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Orthopaedic implant related metal toxicity in terms of human lymphocyte reactivity to metal-protein complexes produced from cobalt-base and titanium-base implant alloy degradation

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Abstract

Metal toxicity from sources such as orthopaedic implants was investigated in terms of immune system hyper-reactivity to metal implant alloy degradation products. Lymphocyte response to serum protein complexed with metal from implant alloy degradation was investigated in this *in vitro* study using primary human lymphocytes from healthy volunteers (n = 10). Cobalt chromium molybdenum alloy (Co-Cr-Mo, ASTM F-75) and titanium alloy (Ti-6Al-4V, ASTM F-136) beads (70 μm) were incubated in agitated human serum at 37 degrees Celsius to simulate naturally occurring metal implant alloy degradation processes. Particulate free serum samples, which were incubated with metal, were then separated into molecular weight based fractions. The amounts of soluble Cr and Ti within each serum fraction were measured and correlated with lymphocyte proliferation response to the individual serum fractions. Lymphocytes from each subject were cultured with 11 autologous molecular weight based serum fractions either with or without added metal. Two molecular weight ranges of human serum proteins were associated with the binding of Cr and Ti from Co-Cr-Mo and Ti implant alloy degradation (at < 30 and 180–330 kDa). High molecular weight serum proteins (≈ 180 kDa) demonstrated greater lymphocyte reactivity when complexed with metal released from Co-Cr-Mo alloy and Ti alloy than with low (5–30 kDa) and midrange (30–77 kDa) serum proteins. When the amount of lymphocyte stimulation was normalized to both the moles of metal and the moles of protein within each fraction (Metal-Protein Complex Reactivity Index, MPCRI), Cr from Co-Cr-Mo alloy degradation demonstrated approximately 10 fold greater reactivity than Ti in the higher molecular weight serum proteins (≈ 180–250 kDa). This *in vitro* study demonstrated a lymphocyte proliferative response to both Co-Cr-Mo and Ti alloy metalloprotein degradation products. This response was greatest when the metals were complexed with high molecular weight proteins, and with metal-protein complexes formed from Co-Cr-Mo alloy degradation. (Mol Cell Biochem **222**: 127–136, 2001)

Key words: bioreactivity, immune response, implants, biocompatibility, metal protein complex, lymphocyte

Introduction

Metal toxicity is not a phenomena typically associated with orthopedic implants. This may be due in large part to the enormous success of total joint replacements in restoring mobility and quality of life to over a million people worldwide every year. However over the long term (> 7 years), total joint replacements have been associated with adverse local and remote tissue responses. Moreover, while these responses are largely ascribed to particulate-based inflammatory reactions of macrophages the degree to which these reactions are mediated by classical metal toxicity responses, is not known.

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The degradation products of prosthetic biomaterials mediate these adverse effects [1, 2]. A significant amount of biomaterial degradation takes place over time resulting in locally and systemically elevated levels of metal [3–6]. Normal human serum levels of prominent implant metals are shown in Table 1 [3–9]. Homogenates of remote organs and tissue obtained postmortem from subjects with cobalt and titanium base alloy total joint replacement components have indicated that significant increases in Co, Cr, Ti and Al concentrations can occur in the heart, liver, kidney, spleen, and lymphatic tissue (see Table 2) [10–12]. This metallic debris may exist as micrometer to nanometer size particles, colloidal and ionic forms of metal (specifically or non-specifically bound by protein) [13], inorganic metal salts/oxides, and/or in an organic storage form such as hemosiderin. Elevated concentrations of circulating metal derived from orthopedic implant degradation may have direct and accumulatory toxicologic effects over the long term. However, the significance of these circulating metal implant degradation products remains unknown in terms of their short and long term toxicologic/pathophysiologic effects and bioavailability *in vivo*. One effect associated with metal implant components, which until now has remained uncharacterized as a form of metal toxicity, is the unpredictable inducement of overaggressive immunologic responses (specifically of type IV, delayed type hypersensitivity) [14–19] leading to untoward clinical effects such as periprosthetic bone loss. Metals reported as sensitizers have included, Ni, Co, Cr and Ti, all constituents of orthopaedic implants [20]. The immunogenic potential of metal degradation products, which purportedly form hapten or hapten-like complexes with serum proteins, has been previously demonstrated [21, 25].

