Leprosy is a chronic infectious disease caused by the gram positive bacterium, *Mycobacterium leprae*, popularly known as Hansen's bacillus. This bacterium is congeneric to the agent causing tuberculosis (*M. tuberculosis*). The disease is not hereditary. It spreads by prolonged contact only. Children are more prone to infection, but no age group is immune. Men are more susceptible than women, the reason remains unknown. Incubation period of the bacterium varies from three months to thirty years.

The disease is reported from several Asian, African, and European countries. It is particularly prevalent in tropical and subtropical countries. In a rough and conservative estimate it is noted that about 15 million people in the world are suffering from leprosy (Goodman and Gilman, 1985). It is prevalent in India affecting approximately 4 million people, and more (\( \sim 10\% \)) in the coastal belt of South India. The disease is characterized by lesions in the skin and/or polyneuritic changes due to involvement of and damage to the peripheral nerve trunk. Leprosy is not a disease of civilization or industrialization but it is one of the oldest scourges of humanity. It was known to occur even 2500 years ago. But we are yet to get an agent for its fast and effective therapy. Earlier it was a general notion, because of their close bacteriological relationship, that both tuberculosis and leprosy could be combated with the
same therapeutic agent. Unfortunately, despite our great success in combating tuberculosis in the recent past, the therapeutic progress for leprosy is not so remarkable. The current therapeutic regimen takes long time to cure the disease; and further, there is a chance of relapse. Only recently vaccine has been developed to immunize people against leprosy following successful culture of the bacteria in armadillo and in mouse foot-pad system. In vitro culture of the bacteria is without any success. That is the reason for the delay in getting the vaccine developed. The vaccine is yet to be popularized and used in large-scale. It is hoped that the leprosy vaccine like all other vaccines will play a great role in combating the disease in near future.

As an antileprotic drug dapsone (DDS) was first introduced in 1941 (see Goodman Gilman, 1985) and was being used earlier as monotherapy. DDS resistance was first proved soon after multiplication of M. leprae in the mouse food-pad had been described (Pettit and Rees, 1954). Clofazimine came later in 1962 to combat the sulfone (DDS) resistant M. leprae. At present triple drug regimen (DDS + rifampicin + clofazimine) is recommended for the treatment of leprosy by WHO (1982).

This chapter deals with the effects of two antileprotic drugs - clofazimine (CLF) and dapsone (DDS).

4.1 Effect of Clofazimine:

4.1.1 Introduction

Clofazimine (S 663) marketed by Ciba-Geigy, U.K. as Lamprone is a substituted iminophenazine dye synthesized first
by Barry et al. in 1937. It is 3-(p-Chloranilino)-10-(p-chloro-phenyl)-2, 10-dihydro-2-(isopropylimino) phenazine and its structural formula is as follows:

\[
\text{Molecular formula: } \text{C}_{27}\text{H}_{22}\text{Cl}_{2}\text{N}_{4}
\]
\[
\text{Molecular weight: } 473.3
\]
\[
\text{Melting point: } 215^\circ\text{C}
\]

In India it is marketed under the trade name 'Hansepran'. It belongs to a chemical class which has not previously been used in therapeutics. The dye forms dark red crystalline prisms, and is insoluble in water but soluble in ethanol and other organic solvents like dioxane, dimethyl formamide, dimethyl sulfoxide, chloroform, etc. The solution is stable.

Because of its wide antibacterial particularly antimycobacterial spectrum the drug is used in a large scale as an antileprotic drug. Its anti-inflammatory property is highly effective against lepra reaction. It is considered to be suitable at any stage and in all forms of the disease. Because of the close relationship of the bacteria \textit{M. tuberculosis} and \textit{M. leprae},
the causative agents of tuberculosis and leprosy respectively, most of the current antileprotic drugs sprang from research on tuberculostatic drugs, and clofazimine (CLF) is not an exception. It was originally being used in the treatment of tuberculosis; its use against vitiligo is also on record (Punshi, 1977). WHO (1982) recommends CLF as the third drug in the triple drug regimen for the treatment of leprosy. Though synthesized long ago it was introduced first in the treatment of leprosy in 1962 by Browne and Hogerzeil. This drug is prescribed for long term use, minimum for two years (WHO, 1982). In human the dose varies from 300 mg/week to 2800 mg/week depending upon severity of the disease. Clofazimine is the only drug of choice for the treatment against sulfone-resistant M. leprae. No report is known as yet about possible development of resistance due to continuous use of it. Its LD 50 in suspension in 1% acacia in the mouse is > 5 g/kg body weight.

One important characteristic side effect of the drug is the development of skin pigmentation. On intake it leads to the development of characteristic orange-red pigmentation of the skin. The higher the dose or longer the period of intake the greater is the intensity of the pigmentation. It is due to the accumulation of the drug in the skin.

The annual consumption of CLF in the state of Orissa, India alone is 50 kg and that in India is 770 kg for the year 1986-87 (OPPI, 1987).

The foregoing paragraphs clearly indicate how important this drug is in the field of internal medicine and how widely it
is being used. Thus, it warrants general consciousness to evaluate if it has any genotoxic potential. Though literature provides a host of information on the pharmacological aspects (see Lamprene, A working party held in 1968; Goodman and Gilman, 1985) and a little on molecular aspects (Morrison and Marley, 1976 a,b; Tsutsumi and Morrison, 1979) of this drug, our knowledge on its potential genotoxic effect is extremely limited. So far as the author is aware there are only two reports in this regard (Morrison and Marley, 1976a; Peters et al., 1983). Both the groups of workers (Morrison and Marley, 1976a; Peters et al., 1983) using the Ames Salmonella assay system with and without S9 mixture have reported no evidence of mutagenicity. But we do not know if it has any genotoxic effect on the eukaryotic system. However, that the drug is cytotoxic and inhibits mitosis and cell growth has already been demonstrated in cultured human fibroblasts (Delhanty et al., 1974). It has no teratogenic effect. In rats it has been shown to cause abortion (Stenger et al., 1970). In human pregnancy there is suggestive evidence that it suppresses the placental production of oestriol (Duncan and Oakey, 1933). Reduction in oestrogen secretion may be a probable cause for abortions; but it is yet to be confirmed in human cases. As mentioned earlier use of mammalian in vivo test system is highly desirable from human exposure and risk assessment point of view. All this prompted us to evaluate the possible toxic effects of CLF at the cytological and genetic levels in mouse in vivo system.

The details of the working parameters followed for this drug are given below.
I. Metaphase chromosome analysis in bone marrow cells.
   A. In adults exposed to the drug directly -
      1. Chronic treatment with a particular dose for different periods (referred to here as time-response study for convenience).
      2. Chronic treatment with different doses for a particular period (referred to as dose-response study).
   B. In youngs exposed to the drug through mother's milk.

II. Micronucleus test.
   A. In bone marrow cells - Dose response as in IA2.
   B. In hepatocytes.

III. Spermatocyte chromosome analysis - Time-response as in IA1.

IV. Sperm test.
   A. Sperm morphology assay - Time -response as in IA1.
   B. Sperm count assay - Time response as in IA1.

4.1.2 Material and Methods

Base-free sample (Batch no. not supplied) was obtained from S.G. Pharmaceuticals, Baroda, India. At the initial stage of our investigation we used ethanol to dissolve the drug and it was practised for metaphase chromosome analysis in both bone marrow cells and spermatocytes. However, later we felt that gum acacia (1%) would be a more suitable vehicle (for making suspension) and it was used in our successive experiments, e.g. in micronucleus tests (both bone marrow and hepatocytes). Accordingly two types of controls were kept : ethanol treated and acacia treated. The drug was fed by intubation. The details of the treatment schedule for different protocols adopted are as follows :
4.1.2.1 Meta phase chromosome analysis in bone marrow cells of mice exposed to the drug directly

Healthy adult mice of both sexes were fed with a daily dose of 40 mg/kg of the drug for different periods (1, 2 and 4 weeks) for time-response study. To evaluate dose-response effect another batch of mice were fed with three different doses of the drug, i.e. 4, 20 and 40 mg/kg/day for one week. The last set of animals treated with the highest dose was common for time-response and dose-response study. The drug was dissolved in required volume of abs. ethanol and the solution was diluted with water to make the final concentration of alcohol 0.2%. The volume of the solution fed to an individual was kept constant (0.25 ml). For dose-response study preparation of the solution was adjusted to keep the volume of the solution administered constant (0.25 ml). The animals were killed 24 h after the last dose treated. For each dose and time level 4-5 mice were employed. Control was run side by side with age and sex matched mice treated with 0.25 ml of 0.2% ethanol in identical fashion.

The collection and processing of the material from control and treated animals, and preparation and staining of the slides were done as described earlier in Materials and Methods (General) (vide 2.1).

4.1.2.2 Meta phase chromosome analysis in bone marrow cells of youngs exposed to the drug through mother's milk

A batch of 7 young mice were exposed to the drug through mother's milk. Their mothers (two) were treated with a dose of
40 mg/kg/day from the date of delivery over the period of lactation (~4 weeks). The youngs thus exposed to the drugs were killed after 2 and 4 weeks of exposure. Control was kept with age matched youngs born to mothers treated identically with 0.2% ethanol.

Cytological preparations of bone marrow cells of youngs were made following the schedule mentioned above (vide 2.1).

4.1.2.3 Micronucleus test in bone marrow cells

As mentioned above here 1% gum acacia, not ethanol, was used as the vehicle for the drug. So control was kept with 1% acacia.

A dose-response study was done and three dose-levels e.g. 4, 20 and 40 mg/kg/day were tested. The animals were treated with the drug in the same way via oral route for 7 consecutive days. For each dose level 5 mice (2 males and 3 females) were employed. The treated and control individuals were killed 24 h after the last dose treated and their bone marrow cells were processed for micronucleus preparation (vide 2.2.1).

4.1.2.4 Micronucleus test in hepatocytes

5 mice (2 males and 3 females) were treated with a dose of 40 mg/kg/day in an identical fashion for one week. However, on the 5th day of the treatment-period hepatectomy was performed in a short while after treatment. The mice were killed 24 h after the last treatment (i.e. at 72 h post-hepatectomy). The treatment schedule is represented diagrammatically below. The age and sex
matched controls fed daily with 0.25 ml of 1% acacia for one week were also hepatectomized and killed following the same schedule. Hepatectomy, collection and processing of the regenerated liver cells, preparation of slides and scoring of micronuclei in hepatocytes have been described earlier (vide 2.2.2).

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
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<tbody>
<tr>
<td>Treatment</td>
<td>Hepatectomy</td>
<td>Killed</td>
<td></td>
<td></td>
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</tbody>
</table>

4.1.2.5 Spermatocyte chromosome analysis

Adult male mice were administered via oral route with a dose of 40 mg/kg of the drug once daily for three different periods (1, 2 and 4 weeks). Control males were fed similarly with 0.2% ethanol (the solvent used for the drug in this experiment). The animals were killed 24 h after the last dose treated, and testes were collected and processed for analysis of spermatocyte chromosomes (vide 2.3).

4.1.2.6 Sperm test (Sperm morphology and count assays)

Vasa deferentia and caput epididymes, the materials for sperm morphology and sperm count assays respectively, were provided by the males used for the spermatocyte chromosome analysis. Details of the processings and scoring have been described elsewhere (vide 2.4.1 and 2.4.2).

4.1.3 Results

4.1.3.1 Metaphase chromosome analysis in bone marrow cells
Qualitative: The drug had in no case any effect on the behaviour of the animal. But the treatment, direct exposure or through mother's milk, led to development of characteristic orange-red pigmentation on the skin and body-hair, and it was more with higher doses or longer period of treatment.

