3,3'-Diindolylmethane Increases Serum Interferon-γ Levels in the K14-HPV16 Transgenic Mouse Model for Cervical Cancer

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Abstract. *While cervical cancer incidence and mortality rates have declined in the United States, this cancer represents a worldwide threat. Human papilloma viral infection causes cervical neoplasia (CIN). 3,3'-Diindolylmethane (DIM) prevents or inhibits the progression from cervical dysplasia to cancer. The aim of this study is to determine the most effective dose of DIM given continuously in food, that significantly increases serum interferon gamma levels (IFN-γ) in the K14-HPV16 transgenic mouse model for cervical cancer. Materials and Methods: Five doses of DIM in food were administered to the mouse model for 20 weeks. Serum Interferon gamma (IFN-γ) levels and estrogen metabolite levels were quantified. Results: At 1000 ppm DIM, serum IFN-γ concentrations were significantly increased (p<0.0396). The estrogen metabolites were unchanged. IFN-γ concentrations in CIN free mice and the percentage of CIN free transgenic mice were well correlated (r=0.88). Discussion: Significant increases in IFN-γ serum concentrations that correlate with the percentage of CIN free mice in each group indicate that 1000 ppm of DIM in food may be the most effective dose for future studies. These results may eventually lead to new and effective vaccination strategies in women already infected with the human papilloma virus.*

Cervical cancer incidence and mortality rates have declined in the United States over the past three decades. Incidence rates for Hispanic women are higher than those for non-Hispanic women. The mortality rate for African American women has declined more rapidly than the one for Caucasian women, yet African American mortality rates continue to be more than double that of Caucasians (1). Cervical cancer strikes nearly

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half a million women each year worldwide, claiming a quarter of a million lives (2). With the development of new vaccines against the two most oncogenic human papillomavirus (HPV16 and HPV18), the incidence of cervical cancer is likely to decrease. It will take decades for vaccination to impact cervical cancer rates (3). Prophylactic vaccines do not block cervical epithelial changes and have no effect on cervical intraepithelial neoplastic (CIN) lesions in those individuals previously infected with HPV. DIM, or indole-3-carbinol, the precursor to DIM, inhibits CIN lesions in women with cervical dysplasia (4-6). This has also been shown in the K14-HPV16 mouse model (7-10). DIM, through multiple estrogenic and non-estrogenic actions, suppresses viral oncogene expression. DIM also suppresses viral infection in children with recurrent respiratory papillomatosis an HPV caused disease (11, 12). DIM inhibits proliferation of viral transcripts E6 and E7 in CaSki cells, an HPV16/HPV18–positive cervical carcinoma cell line (5). DIM can induce a G_1 cell cycle arrest in MCF-7 cells, a human breast adenocarcinoma cell line, that is accompanied by inhibition of cyclin-dependent kinase expression (13). DIM inhibits cell adhesion, spreading and invasion associated with the up-regulation of phosphatase and tensin homolog (*PTEN*, a tumor suppressor gene) and E-cadherin (a regulator of cell cell adhesion) in T-47-D human breast cancer cells (14). DIM also elevates several key cytokines *in vivo*: interferon-gamma (IFN-γ), granulocyte colony stimulating factor (G-CSF), Interleukin-12 (IL-12), and interleukin-6 (IL-6). IFN-γ is responsible for the overall immune response system (15, 16). A reduction in the severity of CIN and in some cases the complete reversal of cervical dysplasia in women taking DIM for four weeks has also been shown (4). The ultimate goal of this research is to determine whether DIM acts to augment the efficacy of preventive HPV vaccines, creating a new therapeutic use for these vaccines in women after HPV infection. The first step was to determine the most effective dose of DIM that inhibits cervical dysplasia and increases the immune response. We have already demonstrated that 1000 ppm of DIM given continuously in food inhibits the

progression of cervical dysplasia to cervical cancer in the K14-HPV16 Transgenic Mouse Model (10). CIS and (SCC) were completely eliminated in the group treated with 1000 ppm DIM compared to the incidence of 21% of cancers in the untreated group and 5% in the group treated with 500 ppm DIM. In this study we examine IFN-γ levels of treated and untreated mice and compare them with cervical histology.

