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Evidence of free radical participation in N-glycolylneuraminic acid generation in liver of chicken treated with gallotannic acid

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The occurrence of N-glycolylneuraminic acid (Neu5Gc) in cancerous tissue and inflammatory diseases, conditions associated with increased oxidative stress suggests the participation of reactive oxygen radicals in Neu5Gc generation, where an oxygen atom is transferred. To study this possibility, we treated two groups of domesticated birds and rabbits with different dosages of gallotannic acid (GTA), a compound known to cause generation of reactive oxygen species (ROS). The antioxidant status and leukocyte capacity, as well as amount and form of sialic acids were assessed in plasma and liver. Results showed that while lipid peroxides were increased, white blood cell (WBC) count was decreased significantly in all treated groups. The increased sialic acids and low protein contents were observed in plasma, possibly as a result of decreased sialic acid cycling crucial for formation of new glycoconjugates in tissues, caused by decreased protein synthesis due to microsomal degranulation. The activities of antioxidant enzymes were also decreased in treated groups, implying increased oxidative stress. The presence of Neu5Gc and apparent absence of Neu5Ac hydroxylase activity in liver of chicken treated with GTA indicate that free radicals might be involved in the non-enzymatic hydroxylation of N-acetylneuraminic acid (Neu5Ac) to Neu5Gc in liver, which normally does not express this sialic acid.

Key words: Gallotannic acid, N-glycolylneuraminic acid, oxidative stress

Sialic acids are important molecules found on cell surface and in secreted glycoconjugates. Their unique distribution in different species, cell types and developmental stages suggest biologically significant functions[1]. They play an important role in endogenous cell-cell recognition as well as host cell-pathogen interaction[2-4]. About 50 forms of these amino sugars derived from neuraminic acid are known and their diversity arises from enzymatic *O*-acetylation, *O*-methylation, *O*-lactylation and sulfation of hydro-xyl groups at positions 4, 7, 8 and 9 of the molecule[4]. The most commonly found forms of these sialic acids are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc).

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Abbreviations: CMAH, CMP-N-acetylneuraminic acid hydroxylase; CMP, cytidine monophosphate; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); EDTA, ethylene diammine tetraacetic acid; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GTA, gallotannic acid; Neu5Ac; N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); PBS, phosphate buffered saline; RBCs, red blood cells; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TBA, thiobarbituric acid; TLC, thin layer chromatography; Tris, [2 amino 2 (hydroxymethyl) propane 1,3 diol]; WBCs, white blood cells.

Neu5Gc is, however, rarely detectable in normal human and chicken tissues[5,6] and is absent in glycolipids of neural tissues of mammals[5]. Interestingly, its presence in traces in certain human and chicken cancer as well as inflammatory diseases has been demonstrated in immunoassays, using Neu5G-specific antibodies and gas chromatography coupled with mass spectrometry[7-9]. This has generated interest and effort towards establishing the mechanism involved in Neu5Gc expression in mammalian tissues. Studies with porcine mandibular gland and murine liver have shown that a soluble multicomponent hydroxylase system is involved in the hydroxylation process that seems to be specific for cytidine monophosphate (CMP) Neu5Ac, free Neu5Ac and glycoconjugate bound Neu5Ac[10-12], as substrates. While the gene for CMP-Neu5Ac hydroxylase (CMAH) is shown to be active in murines and primates, in humans, it has undergone frame shift mutation rendering the enzyme inactive[13,14]. This molecular evidence not only explains the apparent lack of Neu5Gc in human tissues, but also suggests an unknown alternate source of Neu5Gc encountered in cancerous tissues and inflammatory diseases[7]. Chicken is also known to express Neu5Gc in Marek's disease lymphoma[6].

The present study is carried out to explore the possible origin of Neu5Gc, presently established to

exist in traces, in certain pathological cases of humans and chicken[6]. To achieve this, gallotannic acid (GTA), a phenolic compound hydrolysable to glucose and gallic acid[15], was used as it is reported to cause liver cancer[16]. We treated two groups of domesticated birds and rabbits with different dosages of GTA and assessed the antioxidant status and leukocyte capacity, as well as amount and form of sialic acids in plasma and liver. *In vitro* studies with microsomes have shown that it is metabolized into potential carcinogens[17]. GTA and its degradation product, gallic acid are known to induce DNA strand breakages[18]. Its potential to cause free radical generation in tissues[19] was crucial to this study in order to assess the possible association of the resulting biochemical responses with generation of Neu5Gc.