As the chromosome aberrations induced by clofazimine in mice exposed to the drug directly and in youngs exposed through mother's milk did not differ much qualitatively they are considered together. The metaphase chromosomes following treatment, particularly in mice exposed directly, showed some despiralization and the degree of despiralization was more at higher dose levels (Figs. 4a-d). Stretching in the centromeric as well as in the secondary constriction regions was also very common (Figs. 4a,d). The structural aberrations encountered were mainly of chromatid type. Among the break-type aberrations chromatid breaks constituted the most frequently encountered aberrations (Figs. 4a-b,c-h), the acentric chromatid fragments were found to lie either near to its place of origin with a little displacement (Fig. 4c) or somewhere else in the metaphase plate (Fig. 4g). In some cases the break involved the secondary constriction region (Fig. 4i). Not more than one break was observed along a chromatid of the affected chromosome. Occasionally, however, chromosomes were found to have two breaks involving two sister chromatids. Small acentric fragments of untraceable origin were noted in a number of metaphases (Figs. 4j,k); in fact they constituted the second category of break type aberrations to chromatid breaks so far their frequency of occurrence was
Explanation of Fig. 4

Photomicrographs of mouse bone marrow metaphase plates showing various types of structural aberrations induced by clofazimine.

a, b. Metaphases each showing a chromatid break and despiralization of the chromosomes.

c. Chromosomes showing despiralization.

d. A metaphase plate showing despiralized chromosomes and stretching at secondary constriction region of a chromosome.

e. Chromosome with a chromatid break, fragment not displaced.

f. Chromosome with a chromatid break, fragment displaced.
Explanation of Fig. 4 (Contd.)

g. A chromatid break, the broken fragment displaced.

h. Chromosome with a chromatid break, the fragment shows slight displacement.

i. Chromosome with a chromatid break in the secondary constriction region.

j, k. Metaphase plates showing fragments of untraceable origin.

l. A metaphase field showing an iso-chromatid break.
Explanation of Fig. 4 (Contd.)

m.n. Metaphase plates showing polyploidy. Chromosomes with chromatid breaks, sub-chromatid and iso-chromatid and gaps.

o. A metaphase plate with minute paired fragment and gap.

p. A metaphase plate with a ring and paired fragment.
concerned. Iso-chromatid breaks (Fig.41) and gaps (Fig.4n) were also common. A cell was found to contain minute paired fragment probably originating from a chromosome or iso-chromatid break (Fig.4o). A metaphase plate with a centric ring accompanied with a fragment was noted (Fig.4p); it was assumed to have originated by reunion of the broken ends of the sister chromatids. A lone case of translocation showing end to end chromatid interchange was also recorded. Gaps and sub-chromatid breaks together constituted the most common type of aberration (Figs. 4m-6). Though a cell with two break type aberrations was rare two or more aberrations - one break type and others gap type, were noted in several cases (Figs. 4m, o).

The occurrence of polyploid cells though common did not bear any significance in their scoring. Although cells with hypodiploid number of chromosomes were common no aneuploid cell with 41 or 42 chromosomes was recorded.

Quantitative: Tables 4 and 5 summarize the frequencies and kinds of chromosome aberrations in bone marrow cells following direct treatment. The incidences of aberrations including as well as excluding gaps exhibited significant increase over their corresponding control values in all the test weeks (Table 4). They, however, failed to show any correlation with the periods of treatment (r = 0.248, df = 1). Absence of correlation was more marked when total aberrations were considered, the frequencies of total aberrations obtained in three test weeks were very close to each other. Almost the same trend was marked when aberration types like chromatid breaks, fragments and gaps were considered separately.
Table 4. Frequency distribution of different types of structural aberrations of bone marrow chromosomes induced by chronic treatment of clofazimine for different periods with a daily dose of 40 mg/kg. Each of the iso-chromatid break, ring and exchange was counted as two breaks.

<table>
<thead>
<tr>
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<tr>
<td>1</td>
<td>C</td>
<td>400/4</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>5 + 1$	ext{*}$</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>500/5</td>
<td>32</td>
<td>12</td>
<td>-</td>
<td>46 + 2</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>400/4</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>400/4</td>
<td>13</td>
<td>7</td>
<td>-</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>400/4</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>500/5</td>
<td>23</td>
<td>11</td>
<td>1</td>
<td>35 + 1</td>
<td>56</td>
</tr>
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</table>

C = Control, T = Treated, * = absolute figure, € = mean/100 cells + S.E. 't' test: a = p < 0.001.
Table 5. Frequency distribution of different types of structural aberrations of bone marrow chromosomes induced by chronic treatment of clofazimine with different doses for 1 week. Each of the iso-chromatid break, ring and exchange was counted as two breaks.

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<tbody>
<tr>
<td>0.0 (Cont)</td>
<td>400/4</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td></td>
<td>5+1*</td>
<td>7</td>
<td>12+1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.50 ± 0.43°</td>
<td>3.25 ± 0.75</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>400/4</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td></td>
<td>9+1</td>
<td>12</td>
<td>21+1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.50 ± 0.25</td>
<td>5.50 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>400/4</td>
<td>6</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td></td>
<td>14</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.50 ± 0.55°</td>
<td>9.25 ± 0.96°</td>
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</tr>
<tr>
<td>40.0</td>
<td>500/5</td>
<td>32</td>
<td>1</td>
<td>12</td>
<td>-</td>
<td>1</td>
<td>46+2</td>
<td>45</td>
<td>91+2</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>9.60 ± 0.35°</td>
<td>18.60 ± 1.28°</td>
<td></td>
</tr>
</tbody>
</table>

* = absolute figure, ° = mean/100 cells ± S.E.

't' test: c = p < 0.05, a = p < 0.001.

r = +0.949

r = +0.983
Table 6. Frequency distribution of different types of structural aberrations of bone marrow chromosomes in young mice exposed to clofazimine through mother's milk for different periods. Each of the iso-chromatid break, ring and exchange was counted as two breaks.

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</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>300/3</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.66 ± 0.27</td>
<td>2.66 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>400/4</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>14 + 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.00 ± 0.50</td>
<td>9.50 ± 1.15b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>300/3</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>4 + 1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.66 ± 0.54</td>
<td>4.33 ± 1.03</td>
</tr>
</tbody>
</table>

C = Control, T = Treated, * = absolute figure, ° = mean/100 cells ± S.E.

't' test : b = p < 0.01.
The control values for breaks and total aberrations obtained in different test weeks did not vary significantly \( (F = 1.44 \text{ and } 1.13 \text{ respectively}) \). Attempts were made to compare the treated data with the respective pooled control values \( \text{breaks } 0.91\% \text{ and total aberration } 4.66\% \) and the picture remained unaltered. Dose-response analysis of chromosome aberrations obtained with the doses tested revealed a distinct dose related increase of breaks as well as total aberrations (Table 5). Three major aberration types \( \text{e.g. gaps, chromatid breaks and fragments} \) when considered separately also exhibited the same trend. Two higher doses elevated the aberration frequencies significantly over the control value though the lowest dose level failed to do so.

The young mice when exposed to the drug through mother's milk for two weeks yielded significantly higher incidences of breaks as well as total chromosome aberrations, but not after four weeks of exposure (Table 6). The largest increase occurred in chromatid gaps and breaks.

4.1.3.2 Micronucleus test in bone marrow cells

Qualitative: Micronuclei obtained in the erythrocyte line were invariably round in shape, but varied in their size (Figs. 5a-d) suggesting loss of chromosome fragments of various sizes from the nucleus. The position of the micronuclei in the cell was also variable (Figs. 5a-g). The occurrence of more than one micronucleus in one erythrocyte was rare (Figs. 5i,j). The MN recorded in the nucleated cells were in general bigger in size (Fig. 5k). The nucleated cells in most of the cases also
Explanation of Fig. 5

Cut-out photomicrographs of bone marrow smears displaying micromucleated erythrocytes, nucleated cell and abnormal mitotic figure induced by clofazimine.

a,b,c- Polychromatid erythrocytes each containing one micronucleus. The MN vary in size and location.

c,g. Normochromatic erythrocytes each with one micronucleus.

i,j. Polychromatid erythrocytes showing two micronuclei in each case.

k. A nucleated cell having a micronucleus.

l. An anaphase cell with a bridge.
contained one micronucleus. Though some abnormal mitotic figures like lagging chromosome(s)/fragment(s) and anaphase bridge (Fig. 31) formation were found in the smear, their frequency remained at the control level and obviously were not considered for quantitative analysis.

Quantitative: A dose-response study revealed an increased incidence of MN in erythrocyte line (P, + N, ecs.) following treatment of CLP at all the doses tested (Table 7). However the increases for higher two doses only were statistically significant. P. ecs exhibited higher frequencies of MN than N. ecs; in fact in N. ecs the increases though dose related in no case were significant. For P. ecs and erythrocytes as such (P + N. ecs) the increases were not dose related. The frequency of nucleated cells with MN in no case following treatment of the drug differed significantly from control.

Mitotic index dropped down greatly on treatment of the drug and the decreases were very significantly and negatively correlated with the doses \( r = -0.929, \ df = 2 \). The P/N ratio in the treated individuals also showed a tendency of decrease.

4.1.3.3 Micronucleus test in hepatocytes

Qualitative: Our study was restricted to the regenerated hepatocytes only. Other types of cells like Kupffer cells, endothelial cells and fat storing cells were not considered; they are much smaller in size, fewer in number and have a higher nucleus to cytoplasm ratio, and are, thus, easily differentiable from hepatocytes. Characteristic nucleus to cytoplasm ratio (1:4) of
Table 7. Incidences of bone marrow erythrocytes with MN induced by chronic treatment of clofazimine with different doses for 1 week. Values are mean % ± S.E. Figures in parentheses are MN/cells scored.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of P. ec. with MN</th>
<th>N. Ec. with MN</th>
<th>P. + N. Ec. with MN</th>
<th>Nucleated cells with MN</th>
<th>P/N ratio</th>
<th>% of dividing cells (II)</th>
</tr>
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<tr>
<td>0.0 (Cont)</td>
<td>6</td>
<td>0.22 ± 0.05</td>
<td>0.15 ± 0.04</td>
<td>0.18 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>1.16 ± 0.06</td>
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<tr>
<td></td>
<td>(13/6000)</td>
<td>(9/6000)</td>
<td>(22/12000)</td>
<td>(5/6000)</td>
<td>1.16 ± 0.06</td>
<td>1.16 ± 0.06</td>
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<td>0.26 ± 0.05</td>
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<td>0.06 ± 0.02</td>
<td>0.37 ± 0.04</td>
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<tr>
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<td>(13/5000)</td>
<td>(10/5000)</td>
<td>(23/10000)</td>
<td>(3/5000)</td>
<td>0.37 ± 0.04</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>20.0</td>
<td>5</td>
<td>0.65 ± 0.11a</td>
<td>0.28 ± 0.06</td>
<td>0.57 ± 0.04a</td>
<td>0.06 ± 0.03</td>
<td>0.93 ± 0.03c</td>
</tr>
<tr>
<td></td>
<td>(43/5000)</td>
<td>(14/5000)</td>
<td>(57/10000)</td>
<td>(3/5000)</td>
<td>0.93 ± 0.03c</td>
<td>0.93 ± 0.03c</td>
</tr>
<tr>
<td>40.0</td>
<td>5</td>
<td>0.62 ± 0.08b</td>
<td>0.38 ± 0.09c</td>
<td>0.50 ± 0.05a</td>
<td>0.12 ± 0.06</td>
<td>0.95 ± 0.03c</td>
</tr>
<tr>
<td></td>
<td>(51/5000)</td>
<td>(19/5000)</td>
<td>(50/10000)</td>
<td>(6/5000)</td>
<td>0.95 ± 0.03c</td>
<td>0.95 ± 0.03c</td>
</tr>
</tbody>
</table>

* t test: a = p < 0.05, b = p < 0.01, c = p < 0.001.
Explanation of Fig. 6

Photomicrographs of regenerated hepatocytes with micronucleus or nuclear anomalies induced by clofazimine.

a. Hepatocytes showing varying degree of vacuolation in their cytoplasm. A uni-nucleate cell with micronucleus.

b. A trinucleate hepatocyte.

c-h. Uninucleate hepatocytes containing one micronucleus each.

d. A uninucleate hepatocyte with a micronucleus exhibiting the same degree of condensation as the main nucleus.

i-j. Binucleate hepatocytes each with one micronucleus.

k-l. Hepatocytes showing nuclear anomalies.
the hepatocytes helped greatly in identifying them and facilitated easy scoring of MN in them. The hepatocytes are known to vary greatly in their size (1-6 folds) and shape as well as in number and ploidy states of the nucleus (Elias and Sherrick, 1969). The cytoplasm of the hepatocytes exhibited various degrees of vacuolation due to the presence of glycogen (Figs. 6a-h). The vacuolated appearance of the cytoplasm at times posed difficulty in scoring MN. Attempts were made to reduce glycogen content of the cells by keeping the animals on a low carbohydrate diet. As usual the binucleate and uninucleate cells constituted the major bulk of the hepatocytes; multinucleates (represented by trinucleates only) were very few (Fig. 6b).

The particles noted in the present study as MN looked like chromatin bodies in all respects. Besides, they were identical in appearance to the MN recorded in the KC treated positive control. Their nuclear-nature was, thus, ascertained. Interestingly in hepatocytes the MN exhibited the same degree of condensation as the main nucleus (Fig. 6d). The locations of the main nucleus as well as the micronucleus, whenever present, in the cytoplasm varied greatly (Figs. 6c-1). The size of the MN showed a wide range of variation too (Figs. 6c-j). Most of the affected hepatocytes contained only one micronucleus (Figs. 6c-j), a few were found to contain two. Some of the tri- and bi-nucleate cells were found to have one nucleus smaller than the other(s) (~ half) (Figs. 6k,1). They were categorized separately under nuclear anomalies.

Quantitative Analysis of MN in regenerated hepatocytes following clofazimine treatment also exhibited significant increase
Table 8. Incidences of regenerated hepatocytes with MN induced by a daily dose of 40 mg/kg of clofazimine for 1 week. Values are mean % ± S.E. Figures in parentheses are MN/cells scored.

