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Various cells of the immune system and intestine differ in their capacity to reduce hexavalent chromium

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Abstract

The cells of the immune system form a strong line of defence against foreign substances. The present study was undertaken to investigate the capacity of different cells of Wistar rats to reduce potentially carcinogenic hexavalent chromium (Cr-VI) into less toxic trivalent chromium in vitro. 5×10^6 cells were incubated with 10 or 25 μg ml^{-1} of Cr (VI) in the form of $K_2Cr_2O_7$ at 37°C in the presence of 5% CO_2 in air. At various time periods the remaining amount of Cr (VI) was measured and the percentage of Cr (VI) reduced was calculated. Among the single cell suspensions from the splenic cells a peak reduction of 55% was observed with the total spleen cells, 40% with the B-lymphocyte-enriched subpopulation, 10% with T-lymphocytes and 24% with the macrophages. The reduction by splenic and peritoneal macrophages was similar. Total thymocytes reduced 54% of the Cr (VI). Since the most common route of entry of chromium is through drinking water and food, intestinal cells were also investigated. Among the intestinal cells the maximum reduction of 100% (of 10 μg ml^{-1}) was observed with the upper villus cells and 72% with the middle villus cells while reduction was the least (4%) with the crypt cells. The reduction in the intestinal loop in situ was 100%. The time taken by each cell type for the peak reduction to Cr (VI) was markedly different. The findings thus show that the capacity of different cells in the body differs vastly in their capacity and time taken to reduce hexavalent chromium. The most efficient handling of Cr (VI) by the intestine, due to the presence of a variety of cells and bacteria, protects the body from its adverse effects.

Keywords: Chromium (VI) reduction; Macrophage; T-Lymphocyte; B-Lymphocyte; Intestine; Intestinal villus cell; Intestinal crypt cell

1. Introduction

Environmental contamination by chromium (Cr) is common in industrialized areas. Chromium pollution as a consequence of effluent discharge from tanneries and other industries, which include metal plating, manufacturing industries and ferrochrome production, poses a serious problem for environmental quality. In aqueous environments chromium has two oxidation states: hexavalent chromium (Cr-VI) and trivalent chromium (Cr-III). Cr (VI) compounds are generally soluble over a wide pH range and have been shown to exert toxic and carcinogenic effects in humans and experimental animals [1–5]. In addition, Cr (VI) compounds also induce DNA damage such as DNA single-strand breaks and DNA–protein crosslinks in vivo and in cultured cells [6]. Cr (VI) compounds are much more toxic than those of Cr (III) due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids. Non-biodegradability of heavy metals is responsible for their persistence in the environment and their subsequent bioaccumulation in the food chain. The presence of chromate in the environment inhibits most microorganisms, but also promotes the selection of resistant bacteria [7].

Chromium enters the body through the lungs, gastrointestinal tract, and to a lower extent through skin [8]. Inhalation is the most important route for occupational exposure, whereas non-occupational exposure occurs via ingestion of chromium-containing food and water [9,10]. Regardless of the route of exposure Cr (III) is poorly absorbed whereas Cr (VI) is more readily absorbed [8], although less well by the oral route and thus is not very

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toxic when introduced by this route [11]. In contrast, chromium is very toxic by dermal and inhalation routes and causes lung cancer, nasal irritation, nasal ulcer, hypersensitivity reactions and contact dermatitis. Most of the chromium absorbed by inhalation exposure, in comparison to oral administration, is distributed in the lungs, liver, kidneys, red blood cells, plasma, spleen and bone marrow [9]. All the ingested Cr (VI) is reduced to Cr (III) before entering the blood stream [12]. Cr (VI) enters the cells through membrane anionic transporters while Cr (III) does not. Intracellular Cr (VI) is metabolically reduced to the ultimate Cr (III). It is therefore in the interest of the body to reduce the toxic Cr (VI) to the less toxic form, Cr (III). The cells of the immune system form a strong line of defence against foreign substances and the most common route of entry of chromium is through drinking water and food. The present study was, therefore, undertaken to investigate the capacity of different cells of the immune system and intestine of Wistar rats to reduce Cr (VI).

2. Materials and methods

2.1. Animals

The study was carried out on Wistar rats weighing 200–250 g and maintained in the Animal House of the Institute.

2.2. Preparation of spleen cells

The rats were sacrificed by cervical dislocation and the spleens were removed aseptically. The spleen cells were teased out in cold heparinized Eagle's minimum essential medium containing non-essential amino acids (MEM) and a single cell suspension was obtained. The cells were washed and viable cell count done by the trypan blue dye exclusion test [13].