Materials and Methods

White Plymouth domesticated birds (8 months old) were obtained from Punjab Poultry Breeding Institute, Industrial Area, Chandigarh, Punjab. Belgian white rabbits (8 months old) used were obtained from Central Animal House, Panjab University, Chandigarh. Bovine whole blood was kindly donated by Punjab Meat Processors, Dera Bassi, Punjab and human blood was obtained from willing donors.

All chemicals used were of analytical grade (unless stated otherwise). N-acetylneuraminic acid (Neu5Ac) and Bovine serum albumin (BSA) were purchased from Himedia Laboratories Ltd and Sisco Research Laboratories Ltd., India, respectively. Solvents were purchased from E. Merck, India.

Treatment procedure

Two groups of 3 male birds (8 months old) each were treated with gallotannic acid (GTA) in an emulsion of Freunds incomplete adjuvant (FIA) to achieve slow release of GTA. Group I was given 50 mg/kg body wt weekly for 9 weeks, while group II received a single dose of 250 mg/kg body wt, intraperitoneally. Two groups of rabbits were treated similarly, but with higher doses of 250 mg/kg body wt (Group I) and 500 mg/kg body wt. (Group II) in order to compare mammalian and avian response to GTA.

Drawing blood from chicken and rabbits

Blood (9 parts) was drawn from ulna and marginal vein of chicken wings and rabbits ears, respectively into 1 part of anticoagulant (0.115 *M* EDTA, *p*H 7.4) once weekly and plasma was obtained by centrifuging blood at 800 *g* for 10 min. RBCs were washed thrice with 3 vols of 0.9% saline solution and thereafter lysed with 9 vols of distilled water. Aliquots (1 ml) of RBC lysate were stored frozen for enzyme assays.

All birds and rabbits in group II were sacrificed after 3 weeks, while controls and group I after 9 weeks. Animals were fasted overnight and sacrificed by surgical removal of organs under anesthesia.

White blood cell (WBC) count

The test tubes containing drawn blood were incubated, inclined at 37°C for 30 min and plasma (1 ml) containing WBCs was sucked off from the erythrocyte layer into a centrifuge tube. Three vols of 0.87% NH_4Cl solution were added and contents shaken gently for 5 min to lyse the contaminating erythrocytes and centrifuged at 500 g for 3 min and supernatant discarded. The sediment which contains WBCs was washed with 3 ml cold PBS and then suspended in 1 ml ice-cold Tyrode's solution. WBC suspension (20 µl) was mixed with WBC diluting fluid (380 µl) and contents allowed to stand for 5 min to stain the nucleus. The diluted WBC suspension (10 µl) was pipetted into the Nubauer chamber (haemocytometer) and WBCs counted under 500x magnification.

Preparation of liver fractions

This was done using the method as described earlier[12]. Liver tissues were sliced and the small pieces homogenized in the cold (4°C) with 0.01 *M* Tris-HCl buffer (*p*H 7.4) to give a 25% w/v homogenate. Nuclear fraction was obtained by centrifuging homogenate at 1000 *g* for 15 min. The post-nuclear fraction was, thereafter, centrifuged at 10,000 *g* for 20 min to sediment mitochondria and post-mitochondrial supernatant was treated with 8 m*M* $CaCl_2$ to sediment microsomes as reported earlier[20]. Adequate aliquots of all fractions prepared were stored frozen for enzyme and metabolite estimation.

Estimation of lipid peroxides

Lipid peroxides were estimated using the method as described[21]. To 0.2 ml sample, 0.2 ml 8.1% sodium dodecyl sulphate (SDS) was added, followed by 1.5 ml 20% acetic acid (*p*H 3.5). The contents were mixed and 0.8% TBA (1.5 ml) added and made to 4 ml with distilled water. The mixture was heated in water bath at 95°C for 1 hr. The contents were cooled and distilled H_2O (1 ml) was added, followed by 5 ml butanol/pyridine (15/1 v/v). The mixture was shaken

vigorously and centrifuged at 3000 *g* for 10 min. The organic layer was read at 532 nm against blank with homogenizing buffer instead of homogenate.

