Effect of DENA induced hepatocarcinogenesis on neuroendocrine levels in male rats

A S Ghosh1 *, D Bhattacharyya2 †, M Chandra1 & T K Bhattacharyya1 ‡

1Department of Chemical Technology, University College of Science and Technology, Calcutta University, 92, A.P.C.Road, Kolkata 700 009
2Department of Chemistry, Bose Institute, Kolkata

Received 12 March 2007, revised 14 May 2008

Hepatocarcinogenesis was induced in Sprague Dawley rats by injecting diethylnitrosamine (DENA); 150 mg/kg body weight, ip, a well known liver carcinogen and a mutagenic agent. Concurrent with the induction of hepatocarcinoma, psychological stress was also elicited from the changes in brain neurotransmitters. Noradrenaline and dopamine, the neurotransmitters of sympathetic system were estimated from the whole brain and corresponding hormones T3, T4 and prolactin were estimated from the blood of such rats. The neuroendocrine cascade and the marker enzyme gamma glutamyl transferase were estimated at 7, 14, 21 and 30 weeks. A direct relationship between noradrenaline, T3 and T4 and a reciprocal relationship between dopamine and prolactin were observed, which may be correlated to the carcinogenic effect of DENA.

Keywords: DENA, Dopamine, GGT, Male rat, Noradrenaline, Prolactin, T3, T4

Carcinogenicity and mutagenicity of diethylnitrosamine (DENA) is well known1-11. Role of stress on various neurotransmitters, hormones and immunological functions in the genesis and progression of cancer has been reported12. Reports are available on stress as one of the dispositional factors in the clinical progression of malignant disease13.

Carcinogenesis may facilitate a neuroendocrine cascade which may lead to the growth of cancer14-16. The excitatory neuroendocrine action of noradrenaline correlates with the level of hormones, T3 and T4 because tyrosine is a precursor in the synthesis of T3 and T4 and the neurotransmitter, noradrenaline17. This cascade may also be responsible for reward reaction because the tricyclic antidepressant facilitates the increase of the noradrenaline at the receptor sites of CNS by inhibiting the reuptake of noradrenaline into the noradrenergic nerve terminals for a continuous flow of noradrenaline. Thus noradrenaline combats depression and elicits reward18. But with an increase of noradrenaline there may be an increase in sympathetic activity leading to stress. Since noradrenaline is a neurotransmitter of sympathetic nervous system, an excessive release of noradrenaline reveals increased emotional stress due to its increased antidepressant activity leading to aggression19.

Dopamine is an inhibitory neurotransmitter and responsible for punishment reaction. Punishment is a common psychological phenomenon and a causative factor for loss of skill leading to ego-disintegration and schizophrenia20. Most schizophrenias are treated with antidopaminergic medicine21. Increased dopamine mediates the decrease of prolactin and as such it is known to be a prolactin release inhibiting hormone19. Difference between excitatory and inhibitory neuroendocrine cascade in association with the extreme difference between reward and punishment reactions are responsible for generation of stress and be an important factor for the suppression of immunity which may lead to the development of cancer22.

Neurotransmitters have sufficient influence on the release of the endocrine cascade which in turn regulates the immunological functions of the host23. Thus the functions between cascade, noradrenaline, T3-T4 and dopamine and prolactin are assumed to originate from stress in the central nervous system24,25.
Various brain neurotransmitters have important effect on stress, depression, the immune system and cancer.\textsuperscript{26-28}

In the present work, it is proposed to study the role of noradrenaline, dopamine, T\textsubscript{3}, T\textsubscript{4} and prolactin on stress and neuroendocrinal changes in malignancy.

Increased level of GGT activity in the neoplastic changes of liver induced by DENA is already reported\textsuperscript{29,30}. Hence GGT is used as a marker enzyme to study the neoplastic changes in DENA induced hepatocarcinoma.