The specific T cell subpopulations, the cellular mechanism of recognition/activation and the antigenic metal-protein determinants created by these metals remain incompletely characterized. The subsets of participating lymphocytes of nickel sensitive individuals were found to be primarily CD4+ and CD45RO+ cells, whereas CD8+ and CD8+CD11b+ lymphocytes were shown to be underrepresented [26]. Sensitive T-cells have been shown to recognize metals such as nickel in the context of major histocompatibility complex (MHC) Class II molecules [26, 27]. The Langerhans cells of the dermis are well characterized as the primary antigen presenting cells (APC) associated with dermal hypersensitivity. The dominant APC (if any) responsible for mediating an implant related hypersensitivity response remains unknown. Candidate APC's in the periprosthetic region include macrophages, endothelial cells, lymphocytes, langerhans, dendritic cells and to lesser extent parenchymal tissue cells. While there is general consensus implicating the T cell receptor (TCR) in metal induced activation, there are conflicting reports of which region/receptor specificity is responsible for dominating metal reactivity [26–30]. Some investigators report no preferential receptor selection [26], while others have shown the CDR3B region of the VB17+ T cell receptor to be critical in the sense that without this region metal reactivity is abrogated [28, 29]. Metals have also been shown to act as facilitating agents in the crosslinking of receptors (e.g. VB17 of CDR1

Table 1. Approximate average concentrations of metal in human body fluids with and without total joint replacement [3–9]

		Ti	Al	V	Co	Cr	Mo	Ni
		(ng/ml or ppb)						
Serum	Normal	2.7	2.2	<0.8	0.18	0.05	*	0.4–3.6
	THA	4.4	2.4	1.7	0.2–0.6	0.3	*	<9.1
	THA-F	8.1	2.2	1.3	*	0.2	*	*
	TKA	3.2	1.9	<0.8	*	*	*	*
	TKA-F	135.6	3.7	0.9	*	*	*	*
Urine	Normal	<1.9	6.4	0.5	*	0.06	*	*
	TJA	3.55	6.53	<0.4	*	0.45	*	*
Synovial fluid	Normal	13	109	5	5	3	21	5
	TJA	556	654	62	588	385	58	32
Joint capsule	Normal	723	951	122	25	133	17	3996
	TJA	1540	2053	288	1203	651	109	2317
	TJA-F	19173	1277	1514	821	3329	447	5789
Whole blood	Normal	17	13	6	0.1–0.1.2	2.0–4.0	0.5–1.8	2.9–7.0
	TJA	67	218	23	20	110	10	29

Normal – subjects without any metallic prosthesis (not including dental); THA – subjects with well functioning total hip arthroplasty; THA-F – subjects with a poorly functioning total hip arthroplasty (needing surgical revision); TKA – subjects with well functioning total knee arthroplasty; TKA-F – subjects with a poorly functioning total knee arthroplasty (needing surgical revision); TJA – subjects with well functioning total joint arthroplasty; TJA-F – subjects with a poorly functioning total joint arthroplasty (needing surgical revision). *Not tested.

Table 2. Concentrations of metal in body tissue of humans with and without total joint [10–12]

		Cr	Co	Ti	Al	V
		replacements (μg/g)				
Skeletal muscle	Normal	<12	<12	*	*	*
	TJA	570	160	*	*	*
Liver	Normal	<14	120	100	890	14
	TJA	1130	15200	560	680	22
Lung	Normal	*	*	710	9830	26
	TJA	*	*	980	8740	23
Spleen	Normal	10	30	70	800	<9
	TJA	180	1600	1280	1070	12
Psuedocapsule	Normal	150	50	<65	120	<9
	TJA	3820	5490	39400	460	121
Kidney	Normal	<40	30	*	*	*
	TJA	<40	60	*	*	*
Lymphatic tissue	Normal	690	10	*	*	*
	TJA	690	390	*	*	*
Heart	Normal	30	30	*	*	*
	TJA	90	280	*	*	*

TJA – Subjects with a well functioning total joint arthroplasty. *Not tested.

T-cell receptor) to create superantigen-like enhancement of T cell receptor-protein contact [28, 29], whereby metalloproteins or metal-peptide complexes that would not otherwise be antigenic, are able to provoke a response. Furthermore, others have shown that entirely independent of an altered 'self' protein antigen, metal has been reported to crosslink thiols of cell surface proteins of murine thymocytes (i.e. CD3, CD4 and CD45) which have been reported to result in the activation of a tyrosine kinase (p56lck), involved with the activation of T cells through the T cell receptor (TCR) [14, 19, 31, 32]. However, despite reports of non-hapten related mechanisms of metal induced lymphocyte activation, clonal lymphocyte specificity associated with type IV delayed type hypersensitivity remains the dominant mechanism associated with implant related hypersensitivity responses [14, 19, 32]. It remains unknown which metal-protein complexes associated with orthopedic biomaterials are responsible for mediating untoward immunologic effects.

