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A Search for the Genotoxic Principle Associated with the Tubercule Bacillus, *Mycobacterium tuberculosis* Using Mice as Model

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The genotoxic potentiality of some nonpathogenic and pathogenic species of lower group of bacteria was almost exclusively reported by Manna and his collaborators which has been reviewed by him from time to time (Manna 1973, 1980, 1986, 1989). Recently the present authors (Manna and Pal 1989) discovered the mutagenic potentiality of the log culture of tubercule bacillus, *Mycobacterium tuberculosis* in experimentally injected mice by more than one mutagenicity test as recommended (Bochkov *et al.* 1976), like bone marrow chromosome aberrations and micronucleus test in both sexes and germinal chromosome aberrations and sperm with abnormal head morphology in treated males. However, with a view to finding out the factor which could possibly be associated with the bacterium, *M. tuberculosis* for inducing the genotoxic effect in mice has specially been investigated here.

The bone marrow chromosome aberration frequency data in separate sets of mice treated with various samples of *M. tuberculosis* and the BCG vaccine have been used as the indicator of the potentiality of the genotoxic principle against controls.

Materials and methods

Inbred laboratory stock of Swiss albino mice, *Mus musculus* was used. Two male and two female specimens in each set of treated and control were individually injected intraperitoneally with different samples as follows at the rate of 1 ml per 100 gm body weight of individual specimen and the effect in the bone marrow cells was assessed on 7th day after injection.

Preparation of samples for injection

A. Treated series

(Tr. 1. Log. cul.) *Log culture of Mycobacterium tuberculosis.* The pure sample of the bacterium obtained through the courtesy of Dr. A. N. Chakraborty, Department of Pathology, S. S. K. M. Hospital, Calcutta was subcultured in freshly prepared Kirschner's medium which attained the log phase of growth on 7th day of incubation.

(Tr. 2. Cul. fil.) *Culture filtrate.* 10 ml of log culture of *M. tuberculosis* was centrifused at 10,000 r. p. m. for 10 min. The supernatant was gently decanted to another tube and filtered through Sinter's glass filter to remove complete trace of bacterium. The process was repeated to be absolutely sure which was confirmed by culturing a part of the filtrate in sterile medium showing no growth of bacteria. The sample was then injected.

(Tr. 3. Iso. bac.) *Isolated M. tuberculosis suspended in saline.* 10 ml of log culture of *M. tuberculosis* was centrifused at 10,000 r. p. m. for 10 min and the supernatant was discarded. The residual part was mixed with 0.85% normal saline to make the volume of 10 ml again and then centrifused. The supernatant was discarded and the residual part was mixed again with normal saline to make the volume of 10 ml. The process was repeated 3 or 4 times so as to remove the trace of culture medium completely. The sample was then injected into mice

containing live bacterial cells suspended in saline.

(Tr. 4. Aut. kil) *Autoclave-killed isolated M. tuberculosis suspended in saline.* 10 ml sample of isolated *M. bacterium* suspended in saline was prepared as above and it was autoclaved for 15 min in 15 lbs pressure. On cooling down to room temperature, a part of the sample was cultured in sterile medium to make sure that the autoclaving killed all the live bacteria. The other part was injected into mice for the study.

(Tr. 5. BCG vac.) *BCG vaccine.* Anhydrous BCG vaccine (Bacillus of Calmette and Guerin—an attenuated strain of tubercule bacillus prepared for vaccination) was dissolved in 2 ml sterilized distilled water. An equivalent dose for human child (0.2 ml per 5 kg body weight) was injected into the experimental mice for the study.

B. Control series

(Co. 1. Kir. med.) *Kirschner's medium.* Freshly prepared sterile medium was injected into a set of mice to serve as control to specimens treated with log culture of *M. tuberculosis* (Tr. 1) and also the culture filtrate (Tr. 2).