**Materials and Methods**

**Materials**—DENA and Tween 80 were purchased from Sigma Aldrich, USA. MgCl\textsubscript{2}, NaCl, n-butanol, heptane, ethylenediamine, glutamyl p-nitroanilide, glycylglycine, EDTA, KH\textsubscript{2}PO\textsubscript{4}, CH\textsubscript{3}COOH, Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}.5H\textsubscript{2}O and ascorbic acid were procured from BDH, Emerck, India. Radioactive I\textsuperscript{131}-T\textsubscript{3}, I\textsuperscript{131}-T\textsubscript{4} and I\textsuperscript{131}-Prolactin (RIA Kit) were purchased from Diagnostic Products Corp. (DPC), USA.

**Dose selection of DENA for hepatocarcinogenesis induction**—LD\textsubscript{50} of DENA (single dose) in Wistar male rats was reported as 280 mg/kg, when administered orally\textsuperscript{31} and 216 mg/kg, ip. The lowest toxic dose reported was 100 mg/kg, ip in rats\textsuperscript{32}. Hepatocarcinoma was reported by injecting DENA single ip dose of 150 mg/kg body weight\textsuperscript{33}. DENA induced hepatocarcinoma was also reported 100 mg/kg body weight dose administered once a week, for three weeks\textsuperscript{34}. Based on the LD\textsubscript{50} and earlier reported dose inducing hepatocarcinogenesis, in the present study the ip dose of 150 mg/kg body weight was selected.

Emulsion of DENA was prepared with 0.005% Tween 80 in 40% isopropanol (noncarcinogenic and nonmutagenic) and uniformly mixed by needle aspiration. All other materials used in the experiment were of analytical grade.

Male albino rats (Sprague Dawley) weighing 120-150 g were housed in animal room at 30\degree\pm2\degree C and 12:12 hr L/D cycle. Food and water were given ad libitum. Male rats were chosen for these experiments. Females were avoided due to neuro protective effects of estrogen\textsuperscript{35} which may in turn lead to the alterations of neuroendocrine changes.

Rats were divided into following two groups:

(i) DENA treated group: Rats (24) comprising of 4 subgroups of 6 each were administered (once, ip) with DENA (150 mg/kg) in 40% isopropanol with Tween 80 and sacrificed by decapitation at the end of 7, 14, 21 and 30 weeks respectively (indicating time of treatment).

(ii) Control group: Another group of rats (24) comprising of 4 subgroups of 6 each designated as controls were administered ip without DENA with same volume of emulsion with 0.005% Tween 80 in 40% isopropanol and sacrificed by decapitation at the end of same intervals of time.

After decapitation the brains and livers were removed, rinsed and blotted from animals from each subgroup. They were weighed and stored at −20\degree C for further study. Blood was collected immediately after decapitation and kept at 4\degree C for 3 to 4 hr, centrifuged at 2000 rpm for 10 min at cold room temperature. The supernatant was collected and stored in a cold room for hormone assay.

**Estimation of gamma glutamyl transferase (GGT) activity as a hepatocarcinogenic marker**—Livers from DENA treated and control groups were rinsed with buffer (250 mM sucrose, 10 mM triethanolamine, 1 mM EDTA-Na\textsubscript{2}, pH 7.6) and homogenized in 3 ml of buffer/g tissue\textsuperscript{36}. Homogenates were centrifuged at 15,000 g for 5 min. The resulting supernatant (postmitochondrial tissue homogenate) was stored at −20\degree C and the homogenate was used for the assay of GGT. Enzymatic activities were normalized to protein concentrations, and determined by Bradford assay\textsuperscript{37} with bovine serum albumin as standard. Results indicated that cross-contamination with mitochondria, lysosomes, and plasma membranes was <2%.

GGT activity was measured by the method of Strommer \textit{et al}\textsuperscript{38} with glutamyl p-nitroanilide as substrate and glycylglycine as acceptor. Tissues aliquots (0.5 ml) were incubated with 1.5 ml substrate mixture in a cuvette at 37\degree C. The standard final reaction mixture contained 4 mM glutamyl-p-nitroanilide, 10 mM MgCl\textsubscript{2} and 100 mM Tris-HCl (pH 7.6). The reaction rate was measured in a recording spectrophotometer at 405 nm. After 3 min, 50 µl of 50 nM glycylglycine was added and reaction was recorded for another 5 min. One unit of enzyme activity is equivalent to 1 µM of substrate transformed/min at 37\degree C and was calculated with molar extinction coefficient for p-nitroanilide\textsuperscript{38} as 10.820 × 10\textsuperscript{6}.