In the current investigation, we hypothesize that metal degradation products are immunologically reactive when complexed with proteins and that this reactivity is a function of the composition of the metal and the binding protein(s). To test this hypothesis, isolated primary healthy human lymphocyte/monocyte populations were treated with 11 molecular weight ranges of fractionated serum preconditioned with Co-Cr-Mo alloy (ASTM F-75) and titanium alloy (Ti-6Al-4V, F-136) degradation products. Lymphocyte bioreactivity (as measured by lymphocyte proliferation) to serum protein fractions with and without metal degradation products was then determined as was the total protein within serum fractions and the concentration of Cr and Ti within these serum fractions.

Materials and methods

Serum and lymphocytes were obtained from 10 individuals (5 female, 5 male, average age 34, range 23–59) by peripheral venipuncture after obtaining (Internal Review Board approved) informed consent. All subjects were healthy, with no history of metal allergy and no history of receiving a metal implant (other than dental). Serum samples were allowed to complex with metal released from Co-Cr-Mo and Ti alloy degradation, then separated based on molecular size into 11 fractions. These fractions were tested for Cr or Ti metal content, concentrated, and used in cell cultures of lymphocytes isolated from the same subject. Lymphocyte responses were then measured using a proliferation (or lymphocyte transformation) assay.

Blood collection and cell isolation

Human primary lymphocytes and monocytes were isolated from 30 ml of peripheral blood obtained by venous puncture

using a 30 ml heparinized syringe (10 units/ml blood). Density gradient separation (Ficoll-isopaque, Pharmacia, Piscataway, NJ, USA) was used to obtain mononuclear leukocyte cell fractions from heparinized whole blood, yielding between approximately 15–30 × 10^6 mononuclear leukocytes per 30 milliliters of heparinized blood collected per subject.

Metal-protein complex formation

Metal-protein complexes were produced by incubating 0.5 ml of spherical metal particles (70 μm diameter, Starmet Corp., Concord, MA, USA) in 4 ml of serum under constant rocking motion (LabQuake, Sigma Co.) at 37°C for 1 week. For each subject, 4 ml of serum was used to incubate with titanium alloy (Ti6Al4V, ASTM F-136) and Co-Cr-Mo alloy (ASTM F-75) beads (70 μmm diameter). Residual colloidally suspended metal particles were removed from the serum samples using centrifugation at 8,000 rpm for 30 min (Sorvall, DuPont) and sterile filtration through a 0.22 μm filter. Metal-treated and control untreated serum samples were fractionated using FPLC and analyzed for metal content.

Serum fractionation

Serum protein samples were separated into 11 fractions based on molecular size, using fast-protein-liquid-chromatography (FPLC). FPLC, using an all polymer environment, was selected for serum protein separation to minimize metal contamination by processing. The FPLC system was verified to be contamination free through preliminary testing of the eluent. A P-500 Pump (Pharmacia, Piscataway, NJ, USA) was used to pump 0.5 ml samples of serum through two 24 ml Superdex™ 200 columns (Pharmacia, Piscataway, NJ, USA) mounted in series at a flow rate of 5 ml/h, using double deionized water (> 18 MOhms) from a Milli-Q water system (Millipore, Bedford, MA, USA). Other Ultrex grade eluent buffers such as KPO_4 and NaOH were found to contain metal within the parts per million range, precluding their use in these studies. Detection of protein exiting the columns was carried out using ultra-violet absorbence monitoring (UV-1 cell, Pharmacia, Piscataway, NJ, USA). High molecular weight standard protein calibrants (Pharmacia, Piscataway, NJ, USA) were used both with FPLC and in conjunction with polyacrylamide gel electrophoresis to determine the approximate molecular weight of prominent proteins within each FPLC fraction. Metal content within FPLC serum fractions was analyzed using a graphite furnace Zeeman atomic absorption spectrophotometer (GFZ-AAS) (Perkin-Elmer, Norwalk, CT, USA). Serum fractions of 2.5 ml were concentrated using 5 kDa concentration tubes (Centricon, Millipore) at 2000 rpm for 30 min, for a final volume of 50 μl. The method detection limit after FPLC was 0.6 ng/ml for Cr and 40 ng/ml for Ti (detection limits in undiluted serum are 0.03 ng/ml for Cr and 2.0 ng/ml for Ti) [3–6]. All collection containers and apparati were triple acid-washed with Ultrex-grade chemicals (Baker, Chicago, IL, USA) or verified to be contamination free by AAS.