Estimation of sialic acids

Total sialic acids content was estimated in plasma and liver homogenate, using the method described earlier[22]. The sample (1 ml) was mixed with 0.25 ml 0.5 *N* H_2SO_4 (0.25 ml) and heated at 80°C for 1 hr. The solids were sedimented at 10,000 *g* for 20 min. To supernatant (0.4 ml) distilled water (0.4 ml) was added followed by 0.2 ml 4.3% sodium periodate in 1 *N* H_2SO_4. The tubes were incubated for 30 min at 37°C and 0.2 ml 2% sodium arsenite in 0.5 *N* HCl was added. The contents were shaken until the brown colour disappeared and 0.4 ml 6% TBA added and heated in water bath for 8 min. The colour developed was extracted with 1 ml of dimethylsulphoxide and read at 549 nm against blank and Neu5Ac standard.

For TLC analysis of sialic acids, 2 ml of plasma and liver homogenate (25% w/v) were mixed with 0.5 N H_2SO_4 (0.5 ml) separately and contents incubated for 1 hr at 80°C. The sediment obtained was centrifuged at 10,000 *g* for 20 min and supernatant sucked out and then neutralized with NaOH and concentrated by lyophilization. Analysis of concentrated sialic acids was done on cellulose-coated plates. The plates were developed in *n*-propanol/*n*-butanol/0.1 *M* HCl (2/1/1 v/v) solvent and thereafter spots visualized with resorcinol/HCl reagent.

Estimation of reduced glutathione and total thiols

This was done following the method of Ellman[23], as modified by Setlak and Lindsay[24]. The reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by thiol groups gives 2-nitro-5-mercaptobenzoic acid having an intense yellow colour, which absorbs maximally at 412 nm. The absorbance values were used to calculate content of thiol groups and contents expressed in nmols per mg protein.

Estimation of enzyme activities

Neu5Ac hydroxylase activity was determined as in the method of Mukuria *et al*[12], wherein 0.2 ml prepared sample was used in the reaction mixture consisting of 10 m*M* Tris-HCl (*p*H 7.4) containing 1 m*M* GSH, 7.75 m*M*, Neu5Ac and 6 m*M* NADPH. The contents were incubated at 37°C and the decrease in absorbance monitored at 340 nm for 15 min against reference with no Neu5Ac. The time course of the reaction was monitored using double beam spectrophotometer.

Proteins in the prepared samples were determined using the method of Lowry *et al*[25].

Absence of Neu5Ac hydroxylase activity in chicken liver treated with GTA was determined by taking aliquots (2 ml) of post-mitochondrial supernatant from both rabbit and chicken liver and dialysing at 4°C against 3 changes of 10 m*M* Tris HCl (*p*H 7.4) containing 0.1 m*M* GSH and 1 m*M* EDTA for 24 hr to remove endogenous substrates. The dialysate (0.5 ml) was incubated at 37°C with 0.3 ml 10 m*M* Tris HCl buffer (*p*H 7.4) containing 1 m*M* GSH, 7.75 m*M* Neu5Ac and 9.5 m*M* NADPH in a total vol. of 1.0 ml for 2 hr. The reaction was stopped by addition of ice-cold ethanol (2 ml) and the precipitated proteins sedimented at 2000 *g* for 15 min. The supernatant was vacuum evaporated and residue resuspended in 200 µl of distilled water. Analysis of the extracted sialic acids was done on cellulose coated glass plates. The plates were developed in *n*-propanol/*n*-butanol/0.1 *M* HCl (2/1/1 v/v) solvent and spots visualized with resorcinol/HCl reagent.

GR activity was estimated in plasma, erythrocyte lysate and post-mitochondrial supernatant (PMS) of liver from chicken and rabbits[26]. The decrease in absorbance with time due to oxidation of NADPH (molar extinction coefficient, 6.22 M^{-1} cm^{-1}) was monitored at 340 nm and taken to be a direct measure of enzyme activity[27].

GPx assay was done as described[28]. GSH was estimated before and after incubating enzyme reaction mixture for 60 sec[24]. GPx activity was determined by calculating the difference in concentration of GSH at start and end of reaction. Activity was expressed in nmols of GSH consumed/min/mg protein.

SOD activity was assayed using the method of Kono[29]. The assay was based on the ability of SOD to inhibit reduction of nitro blue tetrazolium (NBT) by superoxide radicals, formed by hydroxylamine hydrochloride. The time spent for a fixed absorbance decrease was monitored at 540 nm and units of enzyme activity calculated from concentration of enzyme that gives half-maximum inhibition.