2.3. Preparation of splenic macrophages

Spleen cell suspension was incubated at 37°C for 2 h in an atmosphere of 5% CO_2 and the glass non-adherent cells were decanted gently. The glass-adherent cells were washed thrice with MEM and scraped off with a policeman rod and resuspended as a single cell suspension. More than 95% of the cells were phagocytic [14].

2.4. Separation of T- and B-lymphocytes

T- and B-lymphocytes were separated with glass wool and nylon wool columns by the technique of Julius et al. [15] and Trigio and Cudkowicz [16]. The effluent (T-lymphocytes) and the eluted cells (B-lymphocytes) were centrifuged separately and resuspended in cold MEM and the purity was tested as described elsewhere [17].

2.5. Preparation of peritoneal macrophages

The peritoneal cavity of rats was lavaged with 10 ml of MEM. The aspirated cells were layered in a glass Petri dish and incubated at 37°C in the presence of 5% CO_2 for 2 h. The glass non-adherent cells were removed by washing three times. The glass-adherent cells were scrapped off with a policeman rod. Cells were resuspended in MEM and a single cell suspension was prepared [14].

2.6. Preparation of thymocytes

A single cell suspension of thymocytes was prepared in MEM with 10% fetal calf serum as described by Debetto et al. [18]. The viable cell count was done by the trypan blue dye exclusion test.

2.7. Isolation of intestinal epithelial cells of various differentiation stages from crypt to villus

Rats were fasted overnight and sacrificed by cervical dislocation and decapitation. Small intestine was removed and flushed gently with normal saline containing 1.0 mM dithiothreitol. Intestinal epithelial cells were prepared along the crypt to villus axis on a gradient of differentiation according to Weiser [19]. Briefly, the cecal end of the intestine was ligated and filled with solution 'A' containing 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4 (pH 7.3) after clamping the other end with artery forceps. The intestine was then immersed in solution 'A' and incubated at 37°C for 15 min in a constant water bath. After incubation the intestine was emptied and fluid discarded. The intestine was now filled with solution 'B' containing 1.5 mM EDTA and 5 mM dithiothreitol in phosphate-buffered saline (pH 7.2) and immersed in solution 'B' for incubation. After 4 min incubation, the contents were emptied into a plastic centrifuge tube to recover the first epithelial cell population. The intestine was filled with solution 'B' for different time intervals and the process was repeated for several times to collect cell populations of differentiation stages. Cell populations were centrifuged at $900 \times g$ for 5 min and washed twice with 4 mM EDTA solution containing 15 mM β-mercaptoethanol (pH 7.4) to remove phosphate buffer. For this experiment, epithelial cells in their sequence of dissociation from the intestine were pooled into three fractions on the basis of their protein content and alkaline phosphatase activity as described by Panini et al. [20]. These fractions were designated as upper villi, middle villi and crypt respectively. From each fraction 5×10^6 cells ml^{-1} were used in the tests.

2.8. Experiments on intestinal cells in situ

Laparotomy on each rat was performed by midline incision under light ether anesthesia. The intestine was

washed with normal saline, using a syringe and a blunt needle, through two small cuts. One was made slightly distal to the duodeno–jejunal junction and another at the distal end of the ileum. After washing, the opening was ligated and 10-cm-length loops were prepared from the upper end of the intestine using sterile threads [21]. Test solution was administered into the loops through a proximal opening, which was then immediately ligated. Control loops contained normal physiologic saline solution. The whole intestine was kept in situ and the abdomen stitched immediately. Proper breathing and anesthesia of the animal was maintained throughout the experiment. Loops were removed after 30 min incubation, gently blotted on filter paper, and the contents were drained into graduated tubes. The luminal fluid was made up to the desired volume and centrifuged at $500 \times g$ for 5 min to remove any intestinal debris.

2.9. Estimation of Cr (VI) reduction by the cells

Potassium dichromate (hexavalent chromium; Cr (VI)) was of analytical grade (M/S Qualigens, Lucknow, India). Chromium solution was prepared in MEM containing 10 or 25 μg ml^{-1} of chromium. 5×10^6 cells were suspended in 1 ml chromium solution and incubated at 37°C. At different time periods an aliquot was taken, the cells were removed by spinning and the reduction of Cr (VI) was assayed in the cell-free supernatant spectrophotometrically at 540 nm using diphenylcarbazide reagent [22]. The experiments were setup in triplicate and were repeated thrice. The mean data ± S.D. have been presented as percentage reduction of Cr (VI).

