Evaluation of Immunologic and Intestinal Effects in Rats Administered E171, A Food Grade Titanium Dioxide (TiO_2)

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E171 is a food grade titanium dioxide (TiO_2) commonly used as an additive in many foods. Recent studies have reported that exposure of rats to E171 in water, when first pretreated with 1, 2-dimethylhydrazine (DMH) (a well-known gastrointestinal genotoxic carcinogen, inflammagen, DNA methylating agent and aberrant crypt inducer), induced inflammation in the gastrointestinal tract and suggested enhancement of gastrointestinal pathology related to increased risk of intestinal cancer. To further explore these reported results, we have extensively evaluated rats administered E171 for 7 or 100 days in food *ad libitum,* as opposed to drinking water.

Food grade sample E171-E was supplied by the Titanium Dioxide Manufacturers Association based on an assessment and characterization of the different grades of E171 placed on the market. Chemical analysis, including particle size distribution, was performed and diets (control and E171 containing) were analyzed for TiO_2 content and homogeneity. E171 was provided to male Wistar Han rats, 7-8 weeks of age, in the diet at anticipated levels of 0, 40, 400, or 5,000 ppm for 7 or 100 days. In the 100-day study, rats were pretreated with DMH (as the dihydrochloride, DMH·2HCl) or vehicle (1.5% EDTA in 0.9% NaCl) by intraperitoneal injection and initiated on the experimental diets after a 7-day rest period. For the 7- and 100-day studies, whole blood, small intestinal Peyer's patches, colon, jejunum, and spleen were collected for immunologic evaluations. Cytokines in plasma, tissue extracts and cell culture supernatants were quantified, including the inflammatory mediators, IL-1α, IL-1β, IL-6, IL-12(p70), IL-17A, IL-18, IL-33, CCL2/MCP-1, CXCL1/KC (IL-8), GM-CSF, IFNγ, and TNFα and the anti-inflammatory cytokine, IL-10.

Phenotyping of leukocytes from whole blood, Peyer's patches and spleen was performed by flow cytometry using antibodies directed against rat CD45 to identify rat leukocytes, CD103, MHCII, and CD11b/c to quantify dendritic cells, CD4 to quantify T_{helper} cells, CD25 to quantify activated T_{helper} cells, and FoxP3 to identify $T_{regulatory}$ (T_{reg}) cells. In the 100-day study, tissue segments of small intestine, liver, spleen, lungs, and large intestine were fixed in formalin, and testes were fixed in modified Davidson's solution. Tissues from the 100-day study were fixed and processed for histopathology. Hematoxylin and Eosin-stained sections of distal colon were evaluated for goblet cells, including the number per colonic gland, length of colonic gland, and number per unit length of glands. In addition, segments of proximal, mid, and distal colon were evaluated for aberrant crypt foci (ACF).

Total body weight did not change with E171 administration in either the 7- or 100-day study. Food and water consumption were similar between groups. An increase in spleen cellularity at 5,000 ppm was observed in the 7-day but not in the 100-day study. No significant differences were observed in any E171 treatment groups in the 7-day or 100-day studies in the percentage of dendritic cells, $CD4^+$, activated $CD4^+$ or T_{reg} cells in Peyer's patches, spleen, or peripheral blood, compared to control diet-fed animals. This was also true in the rats pretreated with DMH in the 100-day study. The profile of cytokine production was determined in plasma, sections of jejunum, and colon with no significant changes in the cytokine profile observed in plasma or in tissues analyzed, except for IL-17A in colon (DMH+400 ppm E171) and IL-12p70 in plasma (DMH+40 ppm E171). In addition, two rats exhibited exceptionally elevated plasma IL-6 levels in the DMH+40 ppm E171 treatment group. In the 7-day study, T cells from Peyer's patches, peripheral blood, and spleen were activated using anti-CD3/anti-CD28 and cytokine secretion was also evaluated. No significant changes in cytokine secretion were observed in any of the E171 treatment groups compared to control.

In the 100-day study, no treatment related changes were visualized by histopathology in the small intestine, liver, spleen, testis, or lungs. In the large intestine, one rat in the DMH+control diet group had 2 separate invasive adenocarcinomas. Further, a single rat in each of the DMH+40 ppm E171 and DMH+400 ppm E171 dose groups had adenomas of the large intestine; however, no other animals exhibited histologic changes in the large intestine, whether pretreated with DMH or vehicle. E171 treatment produced no significant changes in ACF or in numbers of crypts per ACF. As expected, there

were significant differences in the number of ACF in the DMH pretreated rats compared to vehicle only, but the number of ACF were not significantly influenced by E171 treatment. Furthermore, there were no differences between groups (even DMH vs. vehicle) in the number of goblet cells per colonic gland, the length of the glands or in the number of goblet cells per unit length of gland.

In summary, with the exception of an increase in spleen cellularity at the 5,000 ppm E171 dose in the 7-day study and an increase in IL-17A in colon (DMH+400 ppm E171) and IL-12p70 in plasma (DMH+40 ppm E171) in the 100-day study, we did not observe any statistically significant changes associated with E171 administration in any other immune parameters in the small intestine, spleen, or blood, whether administered by itself or after pretreatment with DMH. Furthermore, we did not observe histopathologic changes related to E171 treatment in the tissues examined, including tumor formation, ACF, goblet cells, or histologic abnormalities. Our findings support the original observations of the 2-year bioassay (National Toxicology Program) in rats showing no evidence of a carcinogenic effect of TiO_2 in the intestine or other tissues when administered in the diet at levels of 2.5 or 5% (25,000 and 50,000 ppm). Thus, we conclude that there is minimal to no effect of E171 when administered in the diet on the immune system and no effect on the intestinal epithelium with respect to proliferation and cancer.