Catalase (CAT) activity was estimated as described[30]. The rate of decomposition of hydrogen peroxide (H_2O_2) was measured spectrophotometrically at 240 nm and one unit of catalase taken to be the quantity of enzyme that liberates half the peroxide oxygen from a H_2O_2 solution of any concentration in 100 sec at 25°C.

Results

Preliminary assessment of the oxidative status of chicken and rabbits was carried out during treatment with GTA by estimating lipid peroxides content and glutathione reductase (GR) activity weekly in blood. It was observed that while lipid peroxides content in plasma increased in all treated cases (Fig. 1), group II showed decreased GR activity in RBCs (Fig. 2A). Though the activity of this enzyme was seen to be comparatively low in plasma than RBCs, higher activity was observed in plasma of this same group than controls during the treatment period (Fig. 2B). White blood cell count also monitored during this period was low in treated groups (Fig. 3), but in contrast, sialic acid content in plasma increased significantly with dosage of GTA (Fig. 4). Further assessment of the oxidative status was carried out in liver of both chicken and rabbits immediately after the treatment period. As shown in Tables 1 and 2, lipid peroxides content was increased and GR activity decreased in all treated groups, except, group I rabbits, which showed 30% increase in GR activity.

The sialic acids level, unlike in plasma, was reduced by 30% in group II rabbits, while in group II chicken, a 31% increase was observed (Table 1). To determine whether Neu5Ac and Neu5Gc were present in liver and plasma of chicken and rabbits, TLC was performed on cellulose plates. It was observed that rabbit sialic acid isolates showed spots characteristic

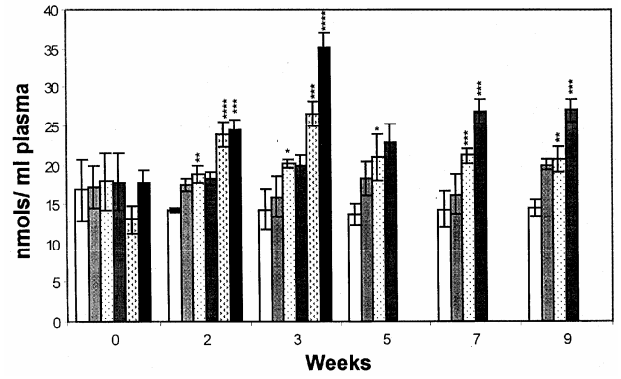

Fig. 1—Lipid peroxides content in plasma of chicken and rabbits treated with GTA [This was determined as described in 'Materials and Methods'. The red colour formed from reaction between peroxide products and TBA (extinction coefficient; 156000 mM^{-1} cm^{-1}) was read at 532 nm and absorbance values used to calculate the quantity of peroxides. Data was expressed as mean ± SD (n=3); (☐) Control rabbit; (▩) Control chicken; (▨) Group I rabbit; (■) Group I chicken; (▨) Group II rabbit; (■) Group II chicken. Significance with respect to control: *p<0.05; **p<0.01; ***p<0.005; ****p<0.001]