(Co. 2. Nor. sal.) *Normal saline.* 0.85 of sodium chloride was dissoled in 100 ml sterilized distilled water and the saline solution was injected into a set of mice to serve as control to saline suspended isolated *M. tuberculosis* (Tr. 3) and also autoclave-killed saline suspended isolated *M. tuberculosis* injected (Tr. 4) specimens.

(Co. 3. Dis. wat.) *Distilled water.* Since sterile double distilled water was used as dilutant in different preparations, a set of normal mice was injected only with the distilled water to serve as control to BCG vaccine treated specimens.

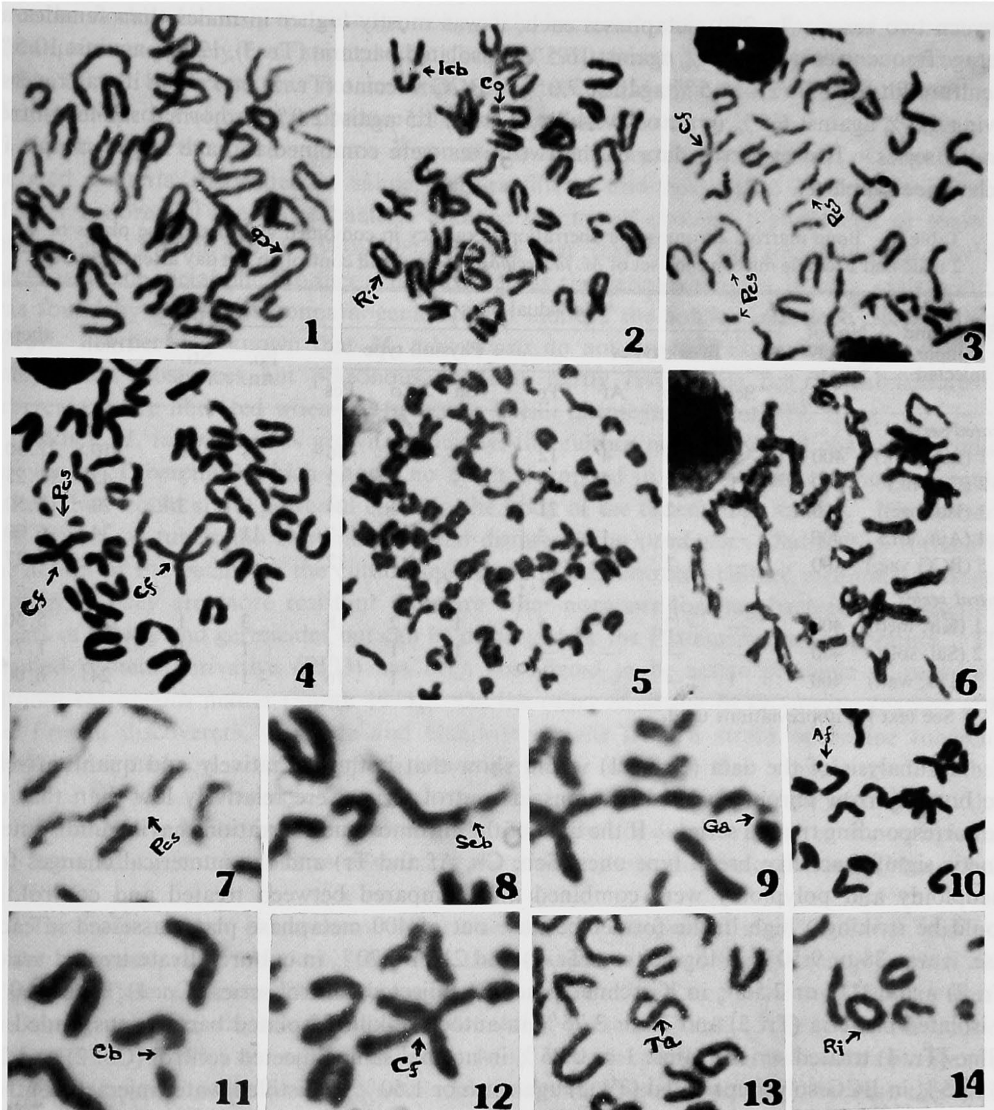
Preparation of bone marrow chromosomes.