Materials and Methods

This study employs a well known transgenic mouse model, K14-HPV16, commonly used in studies involving cervical dysplasia and cervical cancer (7-10, 17, 18). The K14-HPV16 mouse model is transgenic for the entire high risk *HPV16* early region under control of the human keratin-14 promoter, and expresses *HPV16 E6* and *E7* oncogenes in basal squamous epithelial cells. Tumorigenesis in this model is estrogen dependent. Chronic estradiol treatment at 0.1-0.25 mg for 60 days induces invasive squamous carcinoma in the vulva, vagina and outer cervix of K14-HPV16 mice. Epithelial dysplasia and metaplasia are observed after four months of estradiol treatment, leading to high-grade dysplasia and multifocal carcinomas by six months (18). We have found that high-grade dysplasia and multifocal carcinomas can occur as early as three months. The mouse model and the preliminary data that we have obtained are described in greater detail elsewhere (8-10). Once received from the NCI Mouse Repository (MMHCC, Frederick, MD, USA), the mice were housed as breeder pairs. The mice were quarantined until released by the veterinarian based on serological testing and results. Mice were fed irradiated AIN-93M diet (Purina TestDiets, Richmond, IN, USA) and given sterile water. The animals were given daily health checks, including use of a pain scale form. The hemizygote K14-HPV16 was bred with the FVB wild-type to produce the first litter of pups. At weaning, the pups were genotyped. Those mice exhibiting the *E6-E7* exon, (*K14-HPV16* positive) were further bred with FVB wild-type in order to generate progeny for the experimental protocols. All females (*K14-HPV16* and *FVB* wild-type) were trochar implanted with E_2 pellets (0.25 mg/90 day release, Innovative Research of America, Sarasota, Florida) under anesthesia. E_2 pellets were replaced at three month intervals. One group received AIN-93M mouse diet without DIM added and other groups received AIN-93M mouse diet enriched with either 500,1000,1500, 2000 or 2500 ppm of DIM (LKT Laboratories, St. Paul, MN, USA).

Murine IFN-γ levels. Serum IFN-γ concentrations were assayed using a solid phase sandwich enzyme linked immuno sorbent assay (ELISA) (ABCAM, Cambridge, MA, USA). The minimum detectable dose of IFN-γ was 15 pg/ml. The inter-assay and intra-assay coefficients of variation were 4.6% and 5.7% respectively.

Determination of estrogen metabolites in serum. Serum was diluted 1:1 with sodium acetate buffer (pH 4.65) and 20 μl of β-glucuronidase (110, 200 units/ml; Sigma Aldrich, St. Louis, MO, USA). The solution was incubated at 40°C for 24 hours to deconjugate the steroids. After the addition of deuterated estradiol, each sample was thoroughly vortexed and extracted with chloroform. Each sample was derivitized with 10 μl of dry pyridine and 40 μl of bis (trimethylsilyl)-trifluoroacetamide (BSTFA) (Sigma Aldrich, St. Louis, MO, USA), vortexed, and allowed to react at room temperature overnight. One microliter of each sample was injected into a GC-MS (Agilent Technologies 6980N gas chromatograph equipped with an Agilent 5973 mass selective detector, an Agilent 7683 injector and an HP G1701CA MSD Chemstation, Santa Clara, CA, USA) without further treatment. GC-MS conditions and the method have been previously described (19-21).

Statistics. The concentrations of serum IFN-γ and estrogen metabolites were not normally distributed, as indicated by the Shapiro-Wilk test performed in each variable. Therefore, the data are summarized as median [interquartile range (IQR): 25th-75th percentiles] and assessments of the evidence of trends in the DIM dose response, as well as comparisons of the concentrations between any groups was performed using nonparametric tests (trend: the Jonckheere-Terpstra or modified Mann-Whitney *U*-test, as appropriate).

Serum concentrations of IFN-γ are presented as boxplots. The box represents the middle 50% of the data set. The upper boundary locates the 75th percentile of the data set while the lower boundary indicates the 25th percentile. The line inside the box indicates the median. Vertical lines extend from the box to the farthest points that are no more than 1.5 times the IQR away from the top and bottom of the box. Any points lying farther than 1.5 times the IQR are outliers and are represented by small circles. The diamond indicates the mean.