**Brain amine determination**—Rats were sacrificed by decapitation without anesthesia, since anesthesia can alter the level of brain amines\textsuperscript{39}. The brains were
rapidly removed, blotted free from blood, quickly frozen in ice and stored at –20°C. For amine determination the frozen brains were weighed and after thawing they were homogenized with 1.5 ml ice cold 0.01N HCl and 0.1 ml 10% EDTA in a motor driven glass homozenizer containing teflon coated pestle. To the homogenate, 25 ml n-butanol and 4 g NaCl were added. The mixture was shaken for 10 min, centrifuged and kept at room temperature for 20 min. To the supernatant 24 ml n-butanol, 40 ml n-heptane and 1.5 ml 0.5M phosphate buffer (pH 7.3) were added, shaken for 10 min and settled for 10 min, 1.5 ml phosphate buffer layer was taken. It was acidified to pH 3.5 to 4.0 with 3N HCl. Peroxide free ether (20 ml) was added to it and shaken for 10 min. Aqueous acid layer (0.5 ml) was taken for fluorimetric estimation of noradrenaline and dopamine etc., following the method of Welch and Welch40.

**Estimation of noradrenaline**—To 0.5 ml of the sample, 0.5 ml of 2M acetate buffer (pH 6.8), 0.1 ml of 0.1N iodine solution, 0.2 ml of alkaline ascorbic acid ethylene diamine solutions were added. The final volume was 1.45 ml, and the fluorescence was read at 400 nm excitation and 510 nm emission using 2 to 3 nm slit.

**Estimation of dopamine**—Brain extract sample (0.5 ml) was taken. To it 0.5 ml of 2M acetate buffer (pH 6.8), 0.1 ml of 0.1N iodine solution, 0.25 ml of glacial acetic acid/con. HCl (1:1) were added. The test tubes were placed in boiling water bath for 45 min, cooled and fluorescence readings were taken at 325 nm excitation and 380 nm emission using 2 to 3 nm slit.

**Hormone determination**—T341, T442 and prolactin43 were measured by radioimmunoassay (RIA) from blood serum. Standard graph for the hormones T3, T4 and prolactin were prepared by using different concentrations of the radiolabelled (I131) hormones and there radioactivities were plotted against the concentration. Extracted hormones from the blood were made radioactive using the methods of Hunter and Greenwood44 and the concentrations of the hormones were extrapolated from the standard graph. Radioactivity was measured by auto gamma counter and ELISA reader.

**Statistical analysis**—Statistical analysis of results was done by one-way ANOVA45.

---

**Results and Discussion**

GGT level of DENA treated group increased even at 30 weeks of study (Table 1) but not at the same rate when compared with the control indicating progressing of hepatic carcinogenesis proportionately comprising with GGT level (P<0.01). The result suggests that hepatocarcinogenesis stimulates the GGT level almost in a sigmoidal pattern with respect to increasing intervals of time.

Noradrenaline level of whole rat brain and T3/T4 level of blood serum from DENA treated group at different time intervals are presented in Tables 2 and 3 respectively.

**Table 1**—Activity of gamma glutamyl transferase (GGT) in liver tissue homogenate (U/g protein)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Control</th>
<th>DENA treated</th>
<th>Stimulation fold (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 weeks</td>
<td>7.34±1.84</td>
<td>20.56±2.32</td>
<td>~2.8 178.1±6.9</td>
</tr>
<tr>
<td>14 weeks</td>
<td>6.56±3.22</td>
<td>31.04±0.99</td>
<td>~4.7 370.8±13.4</td>
</tr>
<tr>
<td>21 weeks</td>
<td>6.89±2.57</td>
<td>50.71±3.36</td>
<td>~7.4 637.2±36.5</td>
</tr>
<tr>
<td>30 weeks</td>
<td>7.01±3.15</td>
<td>57.30±4.83</td>
<td>~8.2 705.3±40.7</td>
</tr>
</tbody>
</table>