The analysis of metal content within fractionated and unfractionated serum was limited to Cr and Ti as representative metals of Co-Cr-Mo and Ti alloy degradation, respectively. These metals were chosen as representatives because they were the two most prominently elevated metals of each alloy detected in the serum of patients with normally functioning and failed total joint replacements (see Table 1). Other metals such as Co, although more prominent in the composition of Co-Cr-Mo alloy (see Table 3), were not as elevated as Cr in the serum of TJR patients, presumably due to high levels of clearance from the serum.

Proliferation assay (lymphocyte transformation tests)

All of the 15–30 × 10^6 mononuclear leukocytes obtained from each patient were used for proliferation assay analysis. This limited biologic reactivity analysis to this single assay. Proliferation of cells was measured by [^{3}H]-thymidine (Amersham International, Arlington Heights, IL, USA) incorporation into DNA in a 96-well microplate system. The amount of proliferation resulting from each treatment was quantified using the average of three radioactivity incorporation values. This average for each treatment was normalized to that of the negative control (no treatment) producing a ratio, generally termed either a proliferation factor, proliferation index, proliferation ratio or stimulation index, SI. In this paper this ratio will be referred to as the stimulation index (SI). The SI was used to compare lymphocyte reactivity to the two different

Table 3. Weight percent of different metals within the three most prominent implant alloys

Alloy	% Ni	% Ta	% Co	% Cr	% Ti	% Mo	% Al	% Fe	% Mn	% Cu	% V
Stainless steel (ASTM F138)	13–15.5	*	*	17–19	*	2–4	*	Balance	*	0.5	*
Co Alloy (ASTM F75)	1	*	Balance	27–30	*	5–7	*	*	1	*	*
Ti Alloy (ASTM F136)	*	*	*	*	Balance	*	5.5–6.5	*	*	*	3.5–4.5

*indicates less than 0.1.

metals as well as the metal-protein complexes. The lower limit of this stimulation index is zero indicating all cells stopped dividing before addition of [^{3}H]-thymidine, after 5½ days.

Proliferation assays were performed using Ficol separated mononuclear/lymphocyte cell fractions collected from 30 milliliters of peripheral blood per subject. These lymphocytes were cultured in 96-well cell-culture plates (Sigma), at a density of 0.1–0.3 × 10^6 cells/well for a period of 6 days in 150 µL of DMEM/well, 10% autologous serum at 37°C and 0.5% CO_2, with either metal treatments (i.e. 15 µl/well of fractionated and concentrated metal treated serum), a positive control (0.01 mg/ml PHA) and a negative control (untreated). Each treatment was conducted in triplicate (3 wells/treatment). Approximately 72 wells of a 96-well plate were used per subject (11 treatments in triplicate for Co- and Ti-alloy treated serum, 3 positive and negative controls). [^{3}H]-thymidine was added during the last 12 h of incubation after 5½ days of treatment. At day six [^{3}H]-thymidine uptake (1 µCi/culture well) was measured using liquid scintillation. The SI was calculated using measured radiation counts per minute (cpm):

$$\text{Simulation Index} = (\text{mean cpm with treatment})/(\text{mean cpm without treatment}) \quad (1)$$

Six days of incubation were chosen to reproduce, *in vitro*, the time lag associated with *in vivo* lymphocyte proliferation in a DTH response. Radiolabeled lymphocytes were collected onto membranes using a cell harvester (Tomtech Mach 2, Orange, CT, USA) and the amount of differential radiation incorporation was measured using liquid scintillation (Wallac 1205 Betaplate, Gaithersburg, MD, USA).

Total Protein Assay

Assessment of total protein was conducted on serum post fractionation using Pierce Protein reagents (Rockford, IL, USA). Total protein assays were conducted using 10 µl of sample with 200 µl of substrate in 96-well plates, with serial dilutions of bovine serum albumin (Sigma) used as standard calibrants on every plate. The method detection limit was approximately 5 mg/ml total protein.

Statistical analysis

Metal content analysis

By convention, to calculate group means, Cr or Ti concentrations below the detection limit were expressed as one-half the method detection limit. Intergroup comparisons, independent of these means, were made using Kruskall-Wallis non-parametric analysis of variance. The Wilcoxon-Mann-Whitney test was then used if the Kruskall-Wallis test revealed significant differences at $p < 0.05$.

Lymphocyte reactivity

Measured data were subjected to statistical analysis using Student's *t*-tests. Student's *t*-tests for independent samples with unequal or equal variances were used to test equality of the mean values at a 95% confidence interval ($p < 0.05$). Comparisons between group reactivities are limited to individual comparison of reactivity at each specific molecular weight ranges. All treatment specific reactivity measurements were determined to be normally distributed.

