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A preliminary evaluation of immune stimulation following exposure to metal particles and ions using the mouse popliteal lymph node assay

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ABSTRACT

The objective of this preliminary study was to evaluate the threshold for immune stimulation in mice following local exposure to metal particles and ions representative of normal-functioning cobalt-chromium (CoCr) metal-on-metal (MoM) hip implants. The popliteal lymph node assay (PLNA) was used in this study to assess immune responses in BALB/c mice following treatment with chromium-oxide (Cr_2O_3) particles, metal salts ($CoCl_2$, $CrCl_3$ and $NiCl_2$), or Cr_2O_3 particles together with metal salts using single-dose exposures representing approximately 10 days (0.000114 mg), 19 years (0.0800 mg), and 40 years (0.171 mg) of normal implant wear. The immune response elicited following treatment with Cr_2O_3 particles together with metal salts was also assessed at four additional doses equivalent to approximately 1.5 months (0.0005 mg), 0.6 years (0.0025 mg), 2.3 years (0.01 mg), and 9.3 years (0.04 mg) of normal implant wear. Mice were injected subcutaneously (50 μL) into the right hind foot with the test article, or with the relevant vehicle control. The proliferative response of the draining lymph node cells (LNC) was measured four days after treatment, and stimulation indices (SI) were derived relative to vehicle controls. The PLNA was negative (SI < 3) for all Cr_2O_3 particle doses, and was also negative at the lowest dose of the metal salt mixture, and the lowest four doses of the Cr_2O_3 particles with metal salt mixture. The PLNA was positive (SI > 3) at the highest two doses of the metal salt mixture and the highest three doses of the Cr_2O_3 particles with the metal salt mixture. The provisional NOAEL and LOAEL values identified in this study for immune activation corresponds to Co and Cr concentrations in the synovial fluid approximately 500 and 2000 times higher than that reported for normal-functioning MoM hip implants, respectively. Overall, these results indicate that normal wear conditions are unlikely to result in immune stimulation in individuals not previously sensitized to metals.

1. Introduction

In the late 1990s, second generation metal-on-metal (MoM) hip implants became available in the United States as an alternative to metal-on-polyethylene (MoP) hip implants (Hart et al., 2015). This second generation of MoM hip implants offered increased stability, lower volumetric wear, and decreased wear-related osteolysis compared with MoP implants available at that time (Fehring et al., 2015). These devices also provided treatment alternatives for end-stage osteoarthritis in elderly individuals, and were recommended to younger patients hoping to resume an active lifestyle because of their increased resistance to dislocation, as well as, their greater range of hip motion (Bozic et al., 2009; Bucholz, 2014; Drummond et al., 2015; Girard et al., 2010; Hart et al., 2015; Matharu et al., 2015). In 2006, MoM was the most common bearing-type implanted for patients <65 years of age in the United States, with 42% of all primary total hip replacements being performed with MoM devices (Bozic et al., 2009; Bucholz, 2014; Hart et al., 2015). It has been estimated that in total, approximately 1 million MoM hip implants have been implanted worldwide (Hart et al., 2015; SCENIHR, 2014).

The articulating parts of modern MoM hip implants are comprised primarily of cobalt-chromium (CoCr) alloys, in which Co, Cr, and Ni constitute approximately 64%, 28%, and ≤1% of the alloy composition, respectively (ASTM, 2011). It is well understood that all implanted metal alloys, including CoCr alloys, corrode and wear over time (to various degrees), resulting in the release of "wear debris," which is a mixture of metal particles and solubilized metal ions (i.e., Co^{2+}, Cr^{3+}, and Ni^{2+}) (Bauer et al., 2014; Hallab et al., 2001). For normal-functioning

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CoCr MoM hip implants, the volumetric wear rate is <1 mm^3 per year and the majority of the debris is nano-sized (<100 nm) chromium oxide (Cr_2O_3) particles that are round, oval-shaped, or needle-shaped (Madl et al., 2015a, 2015b).

Blood Co and Cr levels in patients with normal-functioning implants are typically about 2–10 times higher than the "background" concentrations present in the general population. Several recent studies have shown that there are negligible systemic health risks associated with the slightly increased blood Co and Cr concentrations reported in patients with normal-functioning implants (Finley et al., 2012a, 2012b; Finley et al., 2013; Monnot et al., 2014; Tvermoes et al., 2014, 2015). However, it is less clear whether low levels of wear debris might result in adverse effects in the periprosthetic tissue of patients with MoM hip implants. Histological evaluation of periprosthetic tissue retrieved at revision surgery of some failed MoM hip implants has revealed a cell mediated immune response characterized by (T and B) lymphocyte infiltration and lymphoid aggregates (primarily CD3+/CD4+ T cells), as well as, the accumulation of plasma cells associated with macrophages containing metallic wear-debris particles in some cases (Campbell et al., 2010; Davies et al., 2005; Willert et al., 2000). Accordingly, it has been hypothesized that metal sensitization may play a role in influencing prosthesis performance, and may contribute to the etiology of certain types of MoM hip implant failures (Campbell et al., 2010; Davies et al., 2005; Hallab et al., 2001; Pinson et al., 2014; Cook et al., 1991). However, the presence of leukocytes, and many of the histological features described in association with adverse local tissue reactions, have been found to be associated with other types of implant failures (Cook et al., 1991; Mirra et al., 1982). Further, questions as to whether allergic sensitization to metal plays a direct role in the failure of the prosthesis, or if loosening of the prosthesis can induce metal allergy as a result of excessive debris generation, have not been fully resolved (Jacobs et al., 2009; Pinson et al., 2014; Watters et al., 2010).

