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REVIEW

Fluorescent humic substances and blackfoot disease in Taiwan

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Blackfoot disease is endemic in Chia Yi and Tainan Counties in south-western Taiwan. Arsenic present in the drinking water taken from wells has been blamed for causing this disease. The discovery in 1975 of fluorescent compounds in these well waters led to the isolation of these substances, to their identification as humic substances containing a large number of elements (among them arsenic), and to the development of an animal model for blackfoot disease. Mice receiving aqueous solutions of these fluorescent substances at a daily dose of 5 mg per 20 g body mass for at least 22 days developed blackfoot and blacktail disease. Work with these fluorescent substances, their chemical properties, and their biological actions is reviewed.

Keywords: Blackfoot disease, Taiwan, arsenic, fluorescent humic substances

INTRODUCTION

Blackfoot disease first appeared in southwestern Taiwan[1] between 1910 and 1920. During the following decades the disease became endemic in the districts of Hsueh-chia, Pei-men, and Hsia-ying in Tainan County, and the district of I-chu and the town of Pu-tai in Chia-yi County. Clinically, blackfoot disease is a progressive deterioration of the extremities involving acroparalysis, coldness, pain, and intermittent lameness. Pathologically, peripheral vascular obstruction causes ulcers and gangrene very similar in appearance to arteriosclerosis obliterans and thrombotic vasculopathy.[2] The disease became a public health concern and the subject of many studies. Publications describing the results of clinical,[3] pathological,[2] epidemiologic,[4,5] and biochemical[6] studies, and investigations of the connection between nutrition and blackfoot disease,[7] are available in the literature. Occurrence of blackfoot disease is closely related to the use of drinking water from artesian wells[4,5] with a depth of more than 100 m. The incidence of the disease ranged in 1960 from 3.4 to 20 per 1000 (depending on occupation) in a total population of approximately 140 000. When determinations of total arsenic in these well waters revealed arsenic concentrations as high as 1.8 mg dm^{-3}, arsenic was assumed to be the cause of blackfoot disease; however, this casual link was not and is not universally accepted. In 1975, the arsenic-containing waters were discovered to fluoresce greenish-blue upon irradiation with ultraviolet light.[8] This paper reviews the results of the studies of the fluorescent compounds and their effects on animals.

CHEMICAL CHARACTERIZATION OF THE FLUORESCENT SUBSTANCES IN WELL WATERS

In 1975, the physical and chemical properties of wellwaters from 53 locations in Chia-yi County and 24 locations in Tainan County were studied.[8,9] Most of the residents in these areas still these waters for drinking. All of the 77 samples collected fluoresced greenish-blue upon irradiation with ultraviolet light. This fluorescence could not be attributed to contamination from plastic containers. The water samples from Chiu Ying Li and Wang Liao Li had the highest fluorescence intensity. These samples were used for a detailed study. The excitation maximum occurred at 325 nm, the emission maximum at 435 nm. Dialysis of the water samples in dialytic bags for 29.5 h caused only small changes in the fluorescence spectrum and intensity. After dialysis for

189.5 h the fluorescence had decreased to 30% of its original intensity. The flourescent substance(s) migrated as one band upon column chromatography on Sephadex G-200. These findings suggest that the fluorescent material is polymeric and remains polymeric during chromatography, but dissociates slowly on prolonged dialysis.[10] The fluoresence is hardly affected when the water samples are refluxed. After 20 min at the boiling point, the fluorescent intensity had increased by 5.6%. Thereafter the intensity decreased slowly to stabilize at 92% of the intensity at room temperature. Exposure of the water samples to ultraviolet light for 30 min did not change the fluorescence.

During the period January through June 1988 water samples were collected from 1189 wells in the blackfoot disease area. Total dissolved solids, pH, fluorescence intensity, and arsenic concentrations were determined. Samples with high arsenic concentrations exhibited intense fluorescence. The severity of symptoms (blackfoot disease, skin disorders) increased with increasing arsenic concentration and increasing fluorescent intensity in the water.