<table>
<thead>
<tr>
<th>Set</th>
<th>Total cells scored/animals</th>
<th>Uninucleate hepatocytes with MN</th>
<th>Binucleate hepatocytes with MN</th>
<th>Uni- + Binucleate ratio</th>
<th>Multi- nucleate hepatocytes with nuclear anomalies</th>
<th>Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>6000/4</td>
<td>0.36 ± 0.02</td>
<td>0.32 ± 0.06</td>
<td>0.34 ± 0.04</td>
<td>0.44 ± 0.01</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(7/1341)</td>
<td>(15/4159)</td>
<td>(22/6000)</td>
<td></td>
<td>(22/6000)</td>
<td>(2/6000)</td>
</tr>
<tr>
<td>T</td>
<td>10000/5</td>
<td>0.70 ± 0.10c</td>
<td>0.53 ± 0.06</td>
<td>0.60 ± 0.04b</td>
<td>0.47 ± 0.03</td>
<td>0.14 ± 0.01b</td>
</tr>
<tr>
<td></td>
<td>(23/3190)</td>
<td>(37/6810)</td>
<td>(60/10000)</td>
<td></td>
<td>(45/10000)</td>
<td>(14/10000)</td>
</tr>
</tbody>
</table>

C = Control, T = Treated.

't' test: c = p < 0.05, b = p < 0.01.
in the incidence of MN (Table 3). The increase was more pronounced in uninucleate hepatocytes. The frequency of nuclear anomaly also increased remarkably in the treated series. The ratios of univalent and multinucleate hepatocytes remained unchanged.

4.1.3.4 Spermatocyte chromosome analysis

Qualitative: So far structural changes were concerned the effects in the control and treated series as well as in different sampling intervals were the same except presence or absence of certain aberration type(s) at certain sampling intervals. The structural aberrations comprised chromatid and chromosome type breaks and fragments of untraceable origin. Chromatid type breaks involved both autosomes (Figs. 7a-e) and sex chromosomes (Figs. 7f, g). In most of the cases the fragments resulted from chromosome type breaks were seen placed far away from their places of origin (Fig. 7h); in some cases, of course, the fragments exhibited a little displacement only (Fig. 7i). In one case the X chromosome was found to be broken into two pieces. In chromosomes were also found to be broken in a similar fashion in 2 pieces. Though chromosome and chromatid type of breaks were of common occurrence in all the sampling intervals, not a single exchange type aberration was encountered. A few cells were found to contain some extra fragments smaller than the smallest univalent (Fig. 7j); because of their smallness their origin could not be traced.

Though cells with less than 20 bivalents were of frequent occurrence only one cell was recorded to contain 21 bivalents.
Explanation of Fig. 7

Photomicrographs of diakinesis - metaphase I spermatocytes showing structural and pairing anomalies of chromosomes induced by clafazimine.

a - e. Diakinesis - metaphase I plates showing chromatid breaks involving autosomes.

f. A diakinesis - metaphase I plate showing break in X-chromosome.
Explanation of Fig. 7 (Contd.)

9. Y-chromosome showing a chromatid break.

h. Chromosome type break, the broken fragment displayed greatly.

i. Chromosome type break, the broken fragment lying near its place of origin.

j. A metaphase I plate showing a minute fragment of untraceable origin.

k. X-Y chromosomes lying wide apart.

l. An autosomal bivalent involved in univalent formation.
Because of the extreme rarity of the occurrence of the latter it was not considered for quantitative analysis. No importance was given to plates with less than 20 bivalents as they might have been originated due to technical shortcomings, and as such they are not presented. Though occurrence of polyploid diakinesis-metaphase I cells was common, but incidence did not vary remarkably from that of control. In an extreme case a cell was noted to contain 80 bivalents (8n).

Both autosomes and sex chromosomes were involved in univalent formation. In several cases X and Y were found to lie wide apart (Fig. 7k). Similar cases were also noted involving autosomes (Fig. 7l). Among the autosomal bivalents the smaller ones were frequently involved.

Quantitative: Data on the abnormalities of meiotic chromosomes induced by clofazimine treated for different periods are presented in Table 9. The total breakage frequencies increased significantly over the control values in all the test weeks. However, the values obtained in different test weeks were very close to each other and did not reveal any correlation with the period of treatment (r = -0.53, df = 1). The individual aberration types did not also exhibit much variation in their frequencies in different weeks. In no week in the control series aberration type other than chromatid break was observed. In contrast, besides chromatid breaks, in the treated series in all the weeks chromosome breaks and fragments were noted.

Frequency of univalent formation, taking autosomes and sex chromosomes together increased significantly in all the test
Table 9. Frequency distribution of different types of structural abnormalities and pairing anomalies of spermatocyte chromosomes induced by chronic treatment of clofazimine for different periods with a daily dose of 40 mg/kg.

<table>
<thead>
<tr>
<th>Samp. Set</th>
<th>Cells scored/animal (wks)</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chrom</td>
<td>Chromat. Frag. Exch. Total</td>
<td>Auto.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>br.</td>
<td>br.</td>
<td></td>
</tr>
<tr>
<td>1 C</td>
<td>200/4</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>250/5</td>
<td>2</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 C</td>
<td>200/4</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>200/4</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 C</td>
<td>200/4</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>T</td>
<td>250/5</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r = -0.53

C = Control, T = Treated, * = absolute figure, ° = mean/100 cells ± S.E.
't' test : c = p < 0.05, b = p < 0.01, a = p < 0.000.
weeks (Table 9). For sex chromosomes only it varied within a limited range (9.6 - 11.2%), while for autosomes it varied to a great extent (2.8 - 13.0%). There was no correlation of the event with the period of treatment.

4.1.3.5 Sperm morphology assay

Qualitative: Though in the present study only intact sperm having head and tail were considered, no sperm with tail abnormality was noted; the abnormalities recorded here involved the head morphology only. However, it was not possible to group the abnormalities under some distinct categories; they were classed into some arbitrary 'types'. The types like triangular, 'axe' shaped, with flat base, with vacuole, with acrosomal spine abnormality, sickle-shaped and amorphous were very common in occurrence (Figs. 9a-i). The sperm heads with acrosomal spine abnormality exhibited a high degree of variation in the spine morphology ranging from a total absence to a long hook shaped one (Figs. 9c, h, j). In addition to the above 'types', semilunar, notched, giant and with swollen middle piece were also available, though rare, in the preparations. All the types of head abnormalities mentioned above were also available in the control individuals. Some abnormal types like spherical, twin and granular were recorded, admittedly rare, in the treated individuals only (Figs. k-m). Sometimes a particular sperm head exhibited a combination of two or more types of abnormalities (Figs. c,d,h,l).

Quantitative: As the grouping of abnormal 'types' was arbitrary it was not possible to have the quantitative analysis

86
Explanation of Fig. 8

Photomicrographs of vasa deferential sperm showing abnormalities in head morphology in mice treated with clofazimine.

a. A triangular sperm head.

b. An axe shape sperm.

c. A sperm containing a vacuole and with flat base and blunt acrosome.

d. A sperm with two vacuoles and short acrosome.

e. A sickle shaped sperm.

f,g. Amorphous sperm heads.

h. An amorphous sperm with flat base and without acrosomal spine.
Explanation of Fig. 8 (Contd.)

1. A plate showing a few amorphous sperm.

j. A sperm with hooked acrosome.

k. Twin sperm.

l. A sperm with spherical head and another triangular sperm with vacuole.

m. A granular sperm.
'type-wise', all the morphologically abnormal sperm heads were taken together under 'abnormal' for the purpose. The frequencies of abnormal sperm ranged from 2.37 to 4.45% in the control series and 4.62 to 7.28% in the treated series (Table 10). If the treated data were compared with the respective week-wise control a trend of increase in the incidence of abnormal sperm heads was clear.

Table 10. Incidences of sperm head abnormalities in mice induced by chronic treatment of clofazimine for different periods with a daily dose of 40 mg/kg. Values are mean per 100 sperm ± S.E. For each point 4-5 animals were employed and 1000 sperm heads were examined from each individual.

<table>
<thead>
<tr>
<th>Set</th>
<th>wk 1</th>
<th>wk 2</th>
<th>wk 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.37 ± 0.25</td>
<td>4.45 ± 0.36</td>
<td>4.17 ± 0.59</td>
</tr>
<tr>
<td>T</td>
<td>7.23 ± 0.78</td>
<td>4.62 ± 0.79</td>
<td>6.10 ± 0.82</td>
</tr>
</tbody>
</table>

C = Control, T = Treated, 
't' test: a = p < 0.001.

But the increase was remarkably significant only at week 1. If the control values for different weeks were pooled the mean value would have been 3.66%, which is very close to the value obtained by earlier workers (Bruce et al., 1974; Kar and Das, 1983) in the same strain of mice. A comparison of the treated data with the pooled control mean (3.66 ± 0.35) exhibited significant increase of abnormality at weeks 1 and 4. The highest value (7.28%) obtained following treatment of the drug was nearly double the pooled
control mean value.

4.1.3.6 Sperm count assay

A week-wise comparative account of sperm counts of treated and control animals is presented in Table 11. In the control series the mean sperm count ranged from 108.28 to 161.09, while in the treated series it ranged from 63.70 to 125.77.

Table 11. Effect of clofazimine given once daily (40 mg/kg) for different periods on the epididymal sperm count. Each value represents the mean number of sperm (heads only) present in one WBC chamber of haemocytometer ± S.E. For each point 4-5 animals were employed and from each animal count was done in 25 WBC chambers. To obtain total count per epididymes each value is to be multiplied by 40,000 (dilution factor).

<table>
<thead>
<tr>
<th>Set</th>
<th>wk 1</th>
<th>wk 2</th>
<th>wk 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>161.09 ± 11.44</td>
<td>108.28 ± 2.04</td>
<td>156.32 ± 25.04</td>
</tr>
<tr>
<td>T</td>
<td>110.09 ± 9.33</td>
<td>126.77 ± 15.95</td>
<td>63.7 ± 12.39</td>
</tr>
</tbody>
</table>

C = Control, T = Treated

't' test: C = p < 0.05.

Week-wise comparison of the control and treated data indicated a little suppression of sperm count in the treated series at weeks 1 and 4. But as a whole no remarkable significant difference was obtained. Apparently the control values showed some difference among themselves but analysis of variance failed to reveal
any variation \((F = 2.51)\). When the treated data were compared with the pooled control mean \((141.90)\) only the data for week 4 showed a significant decrease.

4.1.4 Discussion

4.1.4.1 Metaphase chromosome analysis in bone marrow cells

Both time-response and dose-response analyses of bone marrow chromosomes revealed significantly higher breakage frequencies in the treated series over the control ones. The total aberrations which included both break and gap type aberrations also showed the same picture. All this clearly indicates clastogenic capacity of the drug.

The clastogenic efficiency of the drug is positively correlated with the doses but not with the period of the treatment. Chronic treatment for 1, 2 and 4 weeks did not make much difference in causing chromosome damages as indicated by the production of total aberrations of chromosomes. On the other hand, the frequency of breaks as well as of total aberrations for the highest dose tested \((40 \text{ mg/kg})\) was more than three times the respective value obtained with the lowest dose \((4 \text{ mg/kg})\). The present experiment is based on the chronic treatment of the drug and the minimum period of treatment was one week. No experiment was done with acute treatment. It would have been interesting to know if acute and chronic treatments of the same dose exhibit any difference in production of chromosome damages. Anyway, it is not possible on our part to put forward any explanation at this stage for such lack of influence of the period of treatment.
Clastogenic efficiency of the drug was also demonstrated in the youngs which were allowed to take mother’s milk ad libitum, and whose mothers were receiving the drug regularly. The drug is known to pass very little to the developing youngs through the placental barrier, but considerably to the youngs via mother’s milk (Lamprone - A working party, 1968). The passing of the drug to the youngs through mother’s milk was clearly evident here also from development of characteristic orange-red skin pigmentation and hair colouration in the youngs (Fig. 9). The lower incidence of chromosomal aberrations after 4 weeks of exposure compared to 2 weeks exposure can be attributed to decrease in consumption of mother’s milk and thereby decrease in consumption of the drug with ageing of the youngs. This can be substantiated by the fact that the characteristic skin pigmentation was more intense in youngs exposed for 2 weeks than in youngs exposed for 4 weeks. The results are, thus, in good agreement with those of dose-response study performed in adults exposed directly to the drug.

Qualitatively almost all the aberrations induced by CLF are of chromatid type, even after weeks of chronic treatment. It seems CLF like the great majority of chemical chromosome-damaging agents requires replicative DNA synthesis in order to have the lesion it induces translated into structural aberration.

The chromosomes following CLF treatment exhibited high degree of despiralization of the chromosomes. No quantitative analysis was done for it. However, in general in CLF treated mice, compared to DDS and DEC treated ones (vide infra) and controls, the chromosomes were thinner and longer. The degree as well as
Young mice (1st and 4th) showing colouration in their skin and fur after being exposed to clofazimine through mother’s milk. The 2nd and 3rd are unexposed mice.
incidences of despiralisatioa increased with the increase of dose and treatment period. It is, thus, indicated that the drug affects also the condensation process of the chromosomes greatly.