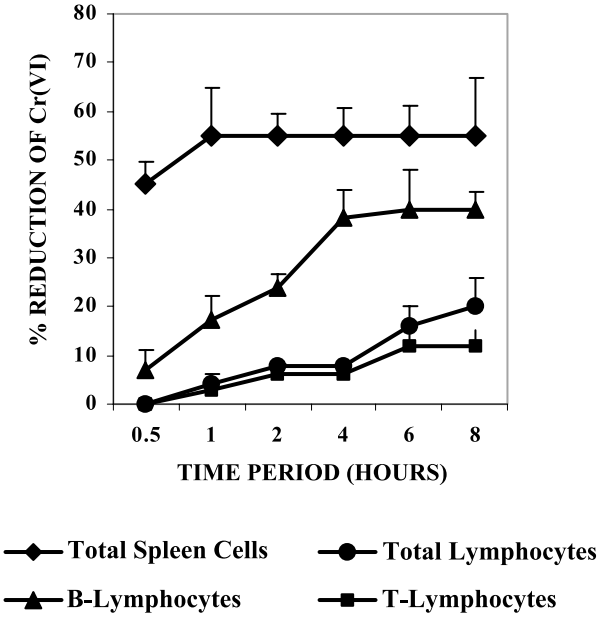

Fig. 1. Reduction of Cr (VI) by rat spleen cells or its enriched subpopulations. A single cell suspension of normal rat spleen was prepared and enriched subpopulations of total lymphocytes were obtained by depleting macrophages with a glass wool column and the T- and B-lymphocyte-enriched subpopulations were prepared with a nylon wool column. 5×10^6 cells ml^{-1} were suspended in MEM containing 10 μg Cr (VI). At different time periods a group of sets were removed and Cr (VI) measured. The findings have been presented as mean value ± S.D. (bars) from repeated experiments as percentage reduction.

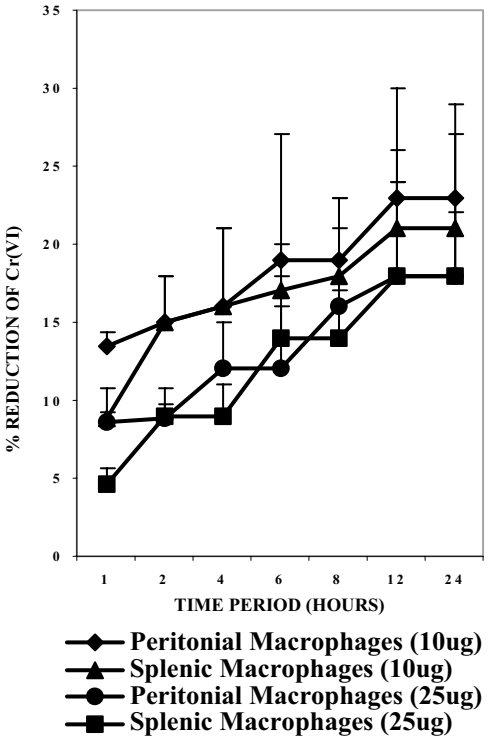

Fig. 2. Reduction of Cr (VI) by macrophage-enriched cells obtained from rat peritoneal lavage cell and the spleen. The peritoneal lavage or the single cell suspension of spleen was layered in a glass Petri dish and incubated at 37°C for 2 h in the presence of 5% CO_2. Then the adherent cells were scrapped off, counted and tested (5×10^6 cells ml^{-1}) as described in Fig. 1 using 10 and 25 μg of Cr (VI).

3. Results

3.1. Reduction of Cr (VI) by splenic lymphocytes

The data summarized in Fig. 1 show that total spleen cells suspended in 10 μg ml^{-1} of Cr (VI) reduced 55 ± 10% of it in 1 h and the same level was maintained throughout the period of observation. The reduction by the enriched subpopulation of B-lymphocytes was 18 ± 5% at 1 h and gradually increased reaching a peak of 40 ± 8% reduction at 6 h of incubation. The least effective cells were the enriched subpopulation of T-lymphocytes that reduced 10 ± 2% of the Cr (VI) at 8 h.