The standard bone marrow chromosome preparations of individual control and treated specimens were made combining the methods of different workers (Ford and Hamerton 1956, Rothfels and Siminovitch 1958, Manna 1985). Individual treated and control specimens were intraperitoneally injected with 0.04% colchicine solution at the rate of 1 ml per 100 g body weight $1\frac{1}{2}$ hr before drawing out the bone marrow cells separately from femurs by pushing warm (37°C) 1.0% sodium citrate solution through the bone-canal after cutting the two ends. The bone marrow cells flushed out with the sodium citrate solution were collected in a centrifusing tube. The cells were further suspended in citrate solution by repeated flushing with a Pasteur pipette fitted with a rubber bulb. After about 15 min incubation at 37°C the cell suspension was centrifused, the supernatant fluid was removed and the material was fixed in acetic-alcohol (1: 3) with one change. The excess fixative was removed and the cells were brought to emulsion-like suspension. A drop of the cell suspension was put on a chilled slide which was stored in 50% alcohol in a refrigerator. The slide was touched to a flame to catch fire and was left in a slanting position to burn out alcohol by the time the cells would stick to the slide. The slide was then stained in diluted stock solution of Giemsa in distilled water (1: 20) keeping the pH adjusted to 7. 2. The frequency of bone marrow chromosome aberrations was assessed in 100 metaphase plates per control and treated specimen separately.

Results

Male and female mice of five treated series (Tr. 1–5) had in their bone marrow cells various types of chromosome aberrations (Figs. 1–14) while they were rather limited in most the control series (Co. 1–3, Table 1). The different bone marrow chromosome aberrations were put under two categories in spite of sometimes the overlapping effects as (1) individual types when some chromosomes of the full complement were affected and (2) gross types when the full metaphase

plate was affected. The individual type aberrations with known genetic significance were subchromatid break (Scb, Fig. 8), chromatid break (Cb, Figs. 1, 11) including some very rare occurrence of isochromatid breaks (Icb, Fig. 2), acentric fragment (Af, Fig. 10), translocations (Tr) in the form of centric fusion (Cf, Figs. 3, 4, 12), ring (Ri, Figs. 2, 14) and terminal fusion/ association between chromatids of two different chromosomes (Ta, Fig. 13), and with debatable



Figs. 1–6 (full) and Figs. 7–14 (magnified affected part). Metaphase plates of bone marrow cells of mice treated with different samples of *M. tuberculosis* showing various types of individual type chromosome aberrations marked by arrows and gross type aberrations. 1, a mid-region chromatid break. 2, an isochromatid break, a terminal constriction and a ring chromosome. 3 and 4, each showing a centric fusion between two different chromosomes and precocious separation of chromatids in some chromosomes. 5, a polyploid metaphase. 6, a metaphase with sticky chromosomes. 7, precocious separation of chromatids in a group IV chromosome. 8, a subchromatid break. 9, a gap. 10, an acentric fragment. 11, a chromatid break in the mid region. 12, a centric fusion between two chromosomes of group III. 13, a terminal fusion/association of chromatids of two chromosomes. 14, a ring chromosome. 1–6: ×ca. 1600, 7–14: ×ca. 2500.

genetic significance were physiological one like gap (Ga, Fig. 9), constriction (Co, Fig. 2), precocious separation of chromatids of some chromosome (Pcs, Figs. 3, 4, 7) etc. Among gross types with known genetic significance were numerical changes like aneuploidy (An) and polyploidy (Po, Fig. 5) and with uncertain significance were stickiness (fig. 6), pycnosis, pulverization etc.