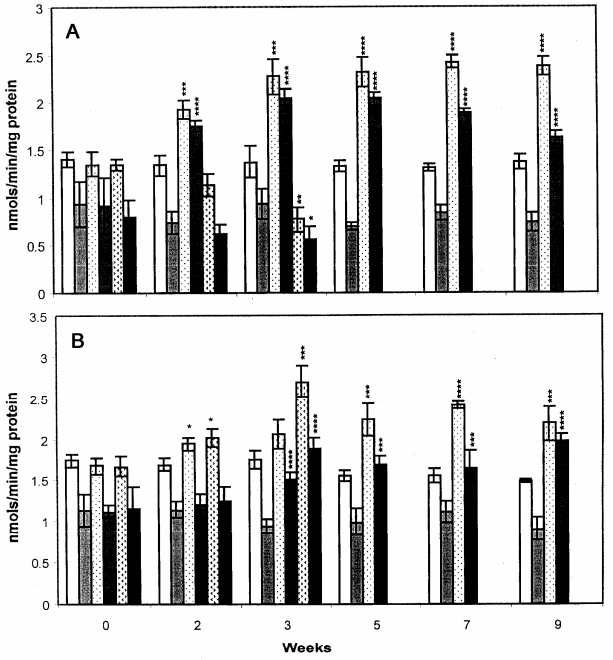

Fig. 2—Glutathione reductase activity (A) in RBCs of chicken and rabbits treated with GTA (B) in plasma of chicken and rabbits treated with GTA [The reaction was initiated by adding 0.6 mM GSSG in a reaction mixture containing 0.1 mM NADPH, 0.05 ml erythrocyte lysate and 0.067 M phosphate buffer containing 0.5 mM EDTA in a total volume of 3.0 ml. The rate of oxidation of NADPH was determined by monitoring change in absorbance at 340 nm against control without enzyme; (☐) Control rabbit; (▩) Control chicken; (▨) Group I rabbit; (■) Group I chicken; (▨) Group II rabbit; (■) Group II chicken. Significance with respect to control: *p<0.05; **p<0.01; ***p<0.005; ****p<0.001]

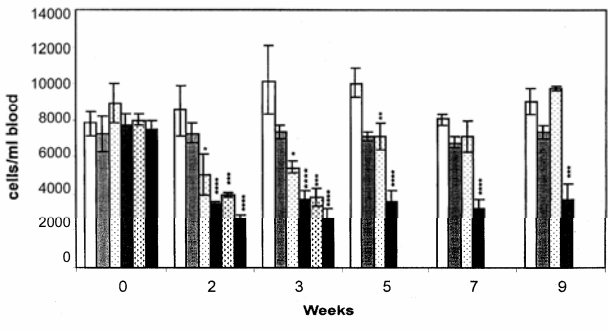

Fig. 3—White blood cell count in blood of rabbits and chicken treated with GTA [WBCs from chicken were isolated from whole blood as described in 'Materials and Methods'. 20 µl of chicken WBC and rabbit whole blood were mixed with 380 µl of dilution fluid separately and 10 µl of mixture pipetted into Nubauer's haemocytometer for counting at 500x magnification; (☐) Control rabbit; (▩) Control chicken; (▨) Group I rabbit; (■) Group I chicken; (▨) Group II rabbit; (■) Group II chicken. Significance with respect to control: *p<0.05; **p<0.01; ***p<0.005; **** p<0.001]

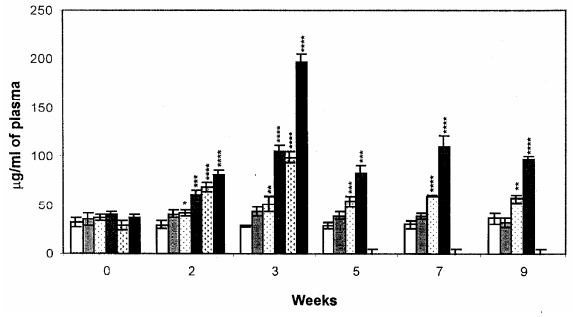

Fig. 4—Sialic acids content in plasma of chicken and rabbits treated with GTA [Sialic acids in plasma were extracted as described in 'Materials and Methods'. The content of sialic acids was determined by extrapolating absorbance values obtained at 549 nm directly from Neu5Ac standard plot. Values obtained are mean±SD from a set of 3 readings. (□) Control rabbit; (▩) Control chicken; (▨) Group I rabbit; (▩) Group I chicken; (▨) Group II rabbit; (■) Group II chicken. Significance with respect to control: *p<0.05; **p<0.01; ***p<0.005; **** p<0.001]

Table 1—Content of selected metabolites in liver and plasma of chicken and rabbits treated with GTA

[Values are expressed as mean ± SD from 3 assays]

Samples	Chicken			Rabbit		
	Control	Group I	Group II	Control	Group I	Group II
Lipid peroxides in liver homogenate (nmols/mg protein)	4.975±0.12	6.16±0.72	7.0±0.27***	0.895±0.009	1.245±0.028***	1.602±0.018****
Total thiols in liver homogenate (nmols/mg protein)	134.03±7.78	132.4±5.29	101.6±7.88*	123.5±7.78	123.5±4.95	104±8.48
SH in liver homogenate (nmols/mg protein	6.065±0.148	4.65±0.1****	3.02±0.21****	8.0±0.042	17.93±0.156***	16.46±1.99*
Sialic acids (µg/g liver)	114±2.83	108.67±12.06	149.67±10.02*	145.5±4.95	142±4.24	102.5±10.61*
Total thiols (nmol/ml plasma)	157±9.9	254±9.7***	298±7.6****	152.5±10.6	274.5±20.6*	295.5±7.8***
GSH (nmol/ml plasma)	15.85±0.5	26±1.73***	32.6±2.86***	14.5±2.97	39.6±4.31*	44.2±1.48**
Protein content (mg/ml of liver PMS)	14.9±1.69	17.8±0.1*	6.4±0.87***	15.3±0.56	13.8±0.14	10±1.13*
Protein content (mg/ml plasma)	45.6±1.01	51.37±1.93*	27.71±1.33****	66±7.07	62.56±2.66	39.57±5.1*

Significance with respect to control.