Results

Serum separation

Eleven fractions of sequential molecular weight ranges were collected from each subject's serum, after treatment with Co-Cr-Mo alloy beads, Ti alloy beads or no metal. The average amount of total protein within each fraction (as measured in pretreated fractionated serum of all 10 subjects) is shown in Fig. 1. Note that over 80% of all serum protein was contained within fractions from 32–77 kDa while 15% was within the 180–250 kDa fraction. The greatest amount of total protein was detected in the 60–68 kDa fraction, which contained over 50% of the serum protein.

Chromium levels within serum

Cr levels were determined within the Co-Cr-Mo alloy treated and non-treated serum fractions of all subjects. The levels of Cr were used as an indicator of which protein fraction(s) preferentially bind soluble metal released from Co-Cr-Mo alloy degradation and to what degree. The average Cr level within unfractionated serum was 680 ng/ml (ppb) after treated with metal beads. The Cr levels within treated fractionated serum were measured and averaged for all subjects and compared to those from non-treated fractionated serum from the same individual (Fig. 2). Chromium released from Co-Cr-Mo particles exhibited a bimodal binding pattern, binding to both low molecular weight protein(s) (< 32 kDa), and to higher molecular weight protein(s) in the 180–330 kDa range (Fig. 2). Cr values within the nontreated serum fractions were two orders of magnitude lower than the treated serum concentrations and demonstrated less distinct peaks in protein binding. Levels of Cr within the serum treated fractions were statistically elevated above that of the nontreated serum fractions (as indicated in Fig. 2). Serum proteins of approximately 77–180 kDa did not appear to bind Cr degradation products.

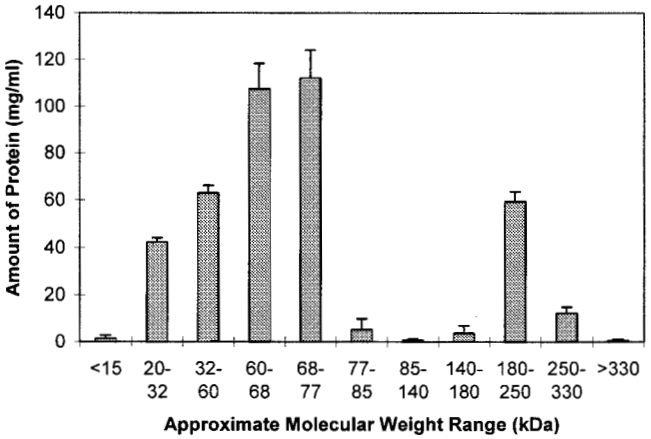

Fig. 1. Mean of total protein content within each of the molecular weight-based serum fractions of all subjects after fast protein liquid chhromatography. Note: The method detection limit is approximately 5 mg/ml. Note: error bars represent S.D.

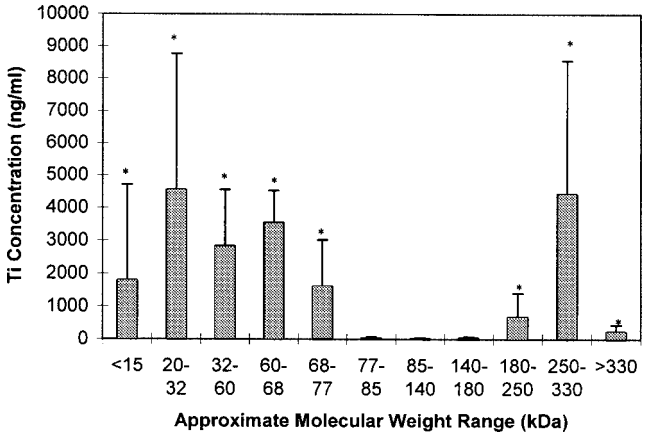

Fig. 3. Average Ti content in serum fractions after Ti-alloy bead treatment. There is a bimodal pattern of protein binding. The Ti concentration within each fraction of non-treated serum was below the method detection limit (40 ng/ml Ti). Note: *indicates significant increase above nontreated fractions (below the detection limit, not shown) at $p < 0.05$. Error bars represent S.D.