4.1.4.2 Micronucleus test in bone marrow cells

Sensitivity, reliability as well as simplicity of the micronucleus test have already been illustrated. Its principle has also been described (vide 1.3.2). In the present investigation poly- and normochromatic erythrocytes as well as nucleated bone marrow cells were considered. However, during scoring of MN in the nucleated cells, as mentioned earlier, due to the presence of large nucleus and comparatively little cytoplasm in certain cells separate existence of MN become doubtful. Similarly confusion arose regarding the reality of MN in certain other cells with lobulated nucleus. Though those doubtful cases were not recorded, the data on nucleated cells were surely influenced by it. For that we do not want to put much importance on the incidence of MN in the nucleated cells. Higher incidence of MN, compared to controls, in erythrocyte line, in which incidence is more relevant (Ledebur and Schmid, 1973; Schmid, 1976) indicates positive effect of the drug. So, the results of MNT agree well with those of cytogenetic assay based on metaphase chromosome analysis.

MN are formed both by clastogens and spindle poisons. Clastogens are known to enhance the frequency of micronucleated P.ees, while spindle poisons enhance the frequency of micronucleated N.ees (Schmid, 1976; Seiler, 1976). However, on the basis of higher incidences of micronucleated P.ees at all the dose levels in the present investigation we can not predict clastogenic
efficiency of the drug, because here chronic treatment was
followed for 7 days. But absence of spindle poisoning effect of
CLF was manifested by non-availability of large sized polychromatic
erthrocytes in the preparation. Such types of erythrocytes are
usually originated due to polyploidy and subsequent elimination
of polyploid nucleus. Though a number of abnormal mitotic figures
like anaphase bridge formation and lagging of chromosome(s) or
chromosomal fragment(s) at anaphases were noted the incidence of
their occurrence was not so remarkable. Further, they are not
quite convincing evidences of spindle inhibition. Nor we got big
sized MN. All these evidences lead us to assume that the MN induced
by the drug resulted not due to its spindle poisoning effect but
due to clastogenic effect.

In treated animals the mitotic index as determined from
smear preparations declined significantly and the decrease was
strongly dose-related. The drug seems to act as an antimetabolite.
Such a characteristic is also indicated by the P/N ratio which
showed suppression in turn-over of bone marrow cells to P.eos.
Our results, thus, confirm the findings of Delhanty et al. (1974)
who have reported substantial inhibition of mitosis and cell growth
in cultured human fibroblasts exposed to CLF at a concentration
as low as 2.5 μg/ml. This antimitotic effect of the drug is believed
to be (Delhanty et al., 1974) due to its anti-mitochondrial activity
which has earlier been demonstrated in yeast cells and in isolated
mitochondria from rat liver cells (Rhodes and Wilkie, 1973). Impair-
ment in DNA template functioning is an alternative explanation for
antimetabolic effect of the drug (Morrison and Barley, 1976a,b;
Tsutsumi and Morrison, 1979).
Though Salamone et al. (1980) have stressed on time-response study for analysis of MN, Yamamoto and Kikuchi (1981) suggest to test a range of doses. However, as we feel, whether one should follow a dose-response or time-response study it depends on the agent to be tested. With multiple-treatment regimen dose-response study seems to be more meaningful. Both single and multiple treatment schedules have been proved to be equally sensitive in detecting alkylating agents and spindle poisons; but multiple treatment is particularly effective for detecting the antimetabolites which require longer period to induce MN (Yamamoto and Kikuchi, 1981). In the present study the highest response in erythrocytes, which was more than 3 times the corresponding control value was obtained with the intermediate dose; the value for the highest dose was very close to it. The maximum effect for the intermediate dose may be explained from the point of antimetabolic effect of the drug as found with methotrexate and 5-fluorouracil (Yamamoto and Kikuchi, 1981).

4.1.4.3 Micronucleus test in hepatocytes

CLF is known to accumulate unchanged in different organs including liver and bone marrow, and the rate of accumulation varies from organ to organ (Fansfield, 1974; Levy, 1974a). The major bulk of it is also excreted out unchanged and very slowly (Warren, 1983). Recently Fern et al. (1981, 1982) have identified 3 metabolites (metabolites I, II and III) in trace amount in urine of patients taking the drug. The idea of analyzing MN in hepatocytes was an attempt to assess genotoxicity of the drug in the cells which take part in the process of metabolism and thereby
to have more information about the active agent responsible for chromosome breakage induced by CLF. The highest dose tested in the bone marrow micronucleus test (40 mg/kg) was also tested for the hepatocyte HNT. The animals were also treated identically (40 mg/kg x 7, oral). Both types of cells - bone marrow and hepatic, show positive response; degree of response, however, differed. In bone marrow cells the incidence of MN in erythrocytes was little less than 3 times the control value; while in hepatocytes it was little less than twice the control value (Fig. 10). Controls for two types of studies were also identical, - acacia treated. The lower incidence of MN in hepatocytes indicates that the metabolites have little role in inducing breaks, rather the drug itself is responsible for that. It is too early to advance speculations on the differential results obtained in two tissues. However, role of differential accumulation of the drug in different organs cannot be ruled out.

4.1.4.4 Spermatocyte chromosome analysis

The importance of germ line cells particularly of mammals in the evaluation of potential genotoxicity of a drug has already been discussed. Significantly higher breakage frequencies for all three sampling periods clearly demonstrate clastogenic capacity of the drug. So the results support our findings based on bone marrow chromosome analysis and micronucleus tests. That period of treatment has little influence on the incidence of chromosome aberrations is also manifested here. As noted in the case of bone marrow cells the frequencies of breakages in spermatocytes following repeated treatment for different periods did not differ markedly.
Fig. 10 Histogram analysis of the incidences of bone marrow erythrocytes and regenerated hepatocytes with micronuclei in mice following chronic treatment of clofazimine for one week.
(Data taken from Tables 7 and 8)
In no instance the incidence of aneuploidy as well as polyploidy exceeded the control range. All these support our findings based on bone marrow chromosome analysis and IFT that CLF has no spindle poisoning effect at least with the doses tested. That the drug has antimitotic effect leading to inhibition of onset of mitosis has, however, been noted in the IFT. But that is partial, and it is documented by the availability of dia-met stages in considerable number in the preparations.

Oakberg (1957, 1960) and Ghosal and Mukherjee (1971) determined the time scale of different gametogenic stages in mice. Accordingly the cells examined after 1, 2 and 4 weeks of treatment were expected to remain at spermatocyte, differentiated spermatogonia and spermatogonial stem cell stages respectively at the time of starting of the treatment, provided there was no inhibitory effect. On obtaining significant positive response after one week-treatment it is reasonable to assume that the drug affected the spermatocyte chromosomes, specifically late spermatocyte chromosomes. A number of chemicals including alkylating agents (e.g. nitrogen mustard, diepoxybutane, ENU, hydroxyurea, etc.) are known to induce chromosome aberrations in spermatocyte stage (see Adler 1982a). As the breakage frequencies obtained after 2 and 4 weeks of treatment remained very close to that for 1 week it is a logical assumption that the spermatogonial stem cells and differentiated spermatogonia remained unaffected, but they got affected while passing through spermatocyte stage (if spermatogonia too - differentiated and stem, would have been affected than after 4 weeks of exposure breakage frequency would have been much more). It may also
happen that the affected spermatogonia were selectively eliminated before their entry in the spermatocyte phase as assumed by Adler (1992a) for several other chemicals. Alternatively, delayed appearance of the affected late spermatocytes may also be a reason for uniformity in the frequencies of breaks in different test weeks. As CLF does not exhibit any inhibitory effect on the cells in dividing phase the last alternative seems to be far from reality. Most of the chemicals including alkylating agents require a round of DNA replication for translation of the damage into a chromosomal event. But surprisingly positive results are obtained as mentioned above with a number of alkylating agents both in the study of anaphase and metaphase chromosomes after treatment of prophase stages. As noted earlier CLF seems to be an S phase dependent chemical, but it does cause chromosome breakage when spermatocytes are exposed to it.

With regard to effect on pairing behaviour both auto-and sex-chromosomes exhibited susceptibility to univalent formation. X-Y bivalents showed higher susceptibility than the autosomal bivalents; in spite of greater number of autosomal bivalents the frequency involving them was in general lower. As mentioned earlier it is not known if such a behaviour results either due to early break down of association or due to complete lack of it. As assumed by many workers (Brewen and Preston, 1973) method of slide preparation is largely responsible for such phenomenon and the incidence in untreated animals remains in the order of 10%. But if one takes autosomes and sex chromosomes together the control values for 3 sampling periods varied from 5.50 - 6.50%; about 2 to 4 times increase of the incidences of univalent formation following
treatment of CLF cannot simply be explained from preparational point of view. It is, therefore, not unlikely that the drug impairs the meiotic pairing of chromosomes. Antirabies vaccine and tetanus toxoid (Das and Nayak, 1987), chlordiazepoxide (a benzodiazepine tranquilizer, Kar and Das, 1987), 3-methyl-4 nitrophenol (Nehez et al., 1985) and MC (Chakrabarti et al., 1986), but not diazepam (Kar and Das, 1981), have been reported to induce such univalent formation in mice. Whatever may be the mechanism, univalent formation involving sex chromosomes (Beechey, 1973; Chandley et al., 1976; Chandley, 1981) as well as autosomes (Burnell, 1973) has earlier been shown to be associated with meiotic break down in male mammals.

4.1.4.5 Sperm morphology assay

So far qualitative aspect is concerned in control and treated mice some common type of abnormalities were recorded. Treated animals produced a few more types of abnormal sperm heads, though their frequencies were low. Similarly occurrence of some particular abnormal types specifically for certain chemicals were noted earlier also (Wyrobek and Bruce, 1975).

In mice, as mentioned earlier, the shape of a normal sperm seems to be genetically controlled and induced abnormalities are suggested to be the result of genetic damage (Wyrobek and Bruce, 1975, 1978; Bruce and Heddle, 1979; Wyrobek et al., 1983a, b). Although the criteria of sperm morphology include different parts of a sperm, analysis becomes easier if limited to head abnormalities only, since head shape is most insensitive to
preparational damage. The frequency of sperm head abnormalities increased in all the test weeks after treatment, but the increases were not remarkably high except at week 1. So we can rank CLF with antimetabolites like hydroxyurea, imuran, I UdR, aminopterine, etc. which are known to produce also less marked effects on sperm morphology (Wyrobel and Bruce, 1975). However, in all those earlier cases the effect was remarkably noticed at week 4. Out of 24 chemicals tested by Wyrobek and Bruce (1975) 10 showed an elevation at week 1, and of them griseofulvin, dichlorvos, trimethylphosphate and I UdR, like CLF, induced the highest effect at week 1. At the highest dose again both metopa (14 mg/kg) and aminopterine (10 mg/kg) induced almost same frequency of abnormalities at weeks 1 and 4. It seems the matured sperm are being affected by CLF.

4.1.4.6 Sperm count assay

An attempt was made to know if CLF causes any adverse effect on sperm production. However, the data obtained in 3 different test weeks were to some extent erratic, significant reduction in count was noted at week 4 only when the treated data were compared with the pooled control mean ( \( \bar{x} = 141.90 \) ). Though a little suppression in count is apparent nothing can be said on the basis of these limited data. Here animals were kept under treatment maximum for a period of 4 weeks which covered mainly the spermiogenesis step for the sperm under study. To get an answer if the drug has any adverse effect on production of sperm treatment for still longer period or long recovery period following treatment is essential.
Thus, our data on chromosome analyses in bone marrow cells and spermatocytes, and on micronucleus test in bone marrow and hepatocytes reveal beyond doubt that CLF has clastogenic effect. So far our knowledge goes it is the first report on the potential mutagenic effect of CLF in eukaryotic system and it turns out to be a positive one. Our findings, however, contradict the reports made by Morrison and Marley (1976a,b) and Peters et al. (1983). To evaluate possible mutagenicity both the groups of workers used the Ames Salmonella assay with and without rat liver microsomes. Morrison and Harley (1976a) employed S. typhimurium strains TA98 and TA100; and Peters et al. (1983) used, in addition to those two, TA1535, TA1537 and TA1538 strains. But no evidence of mutagenicity was detected by any group of workers. Thus, both the earlier works were conducted in bacterial system, and literature provides no other report on possible mutagenicity of CLF. The Ames Salmonella test is no doubt a very sensitive system for detection of potential mutagens (McCann et al., 1975), but a number of potent mutagens are known to escape detection by this system. The best known example is procarbazine which yields positive response in all mammalian systems in vivo and in vitro and also in Drosophila, but is negative in bacterial tests (Adler, 1980). The rodent nasal carcinogen, hexamethylphosphoramide (HMPA), also exhibits similar characteristic (de Serres and Ashby, 1981). The drugs like isoniazid and theophylline also showed negative response in the Ames test, while positive in chromosome test (Ishidate, 1981). Such type of contradiction is, therefore, not uncommon with the chemical mutagens. The reason for such contradiction remains unknown.
Our knowledge on pharmacokinetics of the drug as well as on mechanism of its action at the macromolecular level may help explain the clastogenic capacity of the drug. Though by now we have sufficient knowledge on the former, (Levy, 1974b; Wyngarden and Smith, 1985; Goodman and Gilman, 1985) the latter is still obscure. After oral intake the drug is absorbed in the gastrointestinal tract, passes into the blood stream and from there reaches rapidly into different organs unchanged. It is taken chiefly by cells of the reticuloendothelial system and stored in all organs except brain. In the tissues the drug soon appears as crystals (Conalty, 1956). Because of its lipophilic nature its accumulation is the maximum in fat cells and macrophages. The exact mode of absorption is not yet clear, but it is slow and varies with species, e.g. dogs absorb no clofazimine (Vischer, 1969). The micronized drug is reported to be absorbed at 50% level only, in oil suspension it is more (maximum upto 85%). The drug is retained within the tissue for a very long period even after discontinuation of the treatment. The half-life of the drug is about 70 days (Levy, 1974b). The serum level of the drug remains low soon after administration and increases slightly with the increase of doses. The rate of elimination of the drug is very slow. Only a few micrograms are being excreted daily via the kidneys and still less amount through the sebaceous and sweat glands (Warren, 1968). The orange-red colouration of the fat may persist for months. However, in preliminary investigations based on mass-, UV- and visible spectroscopy Feng et al. (1981, 1982) identified three metabolites of CLP : an unconjugated (Metabolite I)-
3-(P-hydroxy-aniline)-10-(P-chlorophenyl) 2, 10-dihydro-2-isopropyl iminophenazine), a conjugated (Metabolite II) - (3-((\beta-D-glucopyranosiduronic acid)-10-(P-chlorophenyl)-2, 10-dihydro-2-isopropyl iminophenazine and a hydrated (Metabolite III) clofazimine glucuronide in urine of patients taking CLF. All the metabolites are red in colour and formed in very very small amount (3 together constitute < 1% of the dose administered). The most of the drug is excreted unchanged.

