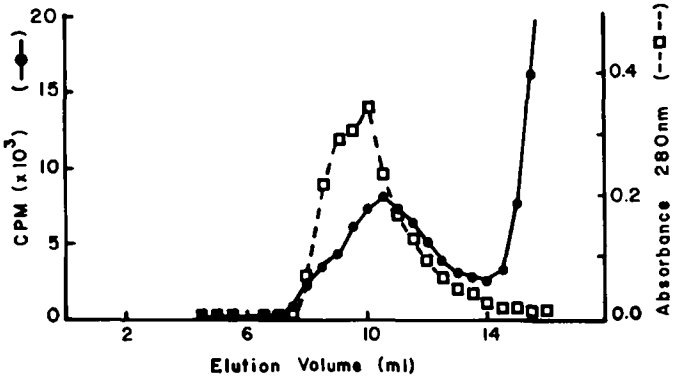

Fig. 4. Fractionation of Cr(III)-serum mixture using Sephadex G-200. $^{51}CrCl_3$ (10 μmol, 7.6 × 10^{10} cpm/mol) was incubated with 100 μl serum. Elution was with 2.7 mM borate–0.1 M NaCl buffer (pH 7.4); 0.5 ml fractions were collected.

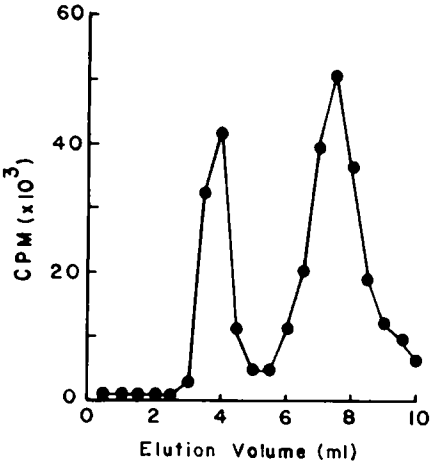

Fig. 5. Fractionation of Cr(III)-serum mixture using Sephadex G-25. $^{51}CrCl_3$ (5 μmol, 1.7×10^{11} cpm/mol) was incubated with 50 μl serum at 37°C, then applied to a 1 x 21.5 cm column. Fractions were eluted with 2.7 mM/borate—0.1 M NaCl buffer (pH 7.4); 0.5 ml fractions were collected. The peak at 4 ml contained protein; that at 8 ml contained chromium salts.

with the protein fractions. Calculation of the amount of Cr bound to protein yielded 2.7×10^{-7} mol Cr/mg protein (Table I). Because protein recovery from this column was less than expected, separation of free Cr from protein-bound Cr was attempted using a Sephadex G-25 column. Two radioactive peaks were detected (Fig. 5). The first peak (eluted at 4 ml) contained protein, whereas the second peak (7 ml) corresponded to chromium salts. Protein recovery was greater than 90%. Replicate experiments yielded 71 960 cpm and 72 170 cpm bound to protein. These values indicated 1.6×10^{-7} mol chromium/mg protein and 2.1×10^{-7} mol chromium/mg protein, respectively (Table I).

A summary of results of cobalt and chromium binding to erythrocytes and plasma proteins is presented in Table I. Under identical incubation conditions, 20 times as much chromium compared with cobalt, was bound to red blood cells ($P < 0.01$) and 25 times more chromium than cobalt was bound to plasma proteins. In the absence of red blood cells, serum bound 8.3×10^{-9} mol cobalt/mg protein (0.3% of that added) indicating a slight preference of cobalt binding to cells as opposed to plasma proteins. In the case of chromium, there was a clear preference for binding to red blood cells.

DISCUSSION

Allergic sensitivity to cobalt has been reported in workers in the cement, pottery and hard metal industries. In addition, cobalt hypersensitivity has been induced in experimental animals. In guinea pigs, cobalt chloride was

determined to be a less potent sensitizer than chromic chloride [6].

Many investigators have reported sensitization following dermal exposure to Cr(VI) [6,8]. However, Siegenthaler et al. [16] recently observed that guinea pigs primed with Cr(VI) gave a better immune response upon challenge with Cr(III)-macrophage than upon challenge with the analogous Cr(VI) complex. In addition, it has been recognized that only Cr(III) compounds are capable of forming covalent bonds with proteins [16]. Since this latter property is generally accepted as a precondition for immunogenicity, Cr(III) was selected for use in the current study.

