Aberrant Crypt Foci Examination in Satellite F0 Animals (EOGRT Study)

In this guide

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This is a paper for discussion.

This does not represent the views of the Committee and should not be cited.

Method:

199. Evaluation of ACF in the colon of a satellite group of F0 animals (10/sex per group) treated with 0, 100, 300 and 1,000 mg E 171/kg bw per day and terminated after weaning was undertaken. The colon was excised, opened longitudinally and the contents removed by rinsing with a 0.9% NaCl solution.

Thereafter, the tissue was divided in parts of a suitable size for fixation by immersion in 5% buffered formalin. A blind examination of these samples stained with 0.5% (w/v) methylene blue in water was performed under a stereomicroscope at 50x magnification for presence of ACF. The Panel noted that the design of the study did not include a positive control group (e.g., treatment with a known gastrointestinal tract tumour initiator such as dimethyl hydrazine (DMH) for the development of ACF.

Results:

200. The definition of ACF used was ‘foci containing more than 2 ABCs’, taken from Shwter et al. (2016). No ACF were found in the colons of the control and the treated groups. A mildly increased morphological variability (increased size and intensity of the staining of a small portion) of the crypts in the two caudal parts of colon was observed in seven animals (See tables 1 & 2 below). These changes were assessed as inconsistent with the appearance and definition of ACF discussed above. Incidence of these single crypts observed in the mid and high doses was not significantly different from the control. The EFSA Panel agreed with this conclusion.

Table 1: Aberrant Crypt Foci Presence in Satellite F0 Animals.

Aberrant Crypt Foci Present

Dosage Group	Control	Low-Dose	Mid-Dose	High-Dose	Total
Females	1/10	0/10	1/10	2/10	4
Males	1/10	0/10	1/10	1/10	3

201. An additional submission of data included photomicrographs of mildly increased variability in crypt morphology from all seven animals. A re-examination was extended to an additional randomly selected nine control animals (4 males and 5 females) and eight high-dose group animals (3 males and 5 females). A mild increased variability in crypt morphology was observed in eight of the nine controls and six of the eight high-dose animals (see Table x). The Panel considered that oral exposure to E 171 at doses up to 1,000 mg/kg bw per day did not induce ACF in the colon.

Table 2: Aberrant Crypt Foci Presence in the Re-Examination of Satellite F0 Animals.

Aberrant Crypt Foci Present

Dosage Group	Control	High-Dose	Total
Females	4/5	3/3	7
Males	4/4	3/5	7

202. With regards to the induction of aberrant crypt foci, EFSA considered additional studies available in the literature in order to form their conclusions. These studies are discussed in detail below.

Bettini et al 2017

Test materials:

203. The test materials used in this study were:

1) E 171, anatase, 20–340 nm (118 nm) (TEM); 44.7% particles100 nm;

2) TiO2 Nanoparticles (NM-105), anatase/rutile, 15–24 nm. Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure) of 1.

3)

Internal exposure:

204. Qualitative measurement in tissues, methodology reliable with some limitations. Adult Wistar rats were administered by gavage 10 mg E 171/kg bw per day for one week or 100 days. In addition, a group of animals were exposed to TiO2 Nanoparticles (NM-105) 10 mg/kg bw per day for one week.

Method:

205. First experiment: 12 male rats were pre-treated with a single injection (180 mg/kg intraperitoneal in isotonic saline) of DMH. The aberrant crypts

average per colon was 190 ACF and 30 large ACF after 100 days. In DMH pre-treated rats also subsequently (7 days later) exposed to either 0.2 or 10 mg/kg bw per day E 171 in drinking water (12 rats/group), there was a statistically significant increase per colon in number of aberrant crypts and large ACF and a statistically non-significant increase in total number of ACF in the high-dose group compared to DMN only controls. No statistically significant differences were observed between the low-dose and control groups. The incidence of ACF was not reported.

206. Second experiment: Male rats received either drinking water (12 controls) or 10 mg E 171/kg bw per day in drinking water (n=11) for 100 days. No ACF were observed in the colons of controls. Four rats in the treated group developed one to three ACF per colon (which in three rats consisted of 1–3 aberrant crypts/ACF with the remaining rat having 12 aberrant crypts in an ACF). The increase in the incidence of rats with ACF (4/11 vs. 0/12 in the control group) was statistically significant.

207. The Panel considered that these data indicate that E 171 has pro-inflammatory potential at the systemic level, paralleled by the development of an inflammatory microenvironment in the intestinal mucosa.

208. The Panel considered that E 171 alone at a dose of 10 mg/kg bw per day may induce development of ACF in male rats. The Panel also noted that E 171 at a dose of 10 mg/kg bw per day increased the number of ACF initiated by a genotoxic carcinogen (not considered further in this paper).