As the metabolites identified recently (Feng et al., 1981, 1982) are produced in very small amount it is highly probable that the drug itself is clastogenic. Relatively lower incidence of MM in liver cells, the site of metabolism, than in bone marrow cells seems to support the view.

Species specific absorption rate as well as differential accumulation rate in different organs are the important factors for the expression of clastogenic effect. Further, accumulation rate depends largely on the dose (Banerjee et al., 1974; Mansfield, 1974). Dose related increase of chromosome aberrations as seen in bone marrow cells clearly indicates the importance of accumulation of the drug in tissue in causing chromosome aberration.

So far as the author is aware literature provides only the reports of Morrison and his co-workers on the mode of action of CLF at the macromolecular level (Morrison and Harley, 1976a, b; Tsutsumi and Morrison, 1979). Morrison and Harley (1976a,b) have reported that CLF binds to all DNA, prokaryotic and eukaryotic. However, no evidence is found to support an intercalating mechanism for binding. It is assumed that the drug binds with the guanine
bases and forms a DNA–clofazimine complex. This binding stops template function of the DNA strands and results in inhibition of DNA dependent RNA polymerase function (Tautsumi and Morrison, 1979).

Further, the drug has already been proved to be a potent inhibitor of mitochondria in yeast cells and of isolated mitochondria of rat liver (Rhodes and Wilkie, 1973). Delhanty et al. (1974) have demonstrated the inhibitory effect of the drug on oxygen uptake in human fibroblasts in culture and subsequent inhibitory effect of it on mitosis and cell growth. The drug, thus, inhibits template functioning as well as impairs energy metabolism. These antimetabolic activities of the drug are also evidenced in this study from significant dose related reduction of mitotic index as marked in the MNP in bone marrow. The clastogenic potency of the drug may be explained from its antimetabolic activity as is found with several other metabolic inhibitors like 5-fluorodeoxyuridine, deoxyadenosine, 6-mercapto purine, methotrexate, etc. (Kihlman, 1966; Matter and Schmid, 1971; Manna and Das, 1975; Yamamoto and Kihuchi, 1981).

Recently Niva et al. (1984) have reported that CLP increases significantly the generation of OH radicals in a dose dependent manner with a subsequent decrease of $H_2O_2$, but has no effect on superoxide dismutase activity of the polymorphonuclear leukocytes and monocytes of leprosy patients. In view of the great role being played by the free radicals in inducing chromosome aberrations by X-rays in dilute solution (Painter, 1970) the possible involvement of OH radicals here leading to chromosome
aberrations cannot be ignored. The foregoing discussion is based on several speculations. The mechanism of aberration production cannot be explained satisfactorily until we know the exact mode of its action at the molecular level.

The lowest dose tested here is four times the minimum recommended human therapeutic dose (50 mg/day) if one calculates on per kg basis. If calculation is done on the basis of the surface area of the individual, the equivalent mouse dose becomes 12 times the human dose (Freidreich et al., 1966). So, on the surface area basis our lowest dose would be one-third of the minimum human therapeutic dose, and the highest dose (40 mg/kg) would be less than half of the maximum human therapeutic dose (400 mg/day). The LD₅₀ of CLP in 1/3 acacia suspension in the mouse is 5 g/kg. The higher two doses tested here elevated the incidences of breaks, total aberrations and MN significantly over the respective control values, though the lowest dose failed to do so. So, from the point of potential genotoxic risk consideration of human therapeutic doses is highly important.
4.2 Effect of Dapsone:

4.2.1 Introduction

4', 4' - diaminodiphenylsulone (DDS) and its other sulphone derivatives belong to the group of sulfonamide. Sulphones have strong bacteriostatic and mild bactericidal effect. Though sulphones were synthesized long back they were introduced as active chemotherapeutic agents against leprosy and tuberculosis in the early 1940s. They are, however, no longer used in the treatment of tuberculosis as more effective drugs are available. But they are still in use as major antileprosy drugs. Among the sulphones DDS is the most potent and with least side effects and is obviously pharmaceutically more important. DDS is commonly known as dapsone. The structural formula of DDS is given below.

\[
\begin{align*}
\text{NH}_2 & \quad \text{SO}_2 & \quad \text{NH}_2 \\
\end{align*}
\]

This compound, originally synthesized as a dye-stuff intermediate, is found to have high antibacterial potency. It is an odourless white crystalline powder soluble in alcohol and dimethylsulfoxide but sparingly soluble in water.

Though leprosy was known to exist in prehistoric times also but therapeutic measures were not known till the use of sulphones. Before the introduction of DDS only plant products and plants like chaulmoogra oil, hydnocarpus oil and their esters,
Centenella asiatica and C. coriacea were used as antileprosy agents. DDS was the first drug to be introduced in the treatment of leprosy and it is the first drug of choice till date in the triple drug regimen recommended by WHO (1982). In case of monotherapy DDS is the only choice. Effectiveness of DDS to combat M. leprae in mouse foot pad system has been studied by Shepard and Chang (1964). The estimated sensitivity of the drug is between 1 and 10 ng/ml for micro-organisms recovered from untreated patients (Levy and Peters, 1976; Shepard et al., 1976). DDS is prescribed for regular use for prolonged period - minimum 2 years. Sometimes it is continued for 10-15 years. The recommended dose of DDS is 100 mg per day, but it may be given in variable doses depending upon requirement and severity of the disease. DDS is known to exert some very common toxicity like haemolysis of varying degree and methaemoglobinemia, which are found to be dose-related. Besides, it causes sulfone syndrome i.e. fever, malaise, exfoliative dermatitis, jaundice, hepatic necrosis, etc. Earlier it was used as monotherapy but the cause of failure of this monotherapy is the emergence of resistant organisms. DDS resistance was first reported by Pettit and Rees (1964). In India Taylor (1976) first identified the DDS resistant cases, and now 20-70 per thousand cases are known to be resistant to DDS in this country. DDS resistant M. leprae now occurs in 10% patients on long-term treatment and occasionally in untreated persons. To combat the DDS-resistant varieties triple-drug regimen (DDS + REN + CLP) has been introduced. Till date, DDS is considered as a pivotal drug in the management of leprosy. On the other hand, production units in India having
specific production quota can hardly meet the total requirement of the country which exceeds more than 50 MT per year (Ind. Pharma. Guide, 1985).

Till recently nobody doubted the carcinogenic potency of DDS. That DDS could be carcinogenic was suggested in 1971 by Mansson who observed malignancies in 9 of 9 patients treated with DDS for several years. Bergel (1973) was the first to show mesenchymal tumours of the abdominal organs and thyroid cancer in 100% Wistar rats which received DDS (0.3%) in the diet for 2 years. Stoner et al. (1973) also reported enhancement of the incidence of lung tumours in a strain of mice treated with dapsone. Later an IARC investigation (Griciute and Tomatis, 1980) recorded spleen sarcoma and thyroid carcinoma in rats fed 10-16 g of DDS each over a period of 104 weeks, and confirmed the findings of Bergel (1973). Literature provides a number of negative reports too (see Griciute and Tomatis, 1980). However, the carcinogenic potency is low. It has no teratogenic activity.

The association of cancers with chromosome aberrations is well established. Naturally with the publication of carcinogenic efficiency of DDS (Bergel, 1973) geneticists got interested to find out if it is clastogenic too. But till date much works have not been done in this line. Beiguelman and his associates studied the possible clastogenic effect of the drug. Peripheral blood lymphocytes exposed in vitro to 4 µg/ml of DDS exhibited significantly higher incidences of aneuploids and achromatic gaps (Beiguelman et al., 1975). Significantly high frequencies of structural chromosome aberrations, but not numerical aberrations,
were also noted in peripheral lymphocytes of patients under DDS therapy (Beiguelman and Pisani, 1976), as well as in cultured skin fibroblasts of patients under DDS alone or combined therapy (Hacket and Beiguelman, 1985). Recently Peters et al. (1983) tested the mutagenic potential of DDS and all its available metabolites and derivatives, and also of other antileprosy drugs using the Ames Salmonella/microsome assay system. All the drugs except the sulfoxide and sulfide analogues of DDS were found to be non-mutagenic.

The works so far done on the possible genotoxic effects of DDS are, therefore, extremely limited and have been carried out either on bacterial system or on human somatic cells exposed in vivo or in vitro. In view of large scale use of DDS regularly for a long period, and of carcinogenic potency recently identified the present study was undertaken to evaluate further mutagenic potential in mouse using different in vivo assay systems.

As the drug is recommended for long term use here in the entire experiment only repeated treatment regimen was followed. For evaluation the assay systems followed by us are given below.

I. Metaphase chromosome analysis in bone marrow cells.
   a. Time - response (chronic treatment with a particular dose for different periods).
   b. Dose - response (chronic treatment with different doses for a particular period).

II. Micronucleus test in bone marrow cells - Dose response as in 1b.
III. Spermatocyte chromosome analysis.
   a. Time - response as in Ia.
   b. Dose - response as in Ib.

IV. Sperm test.
   A. Sperm morphology assay.
      a. Time - response as in Ia.
      b. Dose - response as in Ib.
   B. Sperm count assay.

4.2.2 Materials and Methods

Base free sample of the drug (batch No. 35655) was obtained from Burroughs Wellcome (India) Ltd. The drug is sparingly soluble in water and forms a suspension. The aqueous suspension of the drug was fed to the experimental animals via oral route by gastric intubation. For dose-response study the dilution of the suspension was adjusted and the volume of the suspension received by each animal was kept constant (0.25 ml).

For experiments I, III and IV mentioned above materials were provided by the same animals and adult male mice of the age group 10-12 weeks (at the time of starting of treatment) were used. A dose of 40 mg/kg/day of the drug was fed to a group of mice for 2, 4 or 8 weeks and the animals were killed 24 h after the last dose treated. This regimen was referred to as time-response analysis for convenience.

In another set a batch of 12 mice were fed with a dose of 20, 40 or 80 mg/kg/day of the drug for 4 weeks and the animals were killed 24 h after the last dose. This regimen was referred to as dose-response study.
The treatment schedule 40 mg/kg/day for 4 weeks was common for both time- and dose-response studies. As the treated individuals received the drug with water in suspension age matched untreated males served as controls. 12 h before killing all the animals were treated ip with a dose of 4 mg/kg of colchicine. The bone marrow, testes, caput epididymes and vasa deferentia were collected from each of the control and treated animals for analyses of bone marrow metaphase chromosomes, spermatocyte chromosomes, sperm count and sperm abnormalities respectively. The procedures for collection and processing of the materials and preparation, staining and scoring of slides were described earlier in Material and Methods (General) (vide 2).

For experiment III (MT), however, mice of both sexes (2 males and 2 females for each dose level) were employed and only dose-response study with 3 different doses (20, 40 and 80 mg/kg/day) was conducted. The animals were treated with the drug in the same way, i.e. via oral route, for 15 consecutive days. They were killed 24 h after the last dose treated and their bone marrow cells were processed for micronucleus test (vide 2.2.1). Age and sex matched untreated mice were used as controls.