Only a small fraction of the fluorescent substances could be extracted from the water by chloroform of hexane. The extracted material was separated by thin-layer chromatography into nine fluorescent components. Most of the fluorescent material remained in the water.[8] To isolate the fluorescent substances, 10 l of water from Won-Liao village were filtered to remove suspended material and then passed through an anionexchange column (Amberlite IRA-400). The fluorescent substance sorbed on the column were eluted with aqueous 5% ammonium chloride solution[11] 1 M hydrochloric acid.[12] The brown eluate was evaporated to dryness under vacuum at 50–70°C. The resulting solid was extracted with methanol or acetone, the extract evaporated, and the solid extracted with isopropanol. This procedure was repeated with extractants such as ethyl acetate, chloroform, acetone, and methanol. The final material was then lyophilized. The dark-brown material was soluble in distilled water. Thin-layer chromatography can be used for further fractionation and purification.[36] A sample of well water from Chi Ying Li in Tainan County was treated similarly and four fractions of fluorescent substances isolated on the basis of column chromatography and solubility. three fractions were soluble in water; the fourth was soluble in ethanol. When the isolated solids were disolved in water, the solutions still fluoresced. The solids had to be heated to 600°C for one hour to destroy the ability to fluoresce.[13]

Analysis of the fluorescent substances isolated in this manner by inductively coupled argon plasma emission spectrometry revealed that these substances contained the following elements: Li, Na, K; Mg, Ca, Sr, Ba; B, Al; Si, Sn, Pb; P, As, Sb, Bi; S, Te; Ti, Cr, Mn, Fe, Ni, Cu, Zn; Nb, Mo, Rh, Cd; La and W. Electrothermal atomic absorption spectrometry gave arsenic concentrations of 3019 and 855 $\mu g\,g^{-1}$ in two of the water-soluble fractions and 43.4 $\mu g\,g^{-1}$ in the ethanol-soluble fraction. GC MS revealed the presence of butyl phthalate, benzenedicarboxylic acids, and 2-ethylhexyl phthalate in two of the fractions.[14] Infrared spectroscopy indicated that all the fractions contained C–H, OH, C=O, carboxyl, and Si–O–Si groups. The IR spectra of all the fractions were very similar. ESR spectra revealed the presence of radicals in these substances.[10] The physical and chemical properties of the substances that cause the well waters to fluoresce identify these substances as humic acids.

BIOLOGICAL PROPERTIES OF THE FLUORESCENT HUMIC SUBSTANCES

The biological properties of the fluorescent substances were tested on fertilized chicken eggs, on cell cultures, and on mice. For these purposes the solid substances were dissolved in distilled water.

Solutions of the fluorescent solids in distilled water (0.1 cm^3) of decreasing concentration were injected into fertilized chicken eggs on the eighth day after fertilization. Some of the chicks hatched from the eggs injected with solutions of high concentration showed abnormalities, such as stiff legs extended backwards, feet bent inward and unable to support the body, severely trembling feet, thin feather cover on wings and neck, and sparse coverage of the body by down feathers. Some of the chicks died soon after hatching. The control eggs injected with 0.1 cm^3 distilled water produced healthy chicks.[12]

Experiments with cultured cells[15] revealed the minimal inhibitory concentration of the fluorescent substances to be 31 $\mu g\,cm^{-3}$ for human embryonic kidney cells; 62 $\mu g\,cm^{-3}$ for human embryonic cells; 250 $\mu g\,cm^{-3}$ for continuous HeLa cell lines; 125 $\mu g\,cm^{-3}$ for HEp-2 cells; 250 $\mu g\,cm^{-3}$ for MK-2 cells, and 125 $\mu g\,cm^{-3}$ for

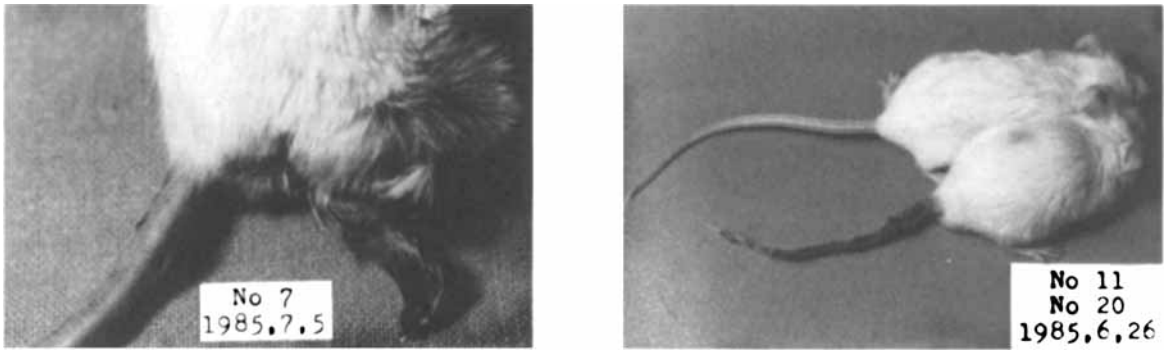

Figure 1 Mice with blackfoot and blacktail disease induced by interperitoneal injection for at least 22 days of an aqueous solution of the fluorescent humic substances isolated from well water.