All statistical tests in this study were conducted so that $p < 0.05$ was considered statistically significant. All data analyses were performed using the SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) (22-24).

Results

IFN-γ concentrations for transgenic and wild-type mice are shown in Figure 1. The results of the Jonckheere-Terpstra test ($p=0.0958$ for the transgenic mice) are also provided. The trend was non-monotonic, hence the Jonckheere-Terpstra test may have had low power detecting a linear trend effect of the dose. In transgenic mice, there was no change in IFN-γ levels in groups treated with 0 ppm or the 500 ppm DIM. A statistically significant increase ($p<0.0396$) was observed in the 1000 ppm DIM group when compared to the 500 ppm group. While IFN-γ levels in all groups receiving DIM above 1000 ppm were increased when compared to those receiving 500 ppm DIM, none of these values was statistically different from the one observed for the 1000 ppm group. A similar trend was observed for wild-type mice receiving increasing doses of DIM continuously in food over 20 weeks.

Serum IFN-γ values for CIN free transgenic mice in each DIM dose group are plotted against the percentage of CIN free mice in each dose group in Figure 2. This cytokine and the cervical histology, that is defined as the percentage of mice in each feeding group that are free of CIN and exhibit normal histology without papillae or gross neoplasia are presented in a recent publication using the same animal model and are part of the same study (10). Both parameters show a similar effect at 1000 ppm DIM and neither biomarker was significantly different from the mice fed DIM
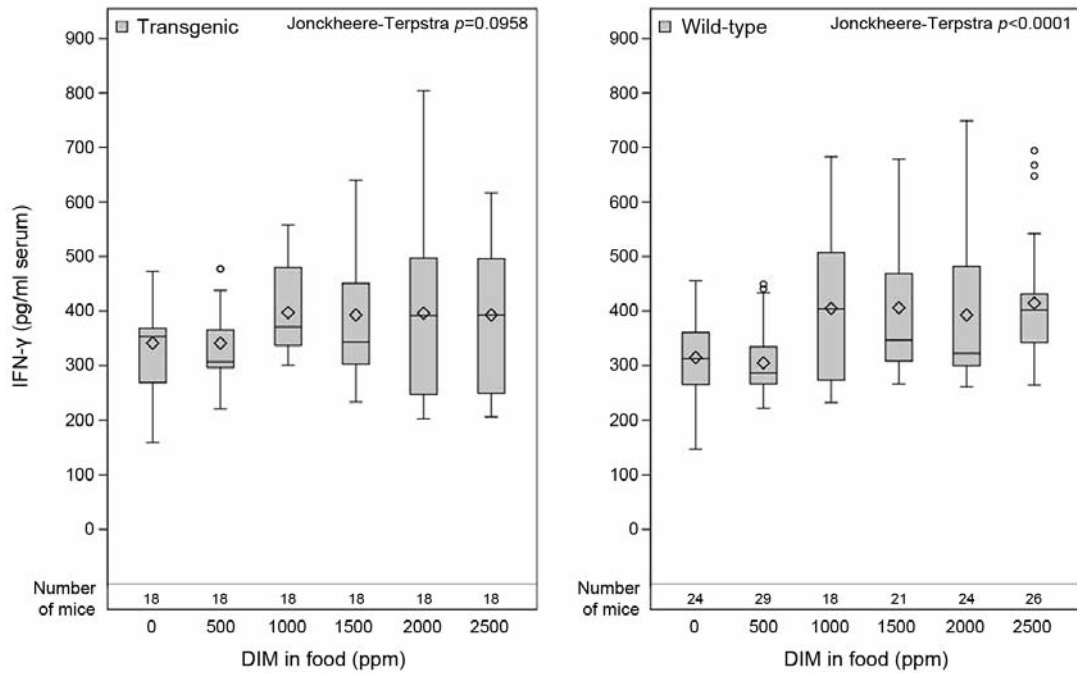
Figure 1. *IFN-γ levels in transgenic and wild-type mice receiving different doses if DIM continuously in food. In the transgenic mice there was a significant increase in IFN-γ concentrations (p<0.0369) at 1000 ppm DIM in food when compared to mice receiving 500 ppm DIM. There was no significant difference between the 0 ppm and the 500 ppm DIM fed mice.*
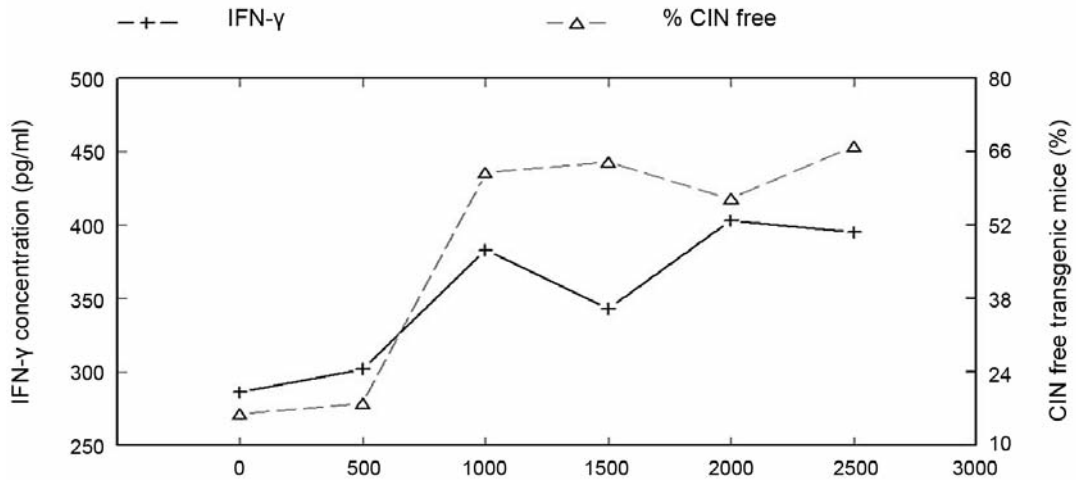