"Sensitization" in this case refers to the induction of a T-cell-mediated immune response to metals such a Co, Cr and Ni (Campbell et al., 2010; Schmidt and Goebeler, 2015; Thomas and Cunningham, 2009; Wang and Dai, 2013). Metal sensitization develops in two phases. The first phase, known as the induction phase, is characterized by immunological priming that results in the acquisition of sensitization. The second phase, or the elicitation phase, occurs when an already sensitized individual is exposed to the same allergen and responds immunologically. The elicitation phase is marked by a more rapid and robust immune response resulting in an allergic reaction. The induction and elicitation phases have different dose-response relationships, and therefore, different thresholds, with the induction of sensitization commonly requiring exposure to higher levels of a particular allergen than elicitation.

Historically, several animal models have been used to evaluate the sensitization potential of medical devices, including the guinea pig maximization test (GPMT), the occluded patch test of Buehler in guinea pigs, and the mouse local lymph node assay (LLNA) (*ISO*, 2010). However, these assays were developed primarily to evaluate skin sensitization potential, and they may not reflect accurately immune reactions within the body that may result from exposures such as those associated with MoM hip implants. The popliteal lymph node assay (PLNA) is an appropriate alternative to the LLNA and guinea pig assays for the assessment of systemic sensitization (rather than skin sensitization) potentially associated with implanted medical devices (e.g., deep tissue exposures) (*ISO*, 2010; WHO, 1999). The PLNA was developed originally for assessing the sensitizing potential of systemically administered drugs (USFDA, 2002), and for this purpose, the assay has been found to be generally reliable, although it has not been used extensively to evaluate the sensitizing potential of metals and/or metal particles (WHO, 2006).

Currently, no information exists regarding the dose of MoM wear debris necessary to induce an internal (i.e., deep tissue) sensitization response. This has not previously been investigated in any detail since, historically, there has been only a low incidence of potential hypersensitivity reactions reported in MoM hip implant patients (Nasser, 2007). While literature is available on threshold doses of Co, Cr, and Ni ions that induce skin sensitization, or elicit allergic contact dermatitis, these data are not necessarily applicable to the threshold dose of metal required to induce or elicit sensitization responses in deep tissue.

The purpose of this study, then, was to investigate the utility of the mouse PLNA as a model for evaluating the induction threshold for immune stimulation associated with exposure to metal particles and ions representative of wear debris generated by normal-functioning MoM hip implants. To this end, the stimulation index (SI) of lymph node cell (LNC) activation was measured and flow cytometric analysis of PLN lymphocyte subsets was performed to evaluate the sensitizing potential of metal particles and/or metal ions injected subcutaneously.

2. Materials and methods

2.1. Animals

Female, nulliparous, experimentally naïve BALB/c mice (Charles River Laboratories), aged 6–8 weeks were group housed in metal-free, disposable plastic cages conforming to the Guide for the Care and Use of Laboratory Animals recommendations (Gerber et al., 2011). The animal room was temperature controlled (range: 68.6 ± 71.0 °F), and equipped with a 12-h light/dark cycle. Mice were acclimatized at least five days prior to initiation of the experiment. Rodent chow and distilled water were available *ad libitum*. All procedures involving laboratory animals and test articles complied with acceptable standards of animal welfare and humane care by the Institutional Animal Care and Use Committee (IACUC) of MB Research (Spinnerstown, PA) or Calvert Laboratories (Olyphant, PA).

2.2. Chemicals and reagents

Nickel chloride ($NiCl_2 \cdot 6H_2O$, CAS # 7791-20-0), chromium chloride ($CrCl_3 \cdot 6H_2O$, CAS # 10060-12-5), cobalt chloride ($CoCl_2 \cdot 6H_2O$, CAS # 7791-13-1), chromium oxide particles (Cr_2O_3, CAS #1308-38-9), 2,4-dinitrochlorobenzene (DNCB, CAS #97-00-7), 2,4-dichloronitrobenzene (DCNB, CAS #611-06-3), sodium dodecyl sulfate (SDS, CAS # 151-21-3), bromodeoxyuridine (BrdU) and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich. TiO_2 particles (TiO_2, CAS # 1317-70-0) were purchased from US Research Nanomaterials, Inc. Potassium dichromate ($K_2Cr_2O_7$, CAS # 7778-50-9) was purchased from Fisher Scientific and gold chloride ($AuCl_3$, CAS # 13453-07-1) was purchased from Acros Organics (Fair Lawn, New Jersey). Phosphate buffer saline (PBS) was purchased from Hyclone and syngeneic vehicle BALB/c mouse serum was obtained from Charles River Laboratory (Raleigh, NC). All antibodies used for flow cytometric analyses were obtained from BD Pharmingen (San Jose, CA) or Acris Antibodies (San Diego, CA).

2.3. Characterization of metallic particles

The size of the Cr_2O_3 particles and TiO_2 particles were determined by RJ Lee Group (Monroeville, PA) using a Hitachi S5500 Ultra-high Resolution Scanning Electron Microscope at an accelerating voltage of 2.0 kV. Secondary electron contrast was used to maximize the surface definition of the particles to aid in sizing measurements. Diameters were measured using the longest chord joining points on the observable perimeter of the particle. Since these particles were sourced commercially, composition of the particulate was periodically checked using a Bruker energy dispersive spectroscopy (EDS) detector at an accelerating voltage of 20 kV. For sterilization, all particles were autoclaved at 127 °C for 30 min. The test particles were shown to be free of endotoxin using the Gel-Clot assay, which has a sensitivity level of 0.03 EU/mL.