GHK and Vero cells from established continuous monkey cell lines; 31 $\mu g\ cm^{-3}$ for primary chicken cells; and 125 $\mu g\ cm^{-3}$ for human lymphocytes. The fluorescent humic substances appear to be more toxic to normal cells than to cells from continuous cell lines.[16]

At 1000 $\mu g\ cm^{-3}$, the fluorescent humic substances reduced the synthesis of [^{3}H]thymidine in HeLa cells to 14% of control. No inhibition was observed at a concentration of 10 $\mu g\ cm^{-3}$. Similarly, the synthesis of glutamic acid (C14) was depressed at 1000 $\mu g\ cm^{-3}$ to 58%, the synthesis of [^{3}H]uracil to 64%.[16] No inhibition was observed at 100 $\mu g\ cm^{-3}$. These experiments indicate that the fluorescent substances inhibit mainly the synthesis of cellular DNA.

The fluorescent substances caused chromosomal aberrations in cultured human (male) lymphocytes from peripheral blood.[17] The fluorescent substances used in this experiment were extracted from well water collected in Chiu Ying Li. The substance was dialysed in a dialytic bag against distilled water. A high-molecular-mass fraction (inside the bag) and a lower-molecular-mass fraction (outside the bag) were collected. At a concentration of 1000 $\mu g\ cm^{-3}$ the lower-molecular-mass fraction inhibited cell division until the mitotic index became $41.8 \pm 1.6\%$, the high-molecular-mass fraction similarly until the index reached $18.4 \pm 2.5\%$. In addition, chromosomal aberrations, such as cracking, breakage, and exchange of one or more chromosomes, were observed.

Peritoneal injection into mice for 13 days of 1.0 cm^3 of an aqueous solution of the fluorescent substance to achieve a daily dose of 200 mg substance per 100 g body mass caused the end of the tail to turn black and the adjoining part to turn red. The animals were sacrificed on the 14th day. Congestion of the blood vessels was observed in the tail. Examination with the microscope revealed necrosis, arterial thrombosis, and adhesion of the thrombus to the walls of the blood vessels.[18]

Mice (balb/c, weighing approximately 20 g) received a daily peritoneal injection of 0.2 cm^3 of an aqueous solution of the first water-soluble fraction of the fluorescent substance (Chiu Ying Li water) to achieve a daily dose of 5 mg per 20 g body mass. The 16 mice were kept in pairs in separate cages and separated as soon as symptoms were observed. After 22–32 daily injections, eight mice developed some of the following symptoms: lameness, swollen limbs, necrotic toes, gangrenous tail.[19] Figure 1 shows two mice with experimentally induced blackfoot and blacktail disease.

FLUORESCENT HUMIC SUBSTANCES AND CANCER

When the incidence of bladder cancer in 19 villages and districts in the blackfoot disease were compared with the fluorescence intensities of water samples from the wells, a close correlation was found. Patients with bladder cancer in the blackfood disease area were clustered along the Chiu Shui river and Pa Chan river. In other regions with blackfoot disease the incidence of bladder cancer was not high.[20] The similarity of the spectral fingerprints of the fluorescent compounds suggest a common source for these materials. The fluorescent compounds could also be connected with the high incidence of bladder cancer.

MUTAGENIC ACTION OF THE FLUORESCENT SUBSTANCES

The four fractions of the fluorescent substances isolated from water from Chiu Ying Li were tested for mutagenicity with the Ames screening test using *Salmonella typhimurium* TA 100 (base replacement test) and TA 98 (frame-shift test). At an initial dose of 500 μg per 0.1 cm^3, the ethanol-soluble fraction from the fluorescent substances was mutagenic to both strains in the presence of S-9. The mutagenicity of this fraction towards TA 100 was weaker in the absence of S-9. A positive mutagenic effect was observed for the third water-soluble fraction at a concentration of 50 μg per 0.1 cm^{-3}. The water-soluble fraction with the highest arsenic concentration (3 mg g^{-1}) had no mutagenicity towards TA 100 without S-9 and towards TA 98 with or without S-9 at 5000 μg per 0.1 cm^3. A very weak effect was observed at 10 000 μg per 0.1 cm^3 in the absence of S-9. The observation that the fraction with the highest arsenic concentration is the least mutagenic suggests a minor or no role for arsenic in mutagenesis. Recently, a fraction that showed high mutagenicity but was low in arsenic was extracted from the fluorescent humic substances with ethyl acetate.[21] This result also questions the complicity of arsenic in this context.