P<0.01

**Table 2**—Noradrenaline levels (ng/g) of whole rat brain

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Control</th>
<th>DENA treated</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 weeks</td>
<td>4092.0 ± 442.8</td>
<td>4818.3 ± 325.7</td>
<td>17.2±1.7</td>
</tr>
<tr>
<td>14 weeks</td>
<td>4158.3 ± 344.3</td>
<td>5220.8 ± 426.4</td>
<td>24.5±2.3</td>
</tr>
<tr>
<td>21 weeks</td>
<td>4210.5 ± 584.3</td>
<td>5330.6 ± 608.2</td>
<td>27.0±3.5</td>
</tr>
<tr>
<td>30 weeks</td>
<td>4240.2 ± 212.7</td>
<td>4919.7 ± 236.6</td>
<td>15.7±2.4</td>
</tr>
</tbody>
</table>

P<0.001

**Table 3**—T3 and T4 levels in blood serum (ng/dl)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Control</th>
<th>DENA treated</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 weeks</td>
<td>T3 80.2 ± 5.1</td>
<td>92.1 ± 2.2</td>
<td>15.2±1.8</td>
</tr>
<tr>
<td>T4 45.3 ± 10.3</td>
<td>51 ± 10.9</td>
<td>13.6±4.3</td>
<td></td>
</tr>
<tr>
<td>14 weeks</td>
<td>T3 72.5 ± 3.8</td>
<td>88.0 ± 7.4</td>
<td>21.5±2.5</td>
</tr>
<tr>
<td>T4 42.0 ± 9.2</td>
<td>50.0 ± 8.4</td>
<td>19.3±2.8</td>
<td></td>
</tr>
<tr>
<td>21 weeks</td>
<td>T3 75.7 ± 4.4</td>
<td>94.3±6.0</td>
<td>25.1±3.1</td>
</tr>
<tr>
<td>T4 38.0 ± 16.2</td>
<td>48.0 ± 7.8</td>
<td>27.4±5.0</td>
<td></td>
</tr>
<tr>
<td>30 weeks</td>
<td>T3 78.0 ± 2.2</td>
<td>93.8±4.3</td>
<td>19.8±0.8</td>
</tr>
<tr>
<td>T4 41.0 ± 13.1</td>
<td>47.0 ± 9.6</td>
<td>14.7±1.9</td>
<td></td>
</tr>
</tbody>
</table>

P < 0.001(T3); < 0.005(T4)
Levels of prolactin from DENA treated rats were significantly increased with increase of time as compared with control but the stimulation showed a maximum increase (265.2%) at 21 weeks whereas stimulation showed a decrease (177.8%) at 30 weeks of interval ($P<0.005$; Table 5).

Since noradrenaline and dopamine can not cross the blood-brain barrier from peripheral region (liver/kidney), the noradrenaline/dopamine reported here is only from whole rat brain and not affected by peripherally derived dopamine/noradrenaline from liver and kidney.

Noradrenaline and dopamine are directly and inversely related with the growth of cancer respectively, but noradrenaline slightly rises at the middle stages (Table 2), perhaps this may be proportionately instrumental for stimulation of the endocrine network like $T_3/T_4$ (Table 3) because tyrosine is the basic precursor for both. The rapid growth of cancer can be attributed for the rise of both noradrenaline and $T_3$, $T_4$. (A)$\Rightarrow$ (Noradrenaline $\leftrightarrow T_3$ or $T_4$). Thus the excess of noradrenaline – $T_3$, $T_4$ is responsible for reward reaction. But in the present study, an increased surge of noradrenaline – $T_3$, $T_4$ in the hepatocarcinogenic model stimulated in a time-dependent manner and attained a level off stage at 21st weeks of study. Therefore inference can be made in such carcinogenesis as there is an increased level of noradrenaline–$T_3$, $T_4$ is responsible for reward reaction which was developed from DENA induction. Consequently, there is an exaggeration of reward reactions, a disposition which may be designated as narcissistic personality disorder in human being as stressed by Kohut Heinz in which restoration of self is lost, causing a stress. Otherwise it may also be explained as a stress caused by sympathetic activation and may be elicited by the excitatory neurotransmitter, noradrenaline.