4.2.3 Results

4.2.3.1 Metaphase chromosome analysis in bone marrow cells

Qualitative: The structural chromosome aberrations encountered in time-response and dose-response studies were mostly of chromatid type. Among the break type aberrations chromatid breaks occurred more frequently (Figs. 11a-f). The
position of breaks varied along the length of the chromatid including the secondary constriction region (Fig. 11g). The broken chromatid fragments of various sizes were found to lie anywhere in the metaphase plate (Figs. 11h,1), but very often they were seen close to their places of origin without much displacement (Figs. 11a-2). A few iso-chromatid breaks were recorded (Fig. 11j); because of their rarity they were not considered as chromosome breaks. Fragments, single (Figs. 11k,l) and paired (Fig. 11a), of untraceable origin were next to the chromatid breaks among the break type aberrations so far their frequency of occurrence was concerned. In general the fragments were small and hence of untraceable origin. A metaphase plate with a ring chromosome accompanied with a paired fragment was noted (Fig. 11n); the ring chromosome was assumed to have originated by the union of the broken ends of sister chromatids (Nüd). A lone case of exchange configuration in which a pair of chromosomes were involved in chromatid interchange (PUId type, Savage, 1975) resulting in the formation of a dicentric chromosome and two acentric fragments lying nearby was noted (Fig. 11o). Gaps and sub-chromatid type breaks (Figs. 11a,b,p) were also common. Though one aberration per affected cell was of usual occurrence, cells (Figs. 11b,p) or chromosomes (Figs. 11a,b) with two types of aberrations (i.e. gap and break) were not rare.

Numerical changes of chromosomes were not marked. Abnormalities like centromeric separation, spindle dysfunctioning and despiralization were also absent. Qualitatively control data did not show much difference from the treated ones, but iso-chromatid breaks and exchanges were altogether absent in the control series.
Explanation of Fig. 11

Photomicrographs of bone marrow cells showing various types of structural chromosomal aberrations induced by dapsone.

a. A chromosome with a chromatid break and a gap involving two chromatids, fragment lying by the side of the chromatid.

b. A chromosome with a terminal chromatid break.

c-f. Metaphase plates showing chromatid breaks, the broken chromatid fragments lying near the chromosomes with slight displacement.
Explanation of Fig. 11 (Contd.)

g. Chromosome with chromatid break in secondary constriction region.

h. Chromosome with chromatid break, the chromatid fragment placed a little apart.

i. A chromosome with a chromatid break, the fragment lying near its place of origin.

j. A chromosome showing iso-chromatid break.

k,l. Metaphase plates showing fragments of untraceable origin.
m. A metaphase plate with paired fragment of untraceable origin.

n. A metaphase with a ring chromosome accompanied with a fragment lying wide apart.

c. A metaphase plate showing a pair of chromosomes involved in chromatid interchange (PUId).

p. A metaphase plate showing chromatid break, sub-chromatid breaks.
Quantitative: Data on different types of chromosomal abnormalities induced by chronic treatment of DDS for different weeks are summarized in Table 12. As age might have been an important factor for induction of aberrations the treated data were compared with age matched week-wise control ones. The incidences of break type aberrations showed significant increase over their corresponding control values in all the test weeks. The same tendency was marked for total aberrations also, but the increases were significant only at weeks 4 and 8. In no case period of treatment correlation of the aberrations induced with the was obtained. As the control values for different weeks did not exhibit any variance (total breaks: F = 6.5; total aberrations: F = 3.05) an attempt was made to compare the treated data with the pooled control value (x total breaks = 0.58 and total aberrations = 1.83), but the picture remained unchanged.

The dose-response study revealed significant increases in the incidences of aberrations including or excluding gaps (Table 13) in the treated series. The increases showed a correlation with the increasing dose levels. But the values for higher two doses for both breaks and total aberrations remained very close to each other.

4.2.3.2 Micronucleus test in bone marrow cells

Qualitative: Qualitatively the micronuclei obtained in dapsone treated series did not differ much from those of the clofazimine treated series. The micronucleated erythrocytes in the treated as well as control series contained only one micronucleus. The micronuclei were invariably round in shape but their
Table 12. Frequency distribution of different types of structural aberrations of bone marrow chromosomes induced by chronic treatment of dapsone for different periods with a daily dose of 40 mg/kg. Each of the iso-chromatid break and exchange was counted as two breaks.

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<tr>
<td>2</td>
<td>C</td>
<td>400/4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>400/4</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6 + 1</td>
<td>5</td>
<td>12</td>
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<td></td>
<td>0.50 ± 0.25</td>
<td>2.00 ± 0.35</td>
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<tr>
<td>4</td>
<td>C</td>
<td>400/4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>8</td>
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<tr>
<td></td>
<td>T</td>
<td>400/4</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>16</td>
<td>21</td>
<td>37</td>
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<td></td>
<td></td>
<td>0.75 ± 0.41</td>
<td>2.00 ± 0.61</td>
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<td></td>
<td>4.00 ± 0.61</td>
<td>9.25 ± 2.27</td>
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<tr>
<td>8</td>
<td>C</td>
<td>400/4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>400/4</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>7 + 4</td>
<td>8</td>
<td>16</td>
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<td></td>
<td></td>
<td>0.50 ± 0.25</td>
<td>1.50 ± 0.25</td>
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<td></td>
<td></td>
<td></td>
<td>2.00 ± 0.35</td>
<td>4.00 ± 0.00</td>
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C = Control, T = Treated, * = absolute figure, * = mean/100 cells ± S.E.
't' test: a = p<0.001, b = p<0.01, c = p<0.05.
Table 13. Incidences of different types of structural aberrations of the bone marrow chromosomes induced by chronic treatment of dapsone with different doses for 4 weeks. Each of the iso-chromatid breaks and rings was counted as two breaks.

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<tr>
<td>0.0 (Cont)</td>
<td>400/4</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00 ± 0.51</td>
</tr>
<tr>
<td>20.0</td>
<td>400/4</td>
<td>7</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>9 + 1</td>
<td>2.29 ± 0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.00 ± 0.51</td>
</tr>
<tr>
<td>40.0</td>
<td>400/4</td>
<td>8</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>4.00 ± 0.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.25 ± 2.27&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>80.0</td>
<td>400/4</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>13 + 3</td>
<td>4.00 ± 0.00&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.00 ± 0.79&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> = absolute figure, <sup>b</sup> = mean/100 cells ± S.E.

't' test: a = p < 0.001, b = p < 0.01, c = p < 0.05.
Explanation of Fig. 12

Cut-out photomicrographs of bone marrow smears displaying micromucleated erythrocytes, nucleated cells and abnormal mitotic figures induced by dapsone.

a-e. Polychromatic erythrocytes each containing one micronucleus.

f-i. Normochromatic erythrocytes, each with one micronucleus.

j. A nucleated cell with one micronucleus.

k,l. Anaphase cells with lagging chromosomes.
size and location varied greatly (Figs. 12a-l). The frequency of occurrence of IM in nucleated cells remained low (Fig. 12j). The incidences of abnormal mitotic figures like lagging chromosomes and anaphase bridges were also rare (Figs. 12k, l).

Quantitative: The dose-response study revealed significantly elevated frequencies of IM, compared to controls, in poly- as well as normochromatic erythrocytes taken separately or together for all the doses tested (Table 14). But the frequencies of nucleated cells with micronuclei remained in the control range. Although percentages of poly-, normo- and poly- plus normochromatic erythrocytes with IM increased significantly for all the doses they failed to show any statistical correlation with the doses (r = 0.704, 0.347, 0.312 respectively). P/N ratios exhibited slight increases in the treated individuals and were only marginally significant over the control value at higher dose levels.

Though the mitotic index calculated showed a tendency of decline the decrease was not remarkable at any dose level tested.

4.2.3.3 Spermatocyte chromosome analysis

Qualitative: With regard to numerical change both dose-response and time-response studies included a few cases of polyploidy. But its frequency remained in the control range. Only one cell was found to contain one extra univalent (Fig. 13a).

Among the structural chromosome abnormalities recorded chromatid breaks were more common in occurrence (Figs. 13b-f).
Table 14. Results of the micronucleus test in bone marrow cells of mice obtained following chronic treatment of dapsone with different doses for 2 weeks. Values are mean per 100 cells ± S.E. Figures in parentheses are MN/cells scored.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of animals</th>
<th>P. ec. with</th>
<th>N. ec. with</th>
<th>P. + N. ec. with</th>
<th>Nucleated cells with</th>
<th>P/N ratio</th>
<th>% of dividing cells (HL)</th>
</tr>
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<tr>
<td>0.0 (Cont)</td>
<td>6</td>
<td>0.21 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.92 ± 0.05</td>
<td>0.49 ± 0.03 (59/12000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(13/6000)</td>
<td>(1/6000)</td>
<td>(14/12000)</td>
<td>(1/6000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>4</td>
<td>0.50 ± 0.01b</td>
<td>0.30 ± 0.03a</td>
<td>0.40 ± 0.03a</td>
<td>0.02 ± 0.02</td>
<td>0.97 ± 0.02</td>
<td>0.45 ± 0.06 (36/8000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20/4000)</td>
<td>(12/4000)</td>
<td>(32/8000)</td>
<td>(1/4000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40.0</td>
<td>4</td>
<td>0.47 ± 0.04a</td>
<td>0.20 ± 0.03a</td>
<td>0.33 ± 0.03a</td>
<td>0.05 ± 0.02</td>
<td>1.20 ± 0.07c</td>
<td>0.37 ± 0.03 (30/8000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(19/4000)</td>
<td>(8/4000)</td>
<td>(27/8000)</td>
<td>(2/4000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80.0</td>
<td>4</td>
<td>0.50 ± 0.05b</td>
<td>0.42 ± 0.04a</td>
<td>0.46 ± 0.01a</td>
<td>0.10 ± 0.03c</td>
<td>1.09 ± 0.04c</td>
<td>0.36 ± 0.07 (29/8000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20/4000)</td>
<td>(17/4000)</td>
<td>(37/8000)</td>
<td>(4/4000)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ r = + 0.704 \quad r = + 0.847 \quad r = + 0.812 \quad r = + 0.639 \]

't' test: a = p < 0.05, b = p < 0.01, a = p < 0.001.
Explanation of Fig. 13

Photomicrographs of diakinesis-metaphase I spermatocytes showing numerical, structural and pairing abnormalities of chromosomes induced in dapsone treated mice.

a. A diakinesis plate with one extra univalent and a gap in X - chromosome.

b-f. Diakinesis plates showing chromatid breaks.
Explanation of Fig. 13 (Contd.)

s.h.  Diakinesis plates showing chromosome type break in autosomal bivalents.

i.  A metaphase I plate showing a fragment of untraceable origin.

j.  A diakinesis plate showing univalent formations involving an autosomal bivalent.
Fig. 13
chromosome breaks (Figs. 13g, h) constituted the second category. Fragments of untraceable origin were not rare (Fig. 13i). No exchange type configuration was noted in either dose- or time-response study. Broken chromatid or chromosomal parts remained in most cases close to their places of origin (Figs. 13b-h) because of synapsis.

In a number of cells univalent formation involving autosomes and/or sex chromosomes was recorded (Fig. 13j).

Quantitative: Table 13 summarizes the data on types and frequencies of spermatocyte chromosome abnormalities induced by DDS treatment for different periods. Although the control animals did not receive any vehicle different sets of age matched males were taken as controls against week-wise treated sets. Chromatid type break was the only type of aberrations encountered in control series, -one each week. Chronic treatment of DDS for 2, 4 and 8 weeks with a daily dose of 40 mg/kg exhibited a tendency of increase of breakage frequencies over the control values in all the sampling weeks (Table 13). However, the data at week 8 only showed a marginally significant increase. The treated values for different weeks did neither differ remarkably from each other nor show any correlation with periods of treatment. The control values for different weeks fluctuated within a narrow range (0.30 - 0.66%). A comparison of the treated values with the pooled control mean (0.54%) did not alter the picture.

Similarly, the incidences of univalent formation involving autosomes or sex chromosomes or both increased in all the test weeks; but the increase for sex chromosomes at week 8
Table 15. Incidences of different types of structural abnormalities and pairing anomalies of spermatocyte chromosomes induced by chronic treatment of dapsone for different periods with a daily dose of 40 mg/kg.

<table>
<thead>
<tr>
<th>Samp. Set Cellscored/animal</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chrom</td>
<td>Chromat. Frag. Exch. Total</td>
</tr>
<tr>
<td></td>
<td>br.</td>
<td>br.</td>
</tr>
<tr>
<td>2 C 150/3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2 T 200/4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4 C 200/4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4 T 200/4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>8 C 200/4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8 T 200/4</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

r = + 0.653

C = control, T = Treated, * = absolute figure, ° = mean/100 cells ± S.E.