The nature of the carrier molecules for induction of metal sensitivity has received considerable attention. Paley and Sussman [17] investigated binding of cobalt to purified proteins. In their study, ^{60}cobalt was mixed with both human serum albumin and ovalbumin and kept at 0°C for 24 h. Analysis by radioautography of electrophoretic strips indicated migration of the radiolabel with both proteins. Cohen [13] added $CrCl_3$ to separate solutions of serum albumin and gamma globulin. He found that the same number of chromium molecules was bound to the 2 proteins. Evidence was obtained that serum albumin and gamma globulin were the actual biologic carriers in patients with cobalt and chromium sensitivities [14]. Patients showed positive intradermal test reactions to serum albumin metal conjugates but not to conjugates formed by reaction of metals with rabbit liver glycogen.

In the experiments reported here, using approximately a 100:1 molar ratio of Co or Cr to protein, cobalt was found capable of binding with serum proteins and blood cells. Duplicate experimental determinations indicated reproducibility of the methods of binding and analysis. Binding of cobalt to serum proteins was non-specific since no one serum protein appeared to bind more cobalt than any other. The extent of cobalt-protein binding was minimally affected by the presence of blood cells. These results are consistent with a non-specific interaction of cobalt with these macromolecules.

Similarly, chromium binding to protein appeared to be non-specific. However, binding of chromium to both proteins and blood cells was 20-fold higher than that seen with cobalt. Using the *t*-test [18], the mean binding values of cobalt and chromium to serum proteins and to blood cells were significantly different ($P < 0.01$). The greater observed chromium binding is consistent with the reported greater sensitizing potency of chromium [6]. These results suggest a correlation between the amount of binding to macromolecules and sensitizing ability. Further studies into the binding characteristics of other metal sensitizers (for example, beryllium, nickel and platinum) and non-sensitizers (cadmium, mercury) and properties of the complexes formed, should provide needed insight into factors that influence ability of metals to act as allergic sensitizers.

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REFERENCES

1 V. Pirila, Sensitization of cobalt in pottery workers, Acta Demato-Venereol., 33 (1953) 193.
2 V. Pirila, On the role of chrome and other trace elements in cement eczema. Acta Dermato-Venereol., 34 (1954) 136.
3 V. Pirila and H. Kajanne, Sensitization to cobalt and nickel in cement eczema. Acta Dermato-Venereol., 45 (1965) 9.
4 L. Schwartz, S.M. Peck, K.E. Blair and K.E. Markuson, Allergic dermatitis due to metallic cobalt. J. Allergy, 16 (1945) 51.
5 I. Sjogren, G. Hillerdal, A. Anderson and O. Zetterstrom, Hard metal lung disease: Importance of cobalt in coolants. Thorax, 35 (1980) 653.
6 R. Hicks, P.J. Hewitt and H.F. Lam, An investigation of the experimental induction of hypersensitivity in the guinea pig by material containing chromium, nickel and cobalt from arc. welding fumes. Int. Arch. Allergy Appl. Immunol., 59 (1979) 265.
7 Th. Maurer, E.G. Weirich and R. Hess, The optimization in the guinea pig in relation to other predictive sensitization methods. Toxicology, 15 (1980). 163.
8 B. Magnusson and A.M. Kligman, The identification of contact allergens by animal assay. The guinea pig maximization test. J. Invest. Dermatol., 52 (1969) 268.
9 H.C. Maguire and M.W. Chase, Exaggerated delayed-type hypersensitivity to simple chemical allergens in the guinea pig. J. Invest. Dermatol., 49 (1967) 460.
10 L. Polak, J.M. Barnes and J.L. Turk, The genetic control of contact sensitization to inorganic metal compounds in guinea pigs. Immunology, 14 (1968) 707.
11 P.R. Gross, S.A. Katz and M.H. Samitz, Sensitization of guinea pigs to chromium salts. J. Invest. Dermatol., 50 (1968) 424.
12 I.L. Bernstein, Isocyanate-induced pulmonary diseases: A current perspective. J. Allergy Clin. Immunol., 70 (1982) 24.
13 H.A. Cohen, Carrier specificity of tuberculin-type reactions to trivalent chromium. Arch. Dermatol., 93 (1966) 34.
14 H.A. Cohen, The role of carrier in sensitivity to chromium and cobalt. Arch. Dermatol., 112 (1976) 37.
15 J.H. Jandl and R.L. Simmons, The agglutination and sensitization of red cells by metallic cations: Interactions between multivalent metals and the red-cell membrane. Br. J. Haematol., 3 (1957) 19.
16 U. Siegenthaler, A. Laine and L. Polak, Studies on contact sensitivity to chromium in the guinea pig. The role of valence in the formation of the antigenic determinant. J. Invest. Dermatol., 80 (1983) 44.
17 K.R. Paley and E.S. Sussman, Absorption of radioactive cobaltous chloride in human subjects. Metabolism, 12 (1963) 975.
18 P. Armitage, Statistical Methods in Medical Research, Wiley & Sons, New York, 1977.