3.2. Reduction of Cr (VI) by macrophages

Macrophages obtained from rat spleen and peritoneal cavity were used. The findings presented in Fig. 2 show that with 10 μg Cr (VI) the peak reduction of 22 ± 5% was

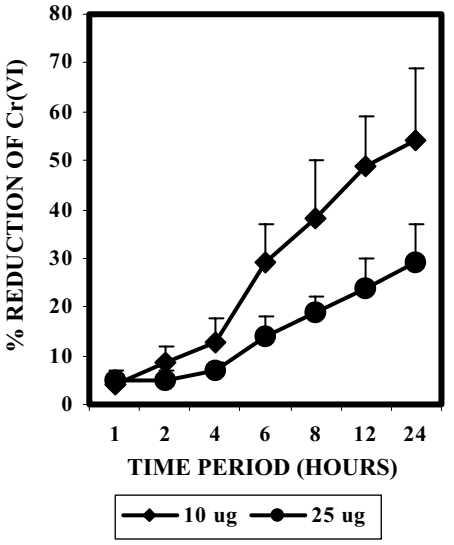

Fig. 3. Reduction of Cr (VI) by rat thymocytes. Total thymus was removed from the rat and a single cell suspension was prepared and tested (5×10^6 cells ml^{-1}) as described in Fig. 1.

attained by splenic macrophages at 12 h while that by the peritoneal macrophages was similar, the peak being $24\pm7\%$ at 12 h. This experiment was repeated using a higher dose of 25 μg chromium. A lower percentage of reduction (nearly half of the 10 μg) was observed with the higher Cr (VI) concentration (Fig. 2).

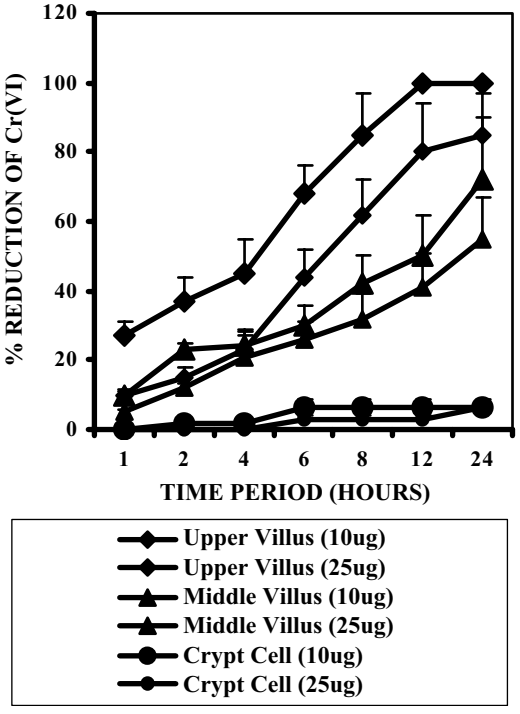

Fig. 4. Reduction of Cr (VI) by rat intestinal cells. Rat intestines were removed and different types of cells were removed and a single cell suspension was prepared and tested (5×10^6 cells ml^{-1}) as described in Section 2.

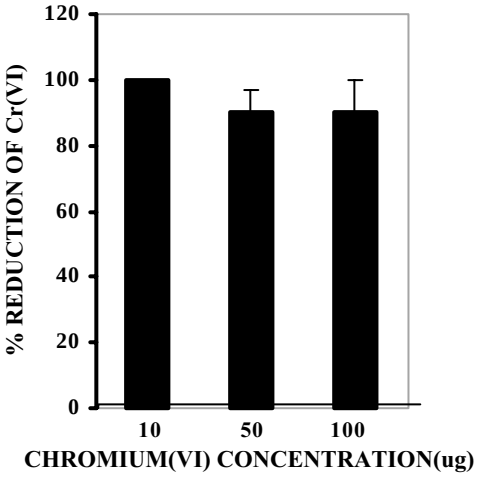

Fig. 5. Reduction of Cr (VI) by rat intestinal loop in situ. Rat intestines were exteriorized, lumen washed and different doses of Cr (VI) injected in lumen, and Cr (VI) reduction tested as described in Section 2.

3.3. Reduction of Cr (VI) by thymocytes

The total cell population obtained from the rat thymus was suspended in 10 and 25 μg concentration of chromium and incubated for different time periods. The data presented in Fig. 3 show that thymocyte gradually reduced Cr (VI) reaching peak reduction at 24 h. The reduction was $54\pm15\%$ with a Cr (VI) concentration of 10 μg and $28\pm8\%$ in 25 μg concentration (Fig. 3).

3.4. Reduction of Cr (VI) by intestinal cells in vitro

Cells obtained from upper and middle villus and the crypts of the intestine were studied for their capacity to reduce Cr (VI). The findings summarized in Fig. 4 show that the reduction was $85\pm12\%$ to 100% by the cells from the upper villus using the two concentrations of Cr (VI). The reduction was minimal with the cells from the crypts while that by the cells from the middle villus was $55\pm12\%$ to $72\pm18\%$ (Fig. 4).