Titanium levels within serum

Titanium levels within each of the serum fractions after treatment with Ti alloy beads demonstrated similar distribution to that of Cr with a bimodal pattern of Ti binding (Fig. 3). The average Ti level within unfractionated serum was 1,700 ng/ml (ppb) after treated with Ti alloy metal beads. Ti levels within all nontreated samples, both pre- and post-fractionation, were below the method detection limit of the atomic adsorption spectrometer (< 40 ng/ml Ti post FPLC). Levels of Ti within the 15–77 and 180–330 kDa fractions were statistically elevated above that of the nontreated serum fractions as indicated in Fig. 3. The greatest levels of Ti were concentrated within the low to mid-molecular weight ranges (15–77 kDa) containing approximately 4800 ng/ml. These concentrations of Ti were over three orders of magnitude above that of nontreated serum. Serum proteins of approximately 77–180 kDa did not bind Ti, in a similar pattern as that found with Cr released from Co-Cr-Mo implant degradation.

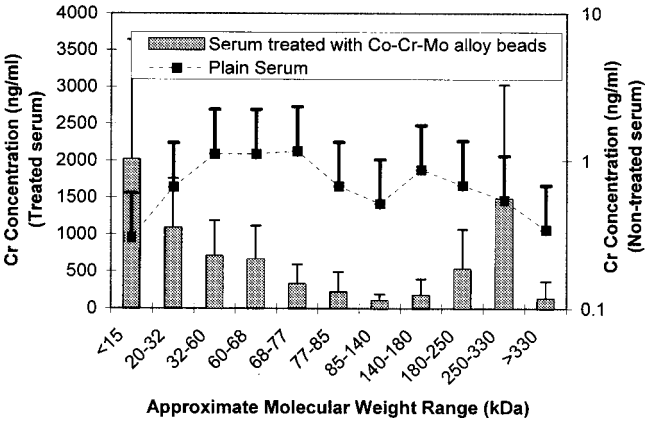

Fig. 2. Comparison of the average Cr content of serum fractions both with and without Co-Cr-Mo alloy bead. A bimodal pattern of binding can be seen for Cr in the Co-Cr-Mo alloy treated serum fractions. Note: The method detection limit is 0.6 ng/ml Cr. All treated fractions had significantly greater Cr than non-treated fractions at a $p < 0.03$. Error bars represent S.D.

Lymphocyte reactivity

The stimulation index for both Co-Cr-Mo and Ti alloy treated fractions were averaged for all subjects and the resulting pattern of activation is shown in Fig. 4. A stimulation index greater than 1.0 indicates lymphocyte activation (i.e. proliferation greater than that of non-treated control serum fractions), while a stimulation index less than 1.0 indicates lymphocyte suppression. Generally, lymphocytes demonstrated greater reactivity to: (1) serum fractions with metal compared to those without, (2) high molecular weight serum fractions (140–330 kDa), and (3) serum fractions with Co-Cr-Mo degradation products as compared with those with Ti alloy degradation products (Fig. 4). Lymphocytes were most greatly stimulated by serum fractions with Cr at 140–330 kDa. All subjects lymphocytes demonstrated increased reactivity to 0.01 mM PHA, exhibiting a range of 10–100 fold increase in thymidine incorporation (cpm) compared to nontreated lymphocytes from the same individual. PHA induced an average SI of 20 (Fig. 4).

Activated lymphocytes demonstrated the greatest proliferation (SI) response to higher molecular weight serum fractions within the 140–330 kDa range, Fig. 4. However, the stimulation index used to measure this proliferation does not take into account the amount of metal or protein within each serum fraction. Normalizing for the amount of metal and pro-

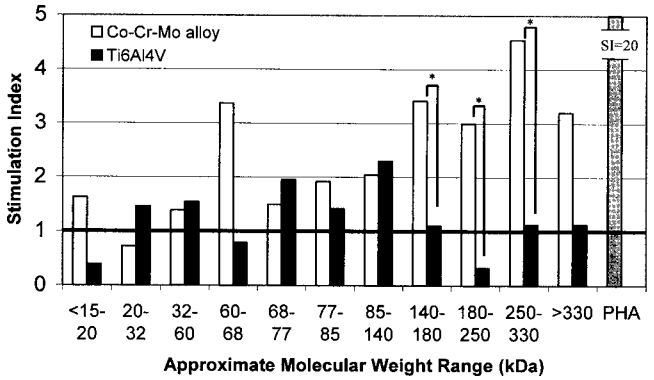

Fig. 4. The mean stimulation index of lymphocytes to autologous serum fractions which were treated with Co-Cr-Mo and Ti alloy beads and compared to nontreated serum fraction. An SI greater than 1.0 indicates a proliferation response. Serum treated with Co-Cr-Mo alloy beads demonstrated significantly greater reactivity within the ranges of 140–330 kDa, *indicates $p < 0.05$, Student's *t*-test. Note: Phytohemaglutin (PHA) was used as a positive control and resulted in an SI = 20, as indicated.