The bone marrow chromosome aberration frequency showed some amount of differences between two sexes. In 200 metaphases each, it was mostly higher in males than females as average frequencies were 20.5% against 16.5% in isolated bacteria (Tr. 3), 12.0% against 10.5% in culture filtrate (Tr. 2), 11.5% against 7.0% in BCG vaccine (Tr. 5) etc. while it was reverse having 5.5% against 6.5% in autoclave-killed (Tr. 4) 1.5 agaist 2.0% in normal saline control (Co. 2) series. However, the data of the two sexes were combined to have larger sample in each series (Table 1).

Table 1. Bone marrow chromosome aberration frequency in combined 400 metaphase plates of 2 male and 2 female mice in each set of *M. tuberculosis* treated and control on 7th day after injection

Series and sample injected	No. of meta-phase	*Individual ones							*Gross ones		Total	aberr. %
		Break types				Physiol. type			Po. An.	Sti. Pyc.		
		Scb	Cb	Af	Tr	Ca	Co	Pcs				
Treated series												
Tr. 1 (Log. cul.)	400	3	15	4	12	11	18	36	4	9	110	27.50
Tr. 2 (Cul. fil.)	400	1	2	3	8	5	4	12	7	3	45	11.25
Tr. 3 (Iso. bac.)	400	1	2	3	21	3	4	24	9	3	70	17.50
Tr. 4 (Aut. kil.)	400	—	—	1	3	—	2	7	11	—	24	6.00
Tr. 5 (BCG vac.)	400	—	2	2	10	—	1	12	9	1	37	9.27
Control series												
Co. 1 (Kir. med.)	400	—	2	—	5	1	2	8	3	1	22	5.50
Co. 2 (Sal. sol.)	400	—	—	—	—	1	1	3	1	1	7	1.75
Co. 3 (Dis. wat.)	400	1	—	—	4	—	1	16	1	1	24	6.00

* See text for abbreviations used.

An analysis of the data (Table 1) would show that both qualitatively and quantitatively the bone marrow chromosome aberrations in control series were relatively low than that of the corresponding treated series. If the data of the chromosome aberrations having undisputed genetic significance like break type ones (Scb, Cb, Af and Tr) and the numerical changes for aneuploidy and polyploidy were combined and compared between treated and control, it would be strikingly high in the former because out of 400 metaphase plates assessed in each case, it was 38 or 9.50% in log culture (Tr. 1) and 21 or 5.20% in culture filtrate treated series (Tr. 2) against 10 or 2.50% in Kirschner's medium injected control series (Co. 1); 36 or 9.0% in isolated bacteria (Tr. 3) and 15 or 3.75% in autoclave-killed isolated bacteria suspended in saline (Tr. 4) treated series against 1 or 0.25% in normal saline injected control (Co. 2) and 23 or 5.75% in BCG solution treated (Tr. 5) against 6 or 1.50% in distilled water injected control (Co. 3) series. Further, if the data of 5 treated series (Table 1) were combined, out of 2,000 metaphase pletes, the frequency of total aberrations was 286 or 14.30% and that of genetically significant ones was 133 or 6.65% against the 3 control series combined, out of 1,200 metaphase plates, 53 or 4.41% were total aberrations and that of genetically significant ones was 17 or 1.40%. Thus, the treated series had much higher chromosome aberration effect than that of control. However, among the different treated series the genotoxic effect varied considerably. It was highest in log culture (Tr. 1), the next isolated bacteria (Tr. 3), then the culture filtrate (Tr. 2), followed by BCG vaccine (Tr. 5) and the lowest in the autoclave-killed saline suspended bacteria (Tr. 4). Among the control series the effect was relatively high in Kirschner's medium

injected specimens than two others, if true genetically significant ones were considered which might suggest that some ingradient in the medium was lowly genotoxic in effect.