Figure 2. *Comparison of the mean number of CIN free mice in each DIM dose group to serum IFN-γ concentrations from that respective group. The histology of CIN free mice has been previously published (8, 10). Number of mice: 0 ppm n=7, 1000 ppm n=11, 1500 ppm n=12, 2000 ppm n=11, 2500 ppm n=13.*

at higher DIM levels. There was a strong correlation between the percentage of CIN free mice and serum IFN-γ serum concentrations (r=0.88). The overall correlation with all IFN-γ concentrations per group was r=0.67. The correlation coefficient between the percentage of mice with CIN in each group and serum IFN-γ concentrations was r=0.35.

Serum estrogens. Results for serum estrogens are presented in Table I. Since the Jonckheere-Terpstra test had indicated that there was a significant trend in E_1 and estriol (E_3), for exploratory purposes we further assessed the successive non-zero DIM dose levels for minimum effective dose with the Hochberg procedure.

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Table I. *Serum concentrations (ng/ml) of estrogen metabolites in K14-HPV16 transgenic mice. All concentrations are presented as the median (IQR: 25th-75th percentile). Jonckheere-Terprstra test for trend was conducted across all (DIM) dose levels for each estrogen metabolite. *p<0.05 was considered significant.*

Estrogen metabolite	DIM (ppm)						*p*-Value
	0	500	1000	1500	2000	2500	
E_1	281.8 (144-2-513.8)	340.6 (267.9-476.1)	170.6 ((81.5-293.8)	149.2 (105.0-214.8)	170.9 (121.1-196.7)	249 (137.1-365.8)	0.0332*
E_2	28.5 (0-47.7)	0 (0-34.8)	5.3 (0-45.1)	11.7 (0-39.5)	17.4 (0-58.7)	0 (0-0)	0.4209
E_3	0 (0-0)	0 (0-1.1)	0 (0-0.7)	0 (0-0.2)	0.5 (0-3.9)	0.2 (0-1.6)	0.0465*
2-OHE_1	2.6 (0-4.5)	4.1 (1.6-5.4)	2.2 (1.7-4.8)	4.6 (1.6-6.0)	3.9 (1.2-5.3)	1.9 (1.2-5.2)	0.691
4-OHE_1	2.6 (1.5-3.7)	1.3 (1.0-4.8)	1.9 (1.4-3.0)	4.0 (1.2-6.3)	4.2 (1.2-5.7)	3.5 (1.0-3.8)	0.4323
16-OHE_1	0.0 (0-7.9)	2.3 (0-11.3)	0.0 (0-1.3)	0 (0-19.5)	0.2 (0-7.7)	0 (0-0)	0.3695

Estrogen (E_1), estradiol (E_2), estriol (E_3), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1) and (16-OHE_1).