The lesser dopamine level (Table 4) indicates, lower the dopamine level higher is the emergence of cancer taking into consideration of the whole brain of the rat. Buckley et al reported that prolactin is an endogenous tumor promoter for chemically initiated cells with a hepatocarcinogen (12-O-tetradecanoyl-phorbol-13-acetate) followed by 6 weeks of increase in ovine prolactin resulting in hepatomegaly and the development of enzyme-altered foci. Promotion with prolactin for 23 weeks further increased the numbers of enzyme-altered foci. Thus prolactin hormone might likewise promote neoplasia.

Prolactin has significant influence in the genesis of cancer. The rise in serum prolactin level, generated from brain stimulated oncogenesis as apparent from the results (Table 5).

Secretion of prolactin by the pituitary is under predominantly negative control by the hypothalamus. A prolactin release inhibiting hormone (PRIH) is carried to the adenohypophysis where it inhibits prolactin secretion. PRIH may in fact be dopamine, and as such prolactin is under dopaminergic control and are inversely related. In the experiment dopamine and prolactin show a reciprocal relationship for a certain period of time and when dopamine level depleted prolactin level went up. Prolactin is known to induce cancer tumors and as such the rise in prolactin level may be regarded as tumorigenic in the experiment.

(B)$\Rightarrow$ (dopamine $\leftrightarrow$ Prolactin).

Thus carcinogenic condition also involves stress due to altered neurotransmitter levels and concurrent release of hormones for the stimulation of cancer.

---

### Table 4—Dopamine levels (ng/g) of whole rat brain

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Control</th>
<th>DENA treated</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 weeks</td>
<td>9022.2 ± 231.7</td>
<td>7619.8 ± 286.3</td>
<td>15.1±3.5</td>
</tr>
<tr>
<td>14 weeks</td>
<td>9431.3 ± 567.5</td>
<td>6225.9 ± 436.4</td>
<td>34.5±4.3</td>
</tr>
<tr>
<td>21 weeks</td>
<td>9400.0 ± 675.1</td>
<td>4794.2 ± 305.7</td>
<td>46.8±4.8</td>
</tr>
<tr>
<td>30 weeks</td>
<td>8638.6 ± 324.4</td>
<td>6652.8 ± 243.2</td>
<td>21.9±2.9</td>
</tr>
</tbody>
</table>

$P<0.005$

### Table 5—Prolactin level in blood serum (ng/ml)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Control</th>
<th>DENA treated</th>
<th>Stimulation fold</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 weeks</td>
<td>10.12± 1.53</td>
<td>20.64 ± 4.52</td>
<td>~ 2.0</td>
<td>101.4± 8.7</td>
</tr>
<tr>
<td>14 weeks</td>
<td>12.96 ± 0.58</td>
<td>40.82 ± 3.46</td>
<td>~ 3.3</td>
<td>210.9± 18.5</td>
</tr>
<tr>
<td>21 weeks</td>
<td>11.45 ± 2.57</td>
<td>42.19 ± 2.58</td>
<td>~ 3.9</td>
<td>265.2± 23.2</td>
</tr>
<tr>
<td>30 weeks</td>
<td>8.94 ± 3.52</td>
<td>25.21 ± 0.01</td>
<td>~ 3.0</td>
<td>177.8± 20.4</td>
</tr>
</tbody>
</table>

$P< 0.005$
Thus it confirms Burnet’s concept of immunosurveillance in which he proposed a psychoendocrine pathway for stress, depressed immunosurveillance and increased incidence of cancer. Thus in the DENA treated group the carcinogenic conditions indicates the involvement of stress due to altered neurotransmitter levels and the concurrent release of the corresponding hormones.