MULTIPLE ACTION HYPOTHESIS

The fluorescent substances in the well waters have as humic acids ionizable groups and donor sites that possess the ability to bind metal ions[10] and a variety of natural and man-made compounds.[11,22] The biological actions of these substances are the sum of the effects exerted by all the constituents (Fig. 2). For instance, the free radicals known to be present in these substances[10] may accelerate the metabolism of fatty acids and the generation of lipid peroxides.[23] Excessive levels of such peroxides inhibit the activity of prostacycline, cause thrombosis,[24] may lead to pathologicfal changes in blood vessels,[25,26] and could be a contributing factor to the appearance of diabetes mellitus,[27] cerebral apoplexy,[25] and retinopathy.[24] Fatty acids may become the source of carbonyl compounds that may build up to excessive concentrations. Malondialdehyde[28] and 4-hydroxynonanal[29] are examples of such carbonyl compounds that are known to be mutagenic and toxic. Malondialdehyde may crosslink proteins and enzymes with concomitant loss of their normal functions.[30] In addition, the free radicals activate carcinogens, such as polycyclic aromatics, azo dyes, and arylamines.[31]

Humic acids are known to contain phenolic components (resorcinol, orcinol, phloroglucinol, pyragallol). These substances are strong inhibitors of thyroid peroxides and prevent the combination of thyroidal iodine with the thyroid hormone. Hence, humic acids may be the cause of endemic goitre.

Within the blackfoot disease area other diseases such as cancer, diabetes mellitus, cardiovascular anomalies, hypertension, cerebral apoplexy,[33] and goitre[34] occurred at statistically significant higher incidences than in areas free of blackfoot disease. The causes of these diseases have not yet been identified with certainty; however, the fluorescent humic substances may be linkable to these diseases. Chlorination of the waters may generate mutagenic substances from the humic acids.[35,36] Therefore, the fluorescent humic substances deserve to be studied in detail on a local scale in Taiwan and also worldwide in respect to their effects on human health.

CONCLUSIONS

Progress from studies of the etiology of blackfoot disease has been very slow. The disease is regionally restricted to Taiwan with no reports from

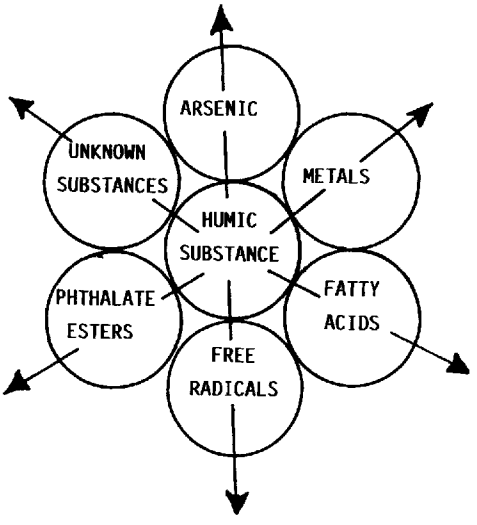

Figure 2 Schematic representation of the multiple action of the fluorescent humic substances on organisms

other locations. The disease appears generally in older people. An animal model for the disease has not been available until very recently. The composition of the well waters is very complex, preventing a detailed chemical characterization. Previous etiological studies attributed blackfoot disease to excess potassium, silicon, arsenic, or soil components. Each of these claims is supported by certain data, but a satisfactory explanation is not achievable on such a single-component basis. All of these components are present in the fluorescent humic substances. The study of the biological effects of these substances led to an animal model for blackfoot disease and to the strong indication that other diseases in the blackfoot disease region with incidences above the norm might also be connected with the fluorescent materials. Further studies of these substances will produce important and far-reaching results for the field of environmental toxicology in Taiwan.

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