Discussion

Since tuberculosis is still a principal infectious disease causing good number of deaths all over the world, obviously the pathogenecity of the bacterium, *M. tuberculosis* and its treatment have quite extensively been studied (Cruickshank 1970, Smith 1973) but the genotoxic potentiality of the bacterium was not investigated before us (Manna and Pal 1989). Practically almost all species of bacteria produce different types of toxins.

Comparing the bone marrow chromosome aberration frequency data of log culture, isolated bacteria suspended in saline, culture filtrate and heat killed bacterial suspension of over a dozen of species of bacteria and the vaccine of cholera, typhoid etc. or toxin of tetanus etc., Manna (1973, 1980, 1986) opined that very likely the toxins liberated by the bacteria as the metabolic product acted possibly as the genotoxic agent in mice system but it was found by them that nonpathogenic species induced the similar genotoxic effect (Manna 1986). Further it is known that *M. tuberculosis* do not produce exotoxins, haemolysins or comparable substances but poisonous products partly responsible for clinical features of tuberculosis are liberated when the tubercule bacilli disintegrate (Smith 1973).

When *M. tuberculosis* is grown artificially, the culture medium would contain a product known as 'Tuberculin' which shows no effect if applied in the body of the non-tubercular animal but would show powerful effect in the body of the tuberculous animal. The bacteria free filtrate of tuberculin contains bacterial disintegration products. Substances formed by the action of the bacilli on the culture medium and concentrated culture medium are largely proteins. They are more resistant than are other nonspore-forming bacteria to deleterious effects of drying and germicides but can be destroyed by the Pasteurization temperature. The purified protein derivative (PPD) has been considered to be active principle of tuberculin without extraneous matter (Smith 1973). On the other hand the BCG vaccine (named after the French discoverers, Calmette and Guerin) is made from a strain of bovine tubercule bacilli cultivated on artificial media containing bile so long they completely lost their virulance for man. Therefore from the above mentioned properties it could be suspected that the surface charged proteins of the tubercule bacilli might not only act as the toxic principle but also act as the genotoxic one as revealed in the present study and the earlier one (Manna and Pal 1989).

The bone marrow chromosome aberration data of the present study, however, did not render unquestionable support solely for the proteins as the active principle because the frequency of chromosome aberration was highest in log culture treated series (27.50%) which was almost equal to the combined data of isolated bacteria (Tr. 3, 17.50%) and the culture filtrate (Tr. 2, 11.25%) series. On the other hand, it was expected that the culture filtrate (Tr. 2) treated series should have shown the maximum aberration frequency because the toxic products are liberated when *M. tuberculosis* bacilli disintegrate (Smith 1973). But in the data the frequency of aberrations was found much lower than that of log culture or isolated bacteria treated series. Further the treatment of BCG vaccine was supposed to induce practically no bone marrow chromosome aberration because it contained the attenuated bacilli, but the frequency was 9.27% which was even higher than that of autoclave-killed bacterial suspension treated specimens (6.0%), while PPD indoced meagrely 3.50% bone marrow chromosome aberrations (Manna and Pal 1990).

Thus it could be suspected that besides the surface proteins of *M. tuberculosis* bacilli, the cellular components could also play a role as the genotoxic principle for which further

studies are in progress.

Summary

The genotoxic principle associated with the tubercule bacilli, *Mycobacterium tuberculosis* has been investigated using bone marrow chromosome aberration frequency as the indicator in different sets of experimentally treated mice against controls. The data scored from 400 metaphase plates of 2 male and 2 female mice on 7th day after injection in each series revealed the average aberration frequency of 27.50% in log culture, 11.25% in culture filtrate, 17.50% in isolated bacteria in saline suspension, 6.0% in autoclave-killed bacterial suspension and 9.27% in BCG treated specimens while it was 5.50% in Kirschner's medium, 1.75% in normal saline and 6.0% in distilled water injected control series. It has been opined that the active principle for the genotoxic effect on mice could be the surface proteins liberated by the disintegration of the bacilli as well as the cellular components, the role of the latter is yet to be tested.

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