*P <0.05; *** P<0.005

**P< 0.01; **** P<0.001

Table 2—Enzyme activities in liver of chicken and rabbits treated with GTA

[Values are expressed as mean±SD from 3 assays]

Enzyme activities	Chicken			Rabbit		
	Control	Group I	Group II	Control	Group I	Group II
Glutathione reductase (nmols/min/mg protein	23±1.8	17.37±1.94**	5.16±1.94****	32.21±1.41	41.78±1.47*	16±2.35**
Superoxide dismutase (µmols/mg protein	0.117±0.004	0.590±0.017***	0.097±0.006*	0.945±0.016	0.819±0.02*	0.277±0.018****
Catalase (units/mg protein)	9.41±0.98	6.18±1.27	4.78±1.41*	7.675±0.163	8.34±0.76	5.25±0.49*
Glutathione peroxidease (nmols/min/mg protein	2.705±0.148	2.44±0.142	1.92±0.08***	2.66±0.014	3.075±0.064*	2.2±0.42

Significance with respect to control

*P < 0.05 *** P < 0.005

**P < 0.01 **** P < 0.001

of Neu5Ac and Neu5Gc mobilities (Fig. 5). Similar spots were observed with plasma of bovines, also known to express both Neu5Ac and Neu5Gc in their tissues[4]. Chicken liver and plasma showed Neu5Ac only. However, analysis of liver from chicken treated with 250 mg/kg body wt. GTA showed presence of Neu5Gc (Fig. 6). Neu5Ac hydroxylase activity was assayed in liver to determine whether the presence of Neu5Gc was a result of the activity of this enzyme in rabbit and chicken. As shown in Table 3, the activity of this enzyme was detected in the soluble portion of the rabbit liver only.

To confirm the absence of this activity in Neu5Gc containing liver of chicken treated with 250 mg/kg body wt. GTA, respective fractions were dialysed to remove free sialic acids and incubated with NADPH and Neu5Ac, as described earlier[12]. Analysis of sialic acids obtained showed hydroxylase activity only in rabbit liver (Fig. 7).

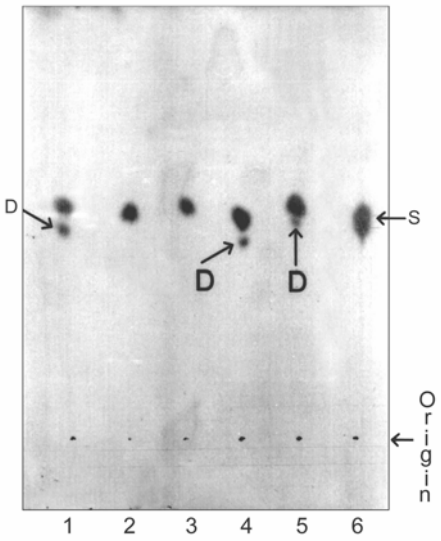

Fig. 5—Cellulose TLC of sialic acids extracted from various samples [Lanes: 1, Bovine plasma; 2, human plasma; 3, chicken plasma; 4, rabbit liver; 5, rabbit plasma; Neu5Ac standard (15 μg); D Mobility of Neu5Gc. The plates were sprayed with resorcinol/HCl reagent]

The contents of reduced glutathione (GSH) and total thiols were estimated in liver. As shown in Table 1, their contents were reduced by 50% and 24% respectively, in group II chicken. In contrast, all the treated groups of rabbits showed over 100% increase in liver GSH levels. The activities of liver GPx, SOD and catalase registered a significant reduction in group II of both chicken and rabbits (Table 2).