't' test: c = p < 0.05, b = p < 0.01.
Table 16. Incidences of different types of structural abnormalities and pairing anomalies of spermatoocyte chromosomes induced by chronic treatment of dapsone with different doses for 4 weeks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Cells scored/animal</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chrom at.</td>
<td>Chrom at.</td>
</tr>
<tr>
<td>0.0 (Cont)</td>
<td>200/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50 ± 0.43°</td>
<td>6.00 ± 2.12</td>
</tr>
<tr>
<td>20.0</td>
<td>200/4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.00 ± 0.70</td>
<td>5.00 ± 1.66</td>
</tr>
<tr>
<td>40.0</td>
<td>200/4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.50 ± 0.43</td>
<td>6.50 ± 2.16</td>
</tr>
<tr>
<td>80.0</td>
<td>250/5</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.80 ± 0.97°</td>
<td>11.00 ± 2.23</td>
</tr>
</tbody>
</table>

r = +0.878

* = absolute figure, ° = mean/100 cells ± S.E.
't' test : b = p<0.01.
only was marginally significant over its corresponding control value, and that too due to nil S.E. for the control value.

Dose-response study showed a positive correlation of frequencies of breaks with the increasing doses (Table 16). In general the incidences kept low and the highest dose (80 mg/kg) only yielded significantly higher frequency over the control value. In no instance the frequency of univalent formation exceeded the control range.

4.2.3.4 Sperm morphology assay

Qualitative: Abnormalities encountered in the sperm morphology in time-response and dose-response studies did not differ qualitatively. Some of the 'types' of the mis-shapen sperm recorded in different test weeks following clofazimine treatment were also noted here. They were triangular, 'axe' shaped, with flat base, with vacuole, with acrosomal spine abnormality, sickle shaped and amorphous (Figs. 14a-k). These varieties were very common in occurrence here also in treated individuals as well as in controls. In addition, some new types like rectangular and 'beaked-tunnel' shaped were found (Figs. 14l-n), though rare, in DDS treated individuals only. However, spherical, twin, granular and with swollen middle piece which were available in CLF treated individuals could not be noted here. Varieties like notched, giant and semilunar were not so common. Here also a particular sperm with a combination of two or more said arbitrary types of abnormalities in its head morphology was not uncommon (Figs. 14c,d,i).
Explanation of Fig. 14

Photomicrographs of vasa deferential sperm showing abnormalities in head morphology in mice treated with dapsone.

a. A sperm with amorphous head.

b. An axe-shaped sperm.

c,d. Sperm with flat base and short acrosome.

e,f. Sperm with vacuoles.
Explanation of Fig. 14 (Contd.)

g,h. Plates showing a few amorphous sperm.

i. One axe-shaped head and another with flat base and without acrosome.

j. An amorphous sperm.

k. A sickle shaped sperm.

l. A rectangular sperm.

m. A rectangular and another triangular sperm.

n. A beaked-funnel shaped sperm.
Quantitative: A trend of increase in the production of abnormalities in sperm morphology was evident for all the treatment periods (Table 17) and for all the doses (Table 18) tested. In no case the treated value exceeded the control value significantly.

Table 17. Incidences of mis-shapen sperm in mice induced by chronic treatment of dapsone for different periods with a daily dose of 40 mg/kg. Values are mean per 100 sperm ± S.E. For each point 4-5 animals were employed and 1000 sperm were examined from each individual.

<table>
<thead>
<tr>
<th>Set</th>
<th>wk 2</th>
<th>wk 4</th>
<th>wk 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3.56 ± 0.61</td>
<td>4.40 ± 0.56</td>
<td>5.12 ± 0.53</td>
</tr>
<tr>
<td>T</td>
<td>6.18 ± 1.83</td>
<td>10.06 ± 3.23</td>
<td>19.55 ± 2.18</td>
</tr>
</tbody>
</table>

C = Control, T = Treated

Table 18. Incidences of mis-shapen sperm in mice induced by chronic treatment of dapsone with different doses for 4 weeks. Values are mean per 100 sperm ± S.E. For each point 4-5 animals were employed and 1000 sperm were examined from each individual.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>wk 2</th>
<th>wk 4</th>
<th>wk 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>4.40 ± 0.58</td>
<td>6.00 ± 1.23</td>
<td>10.05 ± 3.23</td>
</tr>
</tbody>
</table>

4.2.3.5 Sperm count assay

The data on sperm count obtained following treatment of DDS for different weeks fluctuated between 151.37 and 180.11
(Table 19). In no case the treated datum was less than the respective control or the pooled control mean value which was calculated to be $132.09 \pm 7.94$ ($F = 1.85$). The treated value for week 8 only showed marginally significant difference whether compared with the respective control value or compared with the pooled control mean value.

Table 19. Effect of dapsone given once daily (40 mg/kg) for different periods on the epididymal sperm count. Each value represents the mean number of sperm (heads only) present in one WBC chamber of haemocytometer $±$ S.E. For each point 4–9 animals were employed and from each animal count was done in 25 WBC chambers. To obtain total count per epididymis each value is to be multiplied by 40,000 (dilution factor).

<table>
<thead>
<tr>
<th>Set</th>
<th>wk 2</th>
<th>wk 4</th>
<th>wk 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>$130.32 \pm 7.13$</td>
<td>$147.47 \pm 11.5$</td>
<td>$113.46 \pm 16.60$</td>
</tr>
<tr>
<td>T</td>
<td>$151.68 \pm 11.47$</td>
<td>$151.37 \pm 13.02$</td>
<td>$160.11 \pm 13.36$</td>
</tr>
</tbody>
</table>

$C =$ Control, $T =$ Treated

't' test : $c = p \quad 0.05$.

Dose-response study did not reveal any marked difference between control and treated counts for lower two doses (Table 26). The highest dose lowered the count appreciably but the decrease was not statistically significant.
Table 20. Effect of chronic treatment of dapsone with different doses for 4 weeks on the epididymal sperm count. Each value represents the mean number of sperm (heads only) present in one WBC chamber of a haemocytometer ± S.E. For each point 4-5 animals were employed and from each animal count was done in 25 WBC chambers. To obtain total count per epididymis each value is to be multiplied by 40,000 (dilution factor).

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Count (Mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>147.47 ± 11.6</td>
</tr>
<tr>
<td>20.0</td>
<td>145.70 ± 14.62</td>
</tr>
<tr>
<td>40.0</td>
<td>151.37 ± 13.02</td>
</tr>
<tr>
<td>50.0</td>
<td>97.45 ± 19.20</td>
</tr>
</tbody>
</table>

4.2.4 Discussion

4.2.4.1 Metaphase chromosome analysis in bone marrow cells

Significantly higher incidences of breaks as well as total aberrations for all the treatment periods and for all the doses following treatment of the drug clearly reveal clastogenic capacity of DDS, though not severe. The maximum response (breaks only) which was more than 5 times the respective control value and about 7 times the pooled control mean was noted with the intermediate dose and for intermediate time period (4 weeks). However, the incidences of breaks and total aberrations for the intermediate and the highest doses practically did not differ. Positive clastogenic effect of the drug was also demonstrated earlier from metaphase chromosome analysis in human cells. Peripheral blood lymphocytes of leprosy patients under DDS
therapy (50 or 100 mg/day) also showed significantly higher incidences of structural chromosome aberrations comprising chromosome and chromatid breaks and gaps (Beiguelman and Pisani, 1976). Similarly increased incidence of structural chromosome aberrations was also noted in cultured skin fibroblasts of leprosy patients who were under DDS alone or combined therapy (Hackel and Beiguelman, 1985). That the drug does not cause induction of numerical changes of chromosomes in vivo was also documented earlier by Beiguelman and Pisani (1976) and Hackel and Beiguelman (1985). However, peripheral blood lymphocytes of healthy persons when exposed to the drug increased significantly the frequency of aneuploidy, the reason of which remains unknown. In the present investigation we failed to note any correlation between the periods of DDS treatment and the aberration yields. Absence of such a phenomenon was also recorded in lymphocytes and fibroblasts of leprosy patients who were under DDS therapy for different periods (Beiguelman and Pisani, 1976; Hackel and Beiguelman, 1985). On the other hand, role of daily dose in inducing structural changes of chromosomes is quite evident, particularly with reference to lower doses in the mouse (present study) as well as in human in vitro (Beiguelman et al., 1975). Our results are, therefore, in very good agreement with those of Beiguelman and his associates.

4.2.4.2 Micronucleus test in bone marrow cells

MN are known to be formed either from lagging acentric fragments which result from breakage, or from lagging whole chromosomes resulting due to dysfunction of the spindle. The second possibility is associated with the occurrence of large sized
erythrocytes which usually arise due to polyploidy and subsequent elimination of polyploid nucleus; (b) occurrence of abnormal mitotic figures like anaphase bridges, or lagging chromosomes or chromosome fragments at anaphases; (c) occurrence of big sized MN resulting from whole chromosomes; and most important (d) increase of mitotic index in the smear preparation. But none of the above aspects was evident in our preparation. Another important characteristic of the spindle poisons is that the frequency of MN gradually increases with the increase of dose and then declines with further increase of the dose (Schmid, 1976). Our data do not also exhibit such a characteristic curve for MN. Of course, the present study suffers from the limitation of having data for 3 doses only. In view of high incidence of MN in erythrocytes all this clearly indicates that MN were produced by the clastogenic activity of the drug. So, the results of MN are in accordance with those of metaphase chromosome analysis.

The values for 3 different doses for P. egs, N. egs or P. + N. egs, as found with two higher doses in metaphase chromosome analysis, were very high and close to each other. Here the doses also fail to show any influence on the frequency of MN production and, thus, data are in agreement with those of metaphase chromosome analysis.

Since the study involves chronic treatment P/N ratios fail to give any information if the drug has any suppressive effect on the turnover of the bone marrow cells to polychromatric erythrocytes. Slight increase of P/N ratio particularly at higher doses may be attributed to the haemolytic activity of DDS (Goodman and
Gilman, 1935), which is more pronounced on N. ees than on P. ees.
As indicated by the MI data the drug seems to have a little or no inhibitory effect on the cell cycle process.

4.2.4.3 Spermatocyte chromosome analysis

Literature contains, as far as we are aware, no report on the effect of DDS on germinal chromosomes. In the present study only the daily dose of 30 mg/kg for 4 weeks and the daily dose of 40 mg/kg for 8 weeks elevated the breakage frequencies at significant levels. In no other case the breakage frequency exceeded the control limit. The highest effect obtained following the treatment of 30 mg/kg/day for 4 weeks was only about 5 times the control value. Thus, like bone marrow cells spermatocytes also exhibit mild clastogenic effect. Inhibitory effect on cellular propagation of the drug is not evident. In both the instances when significant increases of breaks were noted the cells scored were expected to remain at spermatogonia stem cell stage at the time of starting of the treatment. So, in view of significant effect the question of susceptibility of stem cells cannot be ruled out. However, we have certain reservation in considering this assumption. Because very few chemicals are found to induce cytogenetic effects in stem cells that can be measured in meiotic cells. Adler (1982a,b) has discussed the probable reasons for this at length, but definite answer is yet to be known. Anyway, it needs further analysis of spermatogonial and spermatocyte chromosomes to verify if DDS really affects the stem cells.
The data on univalent formation in DDS treated series were comparable to those obtained from control animals, which indicate the lack of effect of DDS on the pairing behaviour of the chromosomes as judged by the univalent formation. Lack of effect of DDS on univalent formation strengthens our earlier finding that CLF induces univalent formation.

4.2.4.4 Sperm morphology assay

Though the frequencies of mis-shapen sperm in the treated series show a trend of increase in all the test weeks and for all the doses the increases are not significant. It seems that the drug is not effective, at least with the treatment schedule followed here, to elevate the incidence of mis-shapen sperm at a significant level. More work is needed in this line with other dose levels and for longer period of treatment.

4.2.4.5 Sperm count assay

Production of sperm, like sperm morphology, seems to be influenced little by DDS treatment. The value obtained following 8 weeks of treatment differs significantly from the control value, but it is towards the upper side and carries little significance. The highest dose tested here lowers the frequency but not to a significant level.

4.2.4.6 General

The results obtained from bone marrow metaphase chromosome analysis, MN and spermatocyte chromosome analysis reveal a positive, though mild, clastogenic effect of DDS. Our findings...
are in complete accordance with those of Beiguelman and his co-workers (Beiguelman et al., 1975; Beiguelman and Picani, 1976; Bachel and Beiguelman, 1983) who also reported clastogenicity of DDS in human cells exposed \textit{in vitro} and \textit{in vivo} and this aspect has already been discussed in details, but contradict those of Peters et al. (1983). Peters et al. (1983) studied possible mutagenic-carcinogenic effect of not only DDS but also a number of its metabolites and derivatives by using \textit{Salmonella/microsome} assay system. DDS and its derivatives tested were found to be non-mutagenic in absence of rat liver microsomes; only two derivatives—sulfoxide (DDSO) and sulfide (DDSD) analogues yielded positive response in presence of liver microsomes. They (Peters et al., 1983) also demonstrated lack of mutagenic effect of urine concentrates of volunteers taking 50 mg of DDS in the Ames screen, and failed to detect the presence of DDSO and DDSD, the mutagenic derivatives of DDS, in the pharmaceutical preparation of DDS as well as in the urine samples of DDS consumers. That DDS is non-mutagenic was also reported earlier by Lavois et al. (1979) from an identical experiment using \textit{S. typhimurium} strains TA98 and TA100. The sensitivity and reliability of the Ames \textit{Salmonella} test system for the detection of mutagenicity and/or carcinogenicity is well established (Mc Cann et al., 1975). Thus, with regard to potential genotoxicity of DDS the assay based on chromosome analysis in eukaryotic system exhibits positive response, while the Ames bacterial assay system responds negatively. The reason for such contradiction remains unknown. However, in the field of chemical mutagenesis contradiction is not uncommon; a number of chemicals are known to show such type of contradiction. In this
context we can refer to the cases of procarbazine, HMPA, isoniazid, etc., which induce positive effects in several eukaryotic systems but not in the Ames test (Adler, 1980; de Serres and Ashby, 1981; Ishidate, 1981). This point has been discussed in details in connection with clofazimine (vide 4.1.4.7).