Results:

209. Titanium was detected in the immune cells of Peyer’s patches in which patches dendritic cell percentage were increased. Effects were not noted in the spleen. It was noted that this effect was transient (observed at 7 days but not at 100 days). The percentage of regulatory T cells and T-helper (Th) cells were significantly decreased at both time points in E 171 exposed animals. Stimulation of immune cells isolated from Peyer’s patches showed a decrease in T-helper (Th)-1 IFN-c secretion, while splenic Th1/Th17 inflammatory responses sharply increased, as measured in cells taken from exposed rats, stimulated in-vitro with anti CD3/CD28 antibodies.

Intestinal mucosal inflammation:

E 171 treatment:

210. One week = no intestinal inflammation, 100-day = colon microinflammation evidenced by significantly increased IL-1b, IL-8 and TNF-a expression in the colon in addition to increased IL-10. Aberrant crypts were examined in the colon after staining with methylene blue. Data on the effects of TiO2 Nanoparticles on intestinal mucosa were not presented.

211. ACF Definition: The authors did not explicitly give their definition of an aberrant crypt foci (ACF) but the Panel presumed it was 1-or more aberrant crypts/ACF. The authors defined a 'large ACF' as consisting of more than three aberrant crypts per ACF.

Blevins et al. 2019

212. The test material used was E 171, anatase, 110–115 nm (SEM), 36% particles 100 nm. Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure): 3.

213. Internal exposure was not examined.

Method:

214. Six-week-old male Wistar Han IGS rats were exposed to E 171 in a standard diet at 4 concentrations between 0-5,000 mg/kg diet concentration in two studies of 7 days (n=5/group) and 100 days (n=15/group) (equal to 1.8, 4.8, 31.4, 374 mg/kg bw per day) for 7 and (equal to 1.3, 3.5, 22.4 or 267 mg/kg bw per day) for 100 days. The two studies were performed at different Institutions, with the 7-day study performed twice whereas the 100-day study was performed once.

215. The objectives of the study were to evaluate the acute (7 days) and sub-chronic (100 days) effects of dietary E 171 exposure on the immune system of the GI tract and periphery as well as to evaluate effects of the sub-chronic exposure either alone or after pre-administration of a known intestinal genotoxic carcinogen, DMH and an examination of colon for presence of aberrant crypt foci and of aberrant crypt was included.

216. Immune system metrics: Phenotyping of immune cells (i.e. CD103+DC, total and activated T helper cells, total and activated Treg cells) and inflammatory cytokines [(IL-1a,IL-1b, IL-6, interferonc (IFNc), IL-12p70, IL-17A, IL-18, IL-33. CCL2/MCP-1, CXCL1/KC (IL-8), GM-CSF and tumour necrosis factor a(TNF-a)).

217. 7-Day Studies: Rats were randomised into 4 groups of 5 animals and the data from the two studies were pooled. Total food and water consumption were determined at the end of each study. Body weights were determined at the start and end of the 7-day. As calculated by the Panel and taking the mean of the two periods given in the paper: 1.5, 3.9, 25.5, 294 or 1.1, 3, 19, 236 mg/kg bw per day for weeks 1-10 or 11-15, respectively (groups 1–4) and 1.5, 4.1, 25.7, 300 or 1.1, 3.1, 19.2, 237 mg/kg/bw per day for weeks 1–10 or 11–15, respectively (groups 5–8) exposure period at the time of euthanasia.

218. 100-day Study: Animals were randomised into 8 groups of 15 animals each. At the start of the study, animals in groups 5–8 were treated with a single intraperitoneal injection of a sterile dose of 180 mg/kg bw dimethylhydrazine (DMH) dihydrochloride while groups 1–4 were treated with vehicle only. Seven days after intraperitoneal injection, dietary administration of 0, 40, 400 or 5,000 mg/kg diet E 171 was started and continued for 100 days. Bodyweights were determined weekly beginning on day 0 of the study and just prior to euthanasia. Food consumption was determined weekly beginning with administration of the E 171 supplemented diets. Water consumption was determined during weeks 3, 8 and 13 of the study.

Results:

219. No significant changes in food intakes or body weights or liver and spleen weights were found, and no mortality was observed. A trend towards increased food consumption in rats of the high E 171 group was observed. Dietary E 171 produced no general signs of overt toxicity at the highest dose tested, over 100 days.

220. Following the 7- and 100-day feeding periods, rats were euthanised and measurements of inflammatory cytokines (using the LEGEND plex rat inflammation Panel) and phenotyping of immune cells (by-flow cytometry) in the periphery and GI tract were performed. Peyer’s patches, peripheral blood mononuclear cells (PBMC) and spleen cells were analysed for inflammatory and regulatory T-cell responses directly ex-vivo or after in-vitro stimulation with anti-ratCD3 (5lg/ml) and anti-rat CD28 (5lg/ml) for 4 days. Histopathology, ACF, ABC and goblet cell evaluations were performed on rats in the 100-day study.