Comparisons between DIM treated and the untreated groups were conducted using the Mann-Whitney *U*-test. The *p*-values are unadjusted for multiple testing and are shown in Table I. E_1 and E_3 concentrations appear to have evidence of a significant trend with DIM dose, hence a modified Mann-Whitney *U*-test was conducted. Quantitatively, serum estrone concentrations were the highest of all metabolites. Lower concentrations of estrogens were observed when the 0 ppm group was compared to the1000 ppm DIM group ($p<0.0415$). Serum 16-OHE_1, 4-OHE_1 and 2-OHE_1 concentrations were unchanged in all groups

Discussion

This dose response study quantifies serum IFN-γ concentrations in response to increasing doses of DIM in the K14-HPV16 transgenic mouse model for cervical cancer. Possible changes in estrogen metabolism were also assessed. Significant increases in serum IFN-γ were noted in mice given DIM continuously in food at 1000 ppm over 20 weeks. Ruby *et al*. (15) and others (9,16) have shown similar elevations in BALBc mice administered with DIM. For the first time, the minimum effective dose of DIM that elicits an immune response has been identified (1000 ppm). This dose of DIM coincides with the most effective dose of DIM that resulted in a high percentage of mice with normal cervical epithelium (10). CIN free cervical epithelium and serum IFN-γ levels in this mouse model were also well correlated. IFN-γ is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections. Elevated IFN-γ levels caused by DIM may contribute to a more robust overall immune response to virus like particles that are commonly used in HPV vaccines. This observation will be helpful in studies, currently under way, that evaluate vaccination with HPV vaccines after continuous DIM feeding. We also investigated possible changes in estrogen metabolism that would be affected by long term continuous DIM administration. Estrogen exposure is a risk factor for cervical cancer (17, 18). In human studies and in some animal studies, short-term, or bolus administration of DIM caused an increase in C-2 hydroxylation of estradiol (9). No increases in C-2 hydroxylation were observed in this study. Continuous, long term administration of DIM in food (20 weeks) coupled with the implantation of E_2 time released pellets could represent two confounding factors in the determination of estrogen metabolism. Continuous DIM feeding (in combination with dietary components present in the AIN-93M diet) might enhance methylation of estrogen metabolites through induction of O methyl transferase. Smith *et al*. (25) have observed methylation of estrogen metabolites using PC-3 prostate cancer cells. In order to investigate the possibility that methylation occurs in this model, we plan to quantify methylated estrogen metabolites as part of our ongoing studies. No increases over control group values were observed in either 4-OHE_1 (a known carcinogen) or in 16-OHE_1 (an endogenous carcinogen) in any DIM treatment group. In a pilot study of shorter duration (12 weeks) and smaller sample size (n=10), we did observe increased levels of 2-OHE_1 after 12 weeks of Bioresponse DIM administration (BioResponse Inc, Boulder, CO, USA) (9). No other estrogen metabolites were altered. In both studies, there were no changes in other on metabolites. Both IFN-γ serum concentrations and cervical histology are useful biomarkers. Changes in cervical histology are apparent at 1000 ppm of DIM in food and represents the lowest dose that results in a high percentage of CIN free mice. At higher doses of DIM, there is no further increase in the proportion of mice that are CIN free (8, 10). IFN-γ concentrations parallel the histology of CIN free mice and also do not increase above 1000 ppm of DIM continuously fed in the diet. We have now defined the most effective minimum dose of DIM given continuously in food that will be used in future vaccination studies using the HPV mouse model. These results could contribute to new and effective vaccination strategies for women already infected with the human papilloma virus.

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