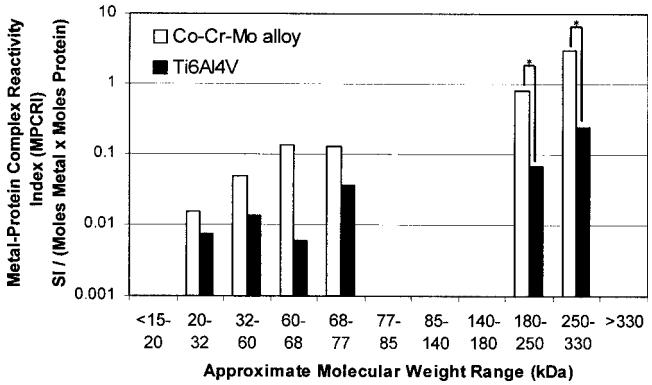

Fig. 5. A comparison of the Metal-Protein Complex Reactivity Index, (MPCRI) for all subjects (n = 10) in reaction to autologous serum fractions which were (1) non-treated, (2) Co-Cr-Mo alloy treated and (3) Ti alloy treated. Note: This is an approximate measure of reactivity based on an average molecular weight determined for each serum fraction and an average of total protein content within that fraction. The MPCRI could not be calculated where concentrations of metal or protein were below method detection limits (i.e. serum fractions < 15, 77, 85, 140, and > 330 kDa). Note: *indicates $p < 0.05$, Student's *t*-test.

tein within each serum fraction facilitates comparative analysis between metal-protein complexes comprised of either different serum proteins or different metals. Therefore, a measure of stimulation/activation was calculated to adjust for both the number of moles of metal and the corresponding moles of protein within the treated fractions. This measure, the Metal-Protein Complex Reactivity Index, (MPCRI), was calculated as shown below.

$$\text{MPCRI} = \frac{\text{(Stimulation index)}}{\text{(Molar concentration of metal moles/ml} \times \text{Molar concentration of protein moles/ml)}} \quad (1)$$

The MCPRI values for Cr and Ti treated lymphocytes are shown in Fig. 5. The MPCRI (Fig. 5) differs from that of the SI (Fig. 4) in the relative levels of activation of Cr and Ti and for both low and high molecular weight proteins. The MPCRI demonstrates less reactivity for midrange proteins (such as albumin and transferrin) due to the large amount of total protein in fractions 3, 4, and 5, (32–77 kD). Cr containing higher molecular weight proteins, dominated by immunoglobulins, are associated with the greatest reactivity.

Discussion

This study demonstrates that metal-protein-complexes produced from the degradation of metal implant alloys can be immunologically reactive *in vitro* in that they are capable of inducing lymphocyte activation through proliferation responses. This hyper-reactivity as a severe or subtle chronic immune response has the potential to promote local and systemic toxicity. Moreover, different serum protein fractions and different metals demonstrate differential patterns of bioreactivity. The specific mechanisms by which metal degradation products activate lymphocytes remains unclear. The three current non-mutually exclusive mechanisms by which metal-protein complexes can activate lymphocytes are: (1) antigen-independent, (2) antigen-dependent and (3) superantigen-like, i.e. a synergistic combination of the first two mechanisms [14, 19]. Experimental support of all three mechanisms has been previously described [14, 19].

Independent of antigen

Metal has been reported to cross-link thiols of cell surface molecules of murine thymocytes (i.e. CD3, CD4 and CD45) which resulted in the activation of a tyrosine kinase (p56lck), involved with the activation of T cells through the T cell receptor (TCR). In addition to these reports of polyclonal activation, other reports implicate anti-chemotactic non-specific immune suppression [14, 19, 32–40].

Antigen dependent metal activation

Metal specific T-cell reactivity remains the primary mechanism purportedly associated with metal-lymphocyte activation [14, 19, 32, 38, 39, 41]. Implant related hypersensitivity reactions have long been considered to be a cell mediated delayed type IV hypersensitivity [25, 50–54], characterized by antigen activation of sensitized T_{DTH} lymphocytes releasing various cytokines which result in the accumulation and activation of macrophages. T_{DTH} lymphocytes are subset

populations of T helper (T_H,) lymphocytes purported to be of the CD4+ T_{H-1} subtype (and in rare instances CD8+, cytotoxic T cells, T_C). In this scheme a metal combines with a self-protein and becomes antigenic after being processed by a macrophage or other antigen presenting cell, (APC). Metal implant alloy degradation products as moieties in immunoactive complexes have been shown to be antigenic in many case studies through temporal association with specific responses such as severe dermatitis, urticaria, vasculitis [23, 35, 36, 42–48]. Additionally a large number of cohort investigations have indicated a correlation between metal implants and metal hypersensitivity reactions [25, 49–60].