Though dapsone was earlier considered to be non-carcinogenic, but recent reports prove that it is weakly carcinogenic (Bergel, 1973; Griciute and Tomatis, 1980). The association between the induction of cancer and the induction of chromosome damage is of long standing, although the debate on the importance of chromosome changes as causal factors in carcinogenesis continues. In view of its carcinogenic efficiency its potential clastogenic capacity cannot be ruled out.

The mechanism of action of dapsone leading to the production of chromosome aberration is not known. An idea on pharmacokinetics of the drug may throw some light in understanding the problem. From the intestinal tract the drug is absorbed rapidly and almost completely. The peak concentration in the blood reaches within 2-3 h. Its half-life of elimination ranges from 10-50 h with a mean of 23 h. An oral dose of 100 mg daily results in a peak serum level that exceeds the minimal inhibitory concentration (MIC) of DDS against H. leprae by a factor of about 500. The peak serum concentration remains above the MIC level for a period of 10 days after administration (WHO, 1982). The drug is distributed in all organs and tends to be retained in liver, kidneys, skin and muscles. Traces of the drug remain present in these organs upto 3 weeks after therapy is stopped. It is also retained in the
circulation for a long time because of intestinal reabsorption from bile. A dose of 100 mg/day produces an average of 2 μg of 'free' drug per gram of blood or non-hepatic tissue. It is acetylated in liver and degree of acetylation is genetically determined (Dutta et al., 1973). About 72% of the drug is eliminated out via kidneys. It serves as a competitive inhibitor of the enzymatic incorporation of para-aminobenzoic acid (PABA) in folic acid synthesis (Seydel et al., 1980).

The metabolism of DDS is not fully understood, its only known metabolite is monoacetyl dapsone which has also been found to be non-mutagenic in the Ames test (Feters et al., 1983). Again the sulfide (DDSD) and sulfoxide (DSSO) analogues of DDS which have been proved to be mutagenic in the Ames test have been detected neither in the pharmaceutical preparation of DDS nor in the urine samples of volunteers taking DDS (Feters et al., 1983). So possibility of involvement of DDSD and/or DSSO in clastogenic activity of DDS is beyond question. Very likely the drug itself or its metabolite is involved in it. It would be interesting to study if the metabolite of the drug is clastogenic. Anyway, the question of differential responses of two systems: chromosome analysis and the Ames test, to the drug remains unanswered.

Though the human therapeutic dose recommended by WHO (1982) is 100 mg/day but depending upon the severity it is sometimes prescribed as high as 300 mg/day. If one calculates on per kg basis the mouse dose would be about 2 mg/kg/day (considering human dose 100 mg/day). The minimum dose tested by us (20 mg/kg/day) would then be 10 times the usual human therapeutic dose.
But if it is calculated on the basis of the surface area of the individual, as usually done for most of the drugs and chemicals, the equivalent dose for mouse should be 12 times the human dose (Freireich et al., 1966); the lowest dose tested would then be close to the human therapeutic dose recommended by WHO. If the mouse dose is calculated on the basis of the surface area from the human dose of 300 mg/day then our highest dose (50 mg/kg) would be close to it. So, the doses tested here are comparable to the human therapeutic dose range.
4.3 Comparative Study of the Effects of Clofazimine and Dapsone on Mitotic and Meiotic Chromosomes:

In the foregoing section the results on the effects of CLF and DDS on mitotic, meiotic and post-meiotic cells have been presented. Since these two drugs are now almost exclusively used in the treatment of leprosy we became tempted to compare the data of these two drugs to have some idea on their relative effectiveness in causing chromosome aberrations. For proper comparison data of only common dose and treatment schedules have been considered and presented through histograms protocol-wise.

For comparison of the data of metaphase chromosome analysis in bone marrow cells the 'breakage' frequencies and 'total aberration' frequencies only were considered. Treatment schedules of a single daily dose of 40 mg/kg for 2 and 4 weeks were common for both CLF and DDS. The incidences of chromosome aberrations, excluding and including gaps, induced by CLF remained at higher level compared to DDS induced ones both for 2 and 4 weeks of treatment (Fig. 15). This clearly indicates higher effectiveness of CLF than DDS in inducing chromosome damage.

Similarly histogram analysis of meiotic chromosomes revealed higher frequencies of structural changes of chromosomes both for 2 and 4 weeks of treatment indicating higher effectiveness of CLF than DDS (Fig. 16). Thus, chromosome analysis following identical treatment on mg/kg basis shows higher clastogenic effect of CLF in mouse in vivo system.
Fig. 15 Comparative analysis of incidences of clofazimine and dapsone induced breaks and total aberrations on bone marrow chromosomes of mice following two and four weeks of chronic treatment (40 mg/kg/day). In each case control value has been subtracted. (Data taken from Tables 4 and 12).

Fig. 16 Comparative analysis of the incidences of clofazimine and dapsone induced breaks on spermatocyte chromosomes of mice following two and four weeks of chronic treatment (40 mg/kg/day). In each case control value has been subtracted. (Data taken from Tables 9 and 15).
Bender et al. (1974) categorized chemicals producing chromosomal aberrations under four heads on the basis of the types of aberrations produced in relation to the stage of the cell cycle. Higher incidence of gaps compared to breaks in CLF treated individuals seems to indicate that CLF comes under category 1 of Bender et al. (1974) which includes compounds producing gaps and deletions in late S and G2 cells. Under this category come FUDR, ADR, Cytosine arabinoside and hydroxyurea and all are inhibitors of biosynthesis of DNA and DNA precursors. Inhibitory effect of CLF on DNA synthesis has already been reported by Morrison and Marley (1976a). In contrast, DDS probably comes under category 3 of Bender et al. (1974) which includes compounds like alkylating agents, nitroso compounds and some antibiotics. These compounds are characterized for production of chromatid aberrations in all types of cells treated in G1 or early S. Almost equal frequencies of break and gap type aberrations in DDS series seem to support our assumption on its inclusion under category 3. However, its action with DNA or DNA precursors is yet to be known.

4.4 Effect of Clofazimine and Dapsone Following Combined Treatment:

4.4.1 Introduction

As mentioned before for the treatment of leprosy, monotherapy was the usual practice in the past and DDS was (is even now) the first choice as antileprosy drug. At present, however, triple drug regimen (DDS + R6M + CLF) is recommended (WHO, 1982) with an intention to combat the DDS resistant M. leprae.
When triple regimen is followed the patients are exposed to three drugs simultaneously. This prompted us to study the genotoxic effect of these drugs following simultaneous treatment. So far as our knowledge goes literature provides only one report in this line. Hackel and Beiguelman (1983) recently recorded higher breakage frequency in the fibroblasts of patients taking DDS along with other antileprosy drugs (but not CLF) than in patients taking DDS only. We have studied the genotoxic effect of CLF and DDS when treated simultaneously on bone marrow chromosomes of mice and the data are presented in this section. For want of time other experiments in this line could not be conducted, and this work should be considered as an initial step for some future elaborate work. The data of combined treatment were also compared with those obtained from separately treated individuals with a view to finding out if these two drugs interact with each other in causing chromosome damage.

4.4.2 Material and Methods

A group of four mice (2 males and 2 females) were employed. Each of the experimental animals received a daily dose of 40 mg/kg of CLF and a daily dose of 40 mg/kg of DDS via oral route at an interval of less than one hour. As done earlier, CLF was dissolved in alcohol (0.2%) while DDS was used as an aqueous suspension. The treatment of both the drugs was continued for 4 weeks, and 24 h after the last dose the animals were killed.

Colchicine-citrate-acetic alcohol-flame drying-Giemsa schedule (vide 2.1) was followed for bone marrow chromosome
preparation. For this experiment no separate control was run. Data from age matched alcohol treated controls corresponding to CLF treatment (from Table 4) and data from age matched untreated controls corresponding to DDS treatment (from Table 12) were pooled and this pooled control values were used for comparison with the treated ones. Values obtained in two types of controls did not differ remarkably.

If the two drugs do not interact with each other, that means if they cause chromosome damage independent of each other, the effect is expected to be simple additive which is calculated as per the formula: \( X + Y - C \) (where \( X \) and \( Y \) stand for individual values for separate treatment and \( C \) for control). This additive value is expressed as expected value. If the observed value for the combined treatment is significantly higher than the expected one it is noted as synergistic, and if significantly lower than antagonistic.

4.4.3 Results

Polyploid and aneuploid cells were of rare occurrence. The structural changes encountered here did not differ qualitatively from those of separate studies described earlier. Hence we do not like to describe them here again.

The frequencies of both break type aberrations and total aberrations exceeded the respective control values significantly (Table 21) indicating positive effect of the drugs following combined treatment. The frequency of break type aberrations following combined treatment was significantly below the expected
Table 21. Frequency distribution of different types of structural chromosome aberrations induced in bone marrow cells of mice treated with clofazimine, dapsone or clofazimine plus dapsone for 4 wks, in each case the animals were fed with a daily dose of 40 mg/kg. Each of the iso-chromatid breaks, rings and exchanges was counted as two breaks.

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</thead>
<tbody>
<tr>
<td>C</td>
<td>300/8</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>5</td>
<td>20</td>
<td>3.12 ± 0.54</td>
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<tr>
<td>CLF</td>
<td>500/5</td>
<td>23</td>
<td>-</td>
<td>11</td>
<td>1</td>
<td>35 ± 1</td>
<td>56</td>
<td>91 ± 1</td>
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<tr>
<td>DDS</td>
<td>400/4</td>
<td>8</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>16</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td>CLF + Obs.</td>
<td>400/4</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>18</td>
<td>15</td>
<td>34</td>
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<tr>
<td>DDS + Exp.</td>
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* = absolute figure, ° = mean/100 cells ± S.E.
't' test: a = p < 0.001, b = p < 0.01, c = p < 0.05, x = p < 0.001. a, b, c significantly higher than control values; x significantly lower than expected value. Data for CLF taken from Table 4 and for DDS from Table 12.
value and was, thus, antagonistic. Similarly the total aberration frequency also showed antagonistic effect (Fig. 17).

4.4.4 Discussion

Possible factor(s) responsible for causing chromosome damage following chronic treatment of CLF or DDS has been discussed in the respective drug section. Here we would like to discuss in general the interaction of these two drugs. Much attention has been paid in the study of conjoint effects of ionizing radiations and chemical agents with a view to finding out some chemoprotective agents against the hazardous effects of the former (see Thomson, 1962; Michaelis and Rieger, 1963). Lot of works have also been done on the interaction of two or more chemicals of different nature in causing chromosome aberrations (see Michaelis and Rieger, 1963). The factors like oxidative phosphorylation, temperature, pH, etc. are also known to influence clastogenic effect of certain chemicals (see Kihlman, 1966, 1977). Here CLF and DDS, two chemically as well as pharmacologically different drugs, interact with each other antagonistically in causing chromosome damage. The dose tested and the time schedule followed for DDS or CLF alone could elevate the 'breakage' as well as 'total aberration' frequencies above the control limit. But how they interact with each other to lower down the chromosome damage (below the expected value significantly) when given simultaneously is not known.

Recently Hackel and Seigelman (1965) analyzed the incidence of chromosome aberrations in fibroblasts of leprosy patients who were under either DDS monotherapy or multiple therapy
Fig. 17 Histogram analysis of the break type and total aberrations induced by clofazimine, dapsone and clofazimine + dapsone in bone marrow cells of mice after four weeks of chronic treatment (40 mg/kg/day). In each case control value has been subtracted. ($\chi$ significantly lower than the expected value, $p < 0.001$)
with DDS and some other antileprosy drugs. They showed that the incidence of chromosome damage was a little higher in the patients who were under multiple therapy than in the patients under DDS monotherapy. In their study no patient taking CLF was, however, included; patients with multiple therapy were taking DDS plus RFM, dexamethazone and/or thalidomide. They did not study the interaction, if any, of these drugs in case of multiple therapy.

In view of potential mutagenic effect of DDS and CLF and of importance of them in human therapeutics combined therapy scores better over monotherapy. However, our finding is based on very limited data and further work is needed in this line.