221. All tissues were collected from well-defined areas, and measurements, procedures and evaluations were performed in a standardised and blinded manner. CD103+dendritic cells (DC) were evaluated in the gut, Peyer’s patches, spleen and in peripheral blood over time period. No change in the percentage of

CD103+DC in peripheral blood, spleen or Peyer's patches due to acute or sub-chronic dietary E 171 consumption alone was observed. The total percentage of CD4+T helper cells, the percentage of T helper cells expressing CD25, an indicator of T helper activation, and the percentage of Treg cells (CD4+FoxP3+) and activated Treg (CD4+CD25+FoxP3+) which could lead to a low-level inflammatory response in the absence of increased inflammatory cells, were quantified in peripheral blood, spleen and Peyer's patches. Dietary E 171 exposure did not change the frequency of CD4+T helper cells systemically or in intestinal Peyer's patches. In addition, there was no detectable impact on the percentage of activated CD4+T helper cells or on the percentage of Treg cells either peripherally or locally in the Peyer's patches of treated rats fed for 7 or 100 days. Collectively, these results suggest that E 171 consumption does not alter T-cell-mediated mechanisms of immune control, either promoting inflammatory CD4+T helper cell activation or in reducing the percentage of anti-inflammatory Treg cells.

222. Regarding the effects on cytokines, the data presented suggest that dietary E 171 does not induce inflammation peripherally or in the GI tract at both time points. In addition, studies were conducted to explore the possibility that E 171 might alter the effector cytokine profile of T helper cells in lymphoid tissue or circulation, which may not be manifest without T cell-specific stimuli. Lymphocytes were isolated from peripheral blood, spleen and Peyer's patches and activated ex-vivo with anti-CD3/anti-CD28 for 4 days to induce T helper cell cytokine production. No effects of E 171 exposure on any of the induced cytokines produced from ex-vivo stimulated T helper cells were observed.

223. In the 100-day study, all animals were treated with E 171, some groups were initiated with 180mg/kg bw DMH before the start of the dietary exposure to E 171 and an additional control initiated with DMH was also included. The same parameters as described above were evaluated, with some differences observed. A modest increase in the relative spleen weight in 22.4 mg E 171/kg bw per day + DMH compared to not initiated animals, an increase in IL-17A in colon (22.4 mg E 171/kg bw per day + DMH) and IL-12p70 in plasma (3.5 mg E 171/kg bw per day + DMH), with no dose-related effects, were observed. There were no changes in spleen cellularity across any of the treated groups. No changes were observed in the percentage of CD103+DC, CD4+T helper cells or total or activated.

224. Safety assessment of the food additive titanium dioxide in peripheral blood, spleen or Peyer's patches in animals exposed to E 171+DMH compared to animals treated with only DMH. No treatment related histopathological changes in

the duodenum, jejunum, ileum, spleen, liver, lung and testes in animals exposed only to E 171 were found. Rats that were initiated with DMH only and those which received 171 in the diet after the initiation displayed several histopathological abnormalities. There were two invasive adenocarcinomas in one animal in the 1.3 mg E 171/kg bw per day + DMH group, and single adenomas in one animal in the 3.5 mg E 171/kg bw per day E 171+DMH group and in one animal in the 22.4 mg E 171/kg bw per day + DMH group.

225. There were no other histopathological changes in the large intestines of the other animals treated with DMH. One rat in the 1.3 mg E 171/kg bw per day +DMH group and one rat in the 22.4mg E 171/kg bw per day +DMH group had subpleural lymphocytes in the lung, but without any evidence of acute inflammatory changes or hyperplasia.

226. Limitations: A significant amount of the epithelial surface of the sampled colon (proximal, middle and distal) was obscured when observed by light microscopy and so were unable to examine the entire surface of the colon samples. The results for the areas of epithelium that were examined indicated an increase in ACF/cm^2 and ABC/cm^2 in groups initiated with DMH compared to the groups that were not initiated with DMH. E 171 treatment administered after DMH did not result in statistically significant increases in aberrant crypt foci or aberrant crypts or any change in the number of aberrant crypt foci or aberrant crypt were observed due to E 171 exposure alone.

227. The Panel noted a considerable variability in the results, which may mask possible effects. Furthermore, the Panel noted that the examination for presence of ACF and ABC was not performed on the whole colon but was limited to three 2 cm long samples (one from the proximal, mid-portion and the distal parts).

228. Dietary E 171, with or without treatment with DMH, had no effect on the length of the colonic glands examined or the number of goblet cells/unit. The Panel considered that this study indicates that acute and sub-chronic dietary intake of E 171 resulted in no significant effects on either peripheral or GI tract immune homeostasis as evidenced by immune cell phenotyping or inflammatory cytokine analysis. Limitations in the pathological examination for ABC and ACF preclude a conclusion on potential for ABC and ACF formation.