Combination of antigen dependent and independent mechanisms

Metals may act with serum proteins to crosslink lymphocyte receptors (e.g. BV17 of CDR1 T-cell receptor) without the presence of an antigen presenting cell, i.e. in a superantigen enhancement of T cell receptor-protein contact [28, 29]. In this circumstance proteins or peptides that would not otherwise be antigenic are able to provoke a response. While this hypothesis has not been extensively supported, the lymphocyte reactions in the current investigation are consistent with such nonspecific mitogenic or 'polyclonal' activation mechanisms (i.e. nonspecific to a single or small population of clonally 'sensitized' or differentiated lymphocytes).

Metal-protein complex induced activation of primary human lymphocytes

The MPCRI is an approximate measure of metal-protein bioreactivity based on an average molecular weight determined for each serum fraction, the moles of metal measured in each fraction, and moles of protein within that fraction. For this analysis it was necessary to assume that all metal was bound to serum proteins and that all binding was between serum proteins and metal ions, not nanometer size particles. This measure of metal-protein reactivity could not be calculated where concentrations of metal or protein were below the method detection limit. Figure 5 demonstrates the utility of this index highlighting the higher reactivity associated with metal-protein complexes containing Cr and the greater reactivity of higher molecular weight metal-protein complexes.

Earlier investigations have indicated that metal degradation products may elicit an immune response and combine with immunoglobulins. Yang *et al*. [21] have shown binding of humoral antibodies (i.e. IgG, IgE, IgM and IgA) to metal complexed with glutathione (GSH) suggesting that humoral hypersensitivity reactions may be induced by elevated levels of circulating metal. However, this has not been demonstrated in a human model. The elevated MPCRI of both Cr and Ti containing high molecular weight fractions (which contain predominantly immunoglobulins) while consistent with the findings of Yang *et al*., suggests a cell mediated response which is either antigen specific (monoclonal stimulation), antigen independent (polyclonal stimulation) or a combination of the two. While not typical of a DTH response, a (clonally) nonspecific activation of lymphocytes does not rule out presence of metal-specific T-cells. However, at present there is incomplete knowledge concerning the degree of metal induced hypersensitivity in patients with total joint replacements and the role which specific T-cells, metal-protein binding and humoral immunity plays in the host response and clinical outcome. The results of this study demonstrate that the most bioreactive metal-protein complexes are those within the higher molecular weight immunoglobulin range.

The majority of released implant metal within and around the joint space and remote from the implant may take the form of particles. Particulate debris is difficult to differentiate from other forms of metal (i.e. soluble metal-protein complexes) when using analytical techniques such as atomic absorption spectroscopy alone [10–12]. Nevertheless, the overlap in the concentration range at which metal ions provoked lymphocyte activation in the current investigation and that reported locally around implants are consistent with the hypothesis that adverse local and remote tissue responses associated with released implant metal particles may be due in part to immunogenic metal ions released from implants.

Conclusions

In this investigation, we have identified two molecular weight ranges of human serum proteins associated with the binding of Cr and Ti from Co-Cr-Mo and Ti implant alloy degradation as potentially toxic. High molecular weight serum proteins (≈ 180 kDa) demonstrated greater lymphocyte bioreactivity when complexed with Cr and Ti than low to midrange (30–77 kDa) serum proteins. This *in vitro* model demonstrates immunogenic potential of both Co-Cr-Mo and Ti alloy degradation products complexed with high molecular weight proteins. A quantifiable measure of metal-protein complex reactivity (MPCRI), demonstrated Co-Cr-Mo alloy degradation products were associated with approximately an order of magnitude greater lymphocyte reactivity than Ti alloy products in the higher molecular weight ranges which are dominated by immunoglobulins (≈ 180–330 kDa). Metal sensitivity may exist as an extreme complication in only a few highly susceptible patients (i.e. less than 1% of joint replacement recipients), or it may be a more common subtle contributor to implant failure. In addition to direct immunogenic responses, metal degradation products may mediate indirect immunologic effects due to immune cell toxicity. Mecha-

nisms by which *in vivo* metal sensitivity occurs have not been well characterized [61, 62]. Thus, we put forward the view of metal implant degradation products as potentially toxic, albeit not by classical interference with cell functionality but through indirect and undesired activation of lymphocytes which then leads to untoward peri-implant and systemic effects. The importance of this line of investigation is growing, as the use of metallic implants is increasing and as expectations of implant durability and performance increase [63, 64].

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