Discussion

The aim of this study was to explore the possible association of increased oxidative stress with the observed appearance of Neu5Gc in liver of domesticated birds, known to lack this sialic acid in normal tissue. Rabbits were used in this study to show that Neu5Gc is expressed in mammalian healthy tissue and to compare their response to GTA with domesticated birds. They were given higher doses of GTA than chicken as determined in our previous trials, which showed that the former exhibited higher tolerance to GTA (unpublished data).

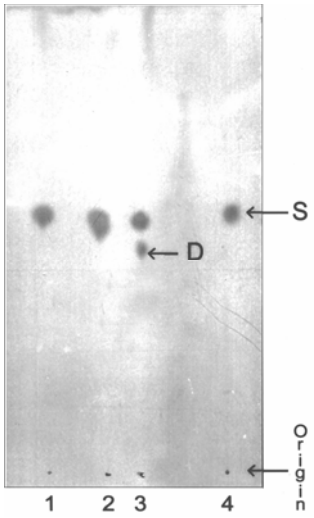

Fig. 6—Cellulose TLC of sialic acids extracted from chicken liver homogenate [Lanes: 1 Chicken control; 2, Group 1 chicken; 3, Group II chicken; 4 Neu5Ac standard (10 μg); D, mobility of Neu5Gc. The plates were sprayed with resorcinol/HCl reagent]

Table 3—Specific activities of Neu5Ac hydroxylase in fractions of liver from chicken treated with GTA

[Values are expressed as mean±SD from 3 assays and are in nmols/min/mg protein; No activity was detected in liver of chicken]

Fractions	Control	Group I	Group II
Post-nuclear fraction	0.012±0.002	0.011±0.003	—
Post-mitochondrial fraction	0.015±0.001	0.015±0.002	0.004±0.0006
Microsomal+Post-microsomal fraction	0.016±0.002	0.015±0.002	0.005±0.0004
Microsomal + 45-70% $(NH_4)_2SO_4$ fraction	0.018±0.0028	0.016±0.002	0.0065±0.0007

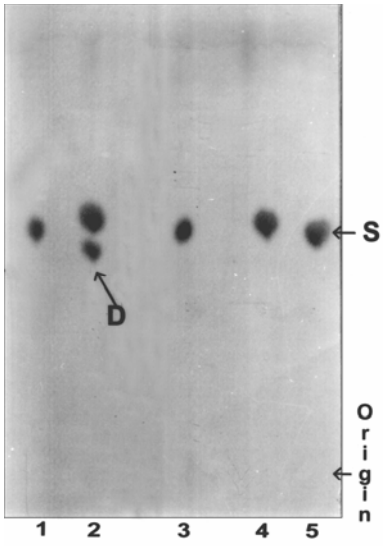

Fig. 7—Cellulose TLC of sialic acids extracted from reaction mixture containing 9.5 m*M* NADPH, 7.75 m*M* Neu5Ac, 1 m*M* GSH and post-mitochondrial supernatants from chicken and rabbit liver [Lanes: 1, Control rabbit liver PMS (No NADPH); 2, control rabbit liver PMS; 3, Group I chicken liver; 4, Group II chicken liver; 5 Neu5Ac Standard (15 μg); D, Mobility of Neu5Gc. The plates were sprayed with resorcinol/HCl reagent]

As given in results, lipid peroxides content and GR activity in plasma were increased in groups treated with GTA, indicating increased oxidative stress. GR activity in RBCs (Fig. 2A) and liver of group II birds and rabbits (Table 2) was decreased despite significant increase in lipid peroxides content in the same group, suggesting that GTA may be inhibiting the enzyme activity. As shown in Table 2, all group II cases showed significant decrease in GPx, SOD and catalase activities in liver, indicating that GTA exerts an inhibitory effect on the antioxidant enzymes activities, thus resulting in an increase in tissue lipid peroxides. GTA is a known astringent and is reported to cause disruption of ion-protein interactions binding ribosomes to endoplasmic reticulum[17]. It is, therefore, possible that the low activities of antioxidant enzymes as well as the decreased total thiol content in liver of group II birds and rabbits (Tables 1 and 2) is a result of the adverse effect GTA has on microsomal functions, a suggestion supported by the significant decrease in protein content observed in plasma and liver (Table 1). It is also possible that by exerting the same effect, GTA disrupts the normal process of haematopoiesis. Low WBCs count observed in all treated groups (Fig. 3) seems to indicate so and also confirm an earlier report of impairment of immune function in birds given tannic acid[31]. The disruption of ribosomal binding to endoplasmic reticulum causes microsomal degranulation, a property possessed by many carcinogens[17]. This is known to disrupt synthesis of glycoproteins, which play a crucial role in immunological response[32], control of cell cycle[33] and plasma membrane function[34]. The affected cells are likely to lose normal growth control often observed in cancer. Low WBCs count may also result from cytotoxic effect of free radicals due to decreased SOD activity, since phagocytosing leukocytes are known to release superoxide anions by activating an NADPH oxidase system localized on their membranes. The free radicals are key to the cell's microbicidal and antigen-degrading activity, while it is protected by its own endogenously produced SOD[35].