3.5. Reduction of Cr (VI) by intestinal cells in situ

The intestinal loops were blotted on filter paper, and the contents were drained into graduated tubes. The luminal fluid was made up to the desired volume and centrifuged to remove any intestinal debris. The Data summarized in Fig. 5 show that the reduction of Cr (VI) was 100%, $90\pm7\%$ and $90\pm10\%$ with 10, 50 and 100 μg Cr (VI) respectively.

4. Discussion

The main finding of the present study is that various cells of the body differ in their capacity to reduce Cr (VI). Among the isolated rat cells tested, peak reduction of

100% was seen by upper villus cells of intestine while the least (4%) was seen with the crypt cells of the intestine, the other cells were in between. Chromium is of significant importance in altering the immune response by immunostimulatory or immunosuppressive mode as shown by its effects on T- and B-lymphocytes, macrophages, cytokine production and the immune response that may induce hypersensitivity reactions [23]. Cells of the immune system form the first line of defense in the body. These cells differed vastly in their capacity and the time taken to reduce Cr (VI). The most effective were the total spleen cells (55 ± 10%) and thymocytes (54 ± 15%), followed by the B-lymphocyte-enriched subpopulation of spleen (40 ± 8%) and least effective were T-lymphocytes (10 ± 2%) while macrophages reduced 24 ± 7% of the Cr (VI). DeFlora et al. [11] have reported similar findings with lung alveolar macrophages.

Cr (VI) enters the cells through membrane anionic transporters but Cr (III) does not. While detoxifying Cr (VI), the cells themselves may get damaged. Chromium-induced damage to DNA both in the gastric mucosa cells and lymphocytes has been studied by comet assay and the effects were found to be similar in both [24,25]. Some of the important factors in determining the biological outcome of chromium exposure include the bioavailability, chemical speciation and solubility of chromium compounds, intracellular reduction, and interaction of chromium with DNA. Intracellular Cr (VI) is metabolically reduced to the ultimate Cr (III). Cr (VI) does not react with macromolecules such as DNA, RNA, proteins and lipids; however, both Cr (III) and the reductional intermediate Cr (V) are capable of co-ordinate, covalent interactions with macromolecules. A series of in vitro and in vivo studies have demonstrated that Cr (VI) induces oxidative stress through enhanced production of reactive oxygen species leading to genomic DNA damage and oxidative deterioration of lipids and proteins. A cascade of cellular events occurs following Cr (VI)-induced oxidative stress including enhanced production of superoxide anion and hydroxyl radicals, increased lipid peroxidation and genomic DNA fragmentation, modulation of intracellular oxidized states, activation of protein kinase C, apoptotic cell death and altered gene expression [23,26,27].

Chromium enters the body through the lungs, gastrointestinal tract, and to a lower extent through skin [8]. The most important route for non-occupational exposure is via ingestion of chromium-containing food and water. Regardless of the route of exposure Cr (III) is poorly absorbed whereas Cr (VI) is more readily absorbed [9,10,12]. Further, Cr (VI) is not very toxic when introduced by the oral route and it was thought to be due to poorer absorption by this route. But our findings show that the reason was efficient detoxification of Cr (VI) by reduction in the intestines. The most efficient reduction (100%) of Cr (VI) was observed by the whole intestinal loop. A number of components in the intestines may be responsible for such efficient handling of Cr (VI). When introduced by the oral route, Cr (VI) is efficiently reduced by upper villus cells (100%), the middle villus cells (72 ± 18%) and the crypt cells (4%) of the intestine as shown in the present study. The intestinal bacterial flora, including *Escherichia coli*, *Lactobacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. have been found to be efficient reducers of Cr (VI) (personal unpublished data). The findings of the present study also suggest that whatever Cr (VI) crosses over the intestinal wall can be efficiently reduced by the lymphocytes and macrophages present in the lymphoid follicles of the intestinal wall and also in the lymph nodes of the omentum. Our findings find support in the study of DeFlora et al. [11] who have shown that saliva and gastric juice and sequestration by intestinal bacteria, blood, liver are efficient Cr (VI) reducers. This is further supported by a number of studies where chronic oral exposure to Cr (VI) exhibited no adverse effects [28–30]. Further, Cr (VI) is reduced to variable degrees in different tissues and organs, such as peripheral lung parenchyma, bronchial tree, kidney, testis, stomach, spleen, plasma and adrenals etc. [31]. The present study is unique in the sense that isolated cell suspensions have been tested. The reduction of Cr (VI) to Cr (III) results in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity, and carcinogenicity of Cr (VI)-containing compounds. These mechanisms of reduction of Cr (VI) explain the lack of genotoxicity, carcinogenicity, and induction of other long-term health effects of Cr (VI) by the oral route.

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