DENA induced hepatic carcinogenicity can lead to neuroendocrinal changes as it is evident from A and B. Thus from the results it is apparent that increase in excitatory noradrenaline or increase in inhibitory dopamine may lead to generation of emotional stresses and the corresponding neuroendocrine changes arising from DENA induced hepatocarcinogenic model. The present experiment involves the excitatory and inhibitory neurotransmitters in the whole brain of the rat.

Increasing level of noradrenaline with T3 and T4 (Tables 2 and 3) when compared with decreasing level of dopamine and increased prolactin (Tables 4 and 5) respectively from whole brain indicates an imbalance of reward and punishment reactions mediated by the difference between excitatory and inhibitory neurotransmitters. Thus an excess of punishment and a decrease of reward reactions may also be held responsible for the difference between the excitatory and inhibitory neuroendocrine cascades, as reported in human beings, and obtained from an experimental rat model on neuroendocrinal changes.

Decreasing level of dopamine in DENA treated group (Table 4) suggested that there is a probable antagonism by increased level of prolactin (Table 5). Prolactin, a natriuretic hormone interacts with the renal dopamine system and interacts with dopamine in the brain. To reinforce this finding, high level of prolactin is also one of the causes of breast cancer as reported in female rats.

There was an increased level of noradrenaline and decreased level of dopamine though tyrosine is the basic precursor for both dopamine and noradrenaline then the increased dopamine will be followed by an increased synthesis of noradrenaline. The present experiment conducted on whole brain of the rats and not exclusive to certain specific receptor of a brain part and if there is an increased synthesis of dopamine in some noradrenergic nerve terminals then there is a concurrent increase of noradrenaline. It may also be true that in certain excitatory area of brain parts there is an increased noradrenaline level followed by an increase of inhibitory dopamine.

Change (%) of neuroendocrine levels at 30 weeks of study were shown to be decreased than that of 21 weeks. This was because of sickness, developed for prolong suffering from carcinogenic condition induced by DENA and hence the rate of biosynthesis of neuroendocrine was decreased whereas exception was found in case of dopamine and GGT level.

Hepatocarcinogenesis may induce the neuroendocrine changes via mediating some signal(s) in brain in such DENA induced model. Besides its known carcinogenicity, DENA may also be responsible for the neuroendocrine changes if it crosses the blood-brain barrier and binds with brain lipid and it may demand further study.

It can be concluded that the neuroendocrine cascade and the marker enzyme GGT as estimated at an interval of 7 weeks within the tenets of the time of 7, 14, 21 and 30 weeks indicating a direct relationship between noradrenaline, T3 and T4 and reciprocal relationship between dopamine and prolactin and both of these neuroendocrine cascades are due to the effect of hepatocarcinogenesis induced by DENA.

Acknowledgement
Thanks are due to CSIR, New Delhi for financial assistance and to Prof Parimal C Sen, Department of Chemistry, Bose Institute, Kolkata for help.

References
8 Druckrey H, Chemical structure and action in transplacental carcinogenesis and teratogenesis, IARC Sci Publ (Lyon, France), 4 (1973) 45.
9 Druckrey H, Specific carcinogenic and teratogenic effects of indirect alkylating methyl and ethyl compounds, and their dependency on stages of oncogenic development, Xenobiotica, 3 (1973) 271.
10 Peto R, Gray R, Brantum P & Grasso P, Nitrosamine carcinogenesis in 5120 rodents: Chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4440 inbred rats, with parallel studies on NDEA alone of the effect of age starting (3, 6 or 20 weeks) and of species (rats, mice, hamsters), IARC Sci Publ (Lyon, France), 57 (1984) 627.
11 Montesano R & Bartsch H, Mutagenic and carcinogenic N-Nitroso compounds: Possible environmental Hazards, Mutat Res, 32 (1976) 179.
16 Ericson G, Neurotransmitters signal aggressive cancer, offer potential for early diagnosis, Record (Washington University in St. Louis), August 12 (2005).