The estimation of sialic acid content in plasma has been used for a long time in clinical diagnosis and prognosis of cancer[36]. Though less sensitive, it has been popular because of its simplicity and safety. It is believed that increased sialic acids content in plasma results due to increased cellular turnover during cancer and inflammatory disease[37]. In this study, we monitored the effect of GTA on the amount of plasma sialic acids. The increased sialic acids content in plasma of all treated groups (Fig. 4) shows that GTA increases shedding of sialic acids from tissue cells. The low protein content in plasma and liver of treated groups indicates that the increase in sialic acids content could be due to the presence of free sialic acids, resulting from increased desialylation of macromolecules and cells headed for destruction in tissues[38].

The presence of Neu5Ac and Neu5Gc was demonstrated in rabbits, but only the former in chicken (Fig. 6) as reported by Varki[1]. Surprisingly, liver of group II chicken was observed to have Neu5Gc. This sialic acid is synthesized from a hydroxylation reaction catalyzed by a hydroxylase enzyme specific for CMP-Neu5Ac[11], free Neu5Ac and glycoconjugate bound Neu5Ac[12]. Humans and chicken are known to lack this sialic acid in their tissues[6]. While humans lack an active hydroxylase protein due to a frame shift mutation in its gene[14], the status of this enzyme in chicken is unknown. Attempts to establish the presence of Neu5Ac hydroxylase in chicken liver failed to detect any activity even in group II, which expressed Neu5Gc. This activity was, however, detected in liver of rabbit,

a mammalian species believed to express Neu5Gc in normal tissues[13].

It has, however, been observed that traces of Neu5Gc are expressed in some forms of human and chicken cancerous tissue[8]. Tissues affected by inflammatory diseases are also shown to express Neu5Gc[7]. Recently, it has been shown that cancer and inflammatory disease are associated with oxidative stress[39]. The reactive oxygen species (ROS), prevalent during oxidative stress, modify lipids, proteins and DNA, resulting in altered cell function. The consequent accumulation of oxidative damage may lead to both acute and chronic cell injury, which is likely to predispose cells to cancer[40]. In this study, there was increased lipid peroxide content in liver and plasma of treated groups. The low antioxidant capacity observed in liver of group II chicken is likely to encourage generation of ROS, which may be harmful to surrounding cells.

The enzymatic hydroxylation of Neu5Ac to Neu5Gc involves the transfer of an oxygen atom to the methyl group bound to C-5 of the sialic acid[11]. Like other sialic acids, the position of Neu5Ac on the cell surface renders it proximal to superoxide anions often generated intercellularly by phagocytosing leukocytes during inflammation and other immunological related responses. GTA is capable of reducing oxygen to superoxide anions. It also reduces Cu^{+2} to Cu^{+1} ions, which participate in Fenton reaction[19]. The hydroxyl ions generated in this reaction, in the presence of H_2O_2, are highly reactive and have been implicated in cellular damage to proteins, DNA and lipids[39]. Against this background and the nature of sialic acid modification involved provides a strong indication that Neu5Gc is generated during increased oxidative stress induced by GTA in both chicken and rabbits.

Conclusion

The high lipid peroxides content and low antioxidant capacity observed in groups treated with GTA demonstrate clearly that a state of increased oxidative stress was induced in both chicken and rabbits. The presence of Neu5Gc in liver of chicken, which normally does not express this sialic acid, suggests non-enzymaticc generation of Neu5Gc, since no Neu5Ac hydroxylase activity was detected after treatment with GTA. Given that increased oxidative stress is associated with cancer and inflammatory disease[39], it can be concluded that the occurrence of Neu5Gc in liver of chicken treated with GTA is formed from non-enzymic transfer of oxygen by reactive oxygen radicals.

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