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Cultured peripheral lymphocytes: one biologic indicator of potential drug hazard

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Summary

Peripheral human lymphocytes (Pls) of three categories of patients under therapy with four drugs: niridazole and tartar emetic for schistosomiasis, diphenylhydantoin for grand mal epilepsy and isoniazid for pulmonary and abdominal tuberculosis were cultured *in-vitro* with and without phytohaemagglutinin (PHA) stimulation. The four drugs revealed a significant incidence of variable degrees of nucleochromosomal pathology under the effect of PHA. Micronuclei, nuclear fragmentation and reduced Pls mitotic response to PHA were specifically notable with tartar emetic. Multinucleation, polyploidy and different grades of chromosome fragility were more conspicuous with niridazole and diphenylhydantoin and to a lesser degree with isoniazid. Niridazole was the only drug that effected blastogenesis and binucleation of Pls in absence of PHA and induced high incidence of giant polynucleated cells on stimulation with PHA. Those niridazole induced effects are reported for the first time.

In view of the hazards of such *in-vitro* nucleochromosomal findings which might serve as biologic indicators of an oncogenic, teratogenic or mutagenic potential of those drugs, any extrapolation as to their *in-vivo* significance should be critically evaluated.

The list of environmental agents physical, chemical and biologic which are known to break chromosomes is steadily growing. Evidence is accumulating that some of those agents may be oncogenic, others may well be mutagenic or teratogenic. Genetic diseases known to manifest early somatic cell chromosome breakage of some tissues are prone to develop cancer in the same tissues e.g. ataxia telangiectasia, Fanconi's anaemia, Bloom's syndrome and xeroderma pigmentosum.

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Hence as the number of pharmaceutical drugs and chemicals which are ingested by man or which pollute his environment increases, biologic safety against an anticipated increase in the incidence of breakage of somatic and may be germinal cells chromosomes should be ensured.

The present study was undertaken to determine any *in-vitro* detectable effect on cultured peripheral lymphocytes, of some drugs used for therapy of three highly prevalent diseases namely: schistosomiasis, tuberculosis and epilepsy.

Material and methods

The study material is composed of three groups of patients who attended Ain-Shams University Hospitals in the interval between 1969–1973 and a control group selected from hospital working staff and their families. Study group 'A' consisted of thirty-five bilharzial patients aged 8–18 years (twenty males and fifteen females) who had mixed urinary and intestinal bilharziasis for a period ranging between 3 and 12 years. Twenty patients were treated orally with niridazole (Ambilhar (R) Ciba) in a dosage of 25 mg/kg/day for 7–9 days. Fifteen patients were treated with potassium antimony tartarate (Tartar Emetic Cid) in a dosage of 2 mg/kg/injection/twice weekly for 6 weeks. Study group 'B' consisted of twenty tuberculous patients aged 4–12 years (twelve males and eight females) who received isoniazid (Remifon-Roche) therapy for pulmonary tuberculosis and/or tuberculous peritonitis in a dosage of 10 mg/kg/day for a duration of 6 months–3 years. Study group 'C': consisted of twenty-one epileptic patients aged 3–12 years (eleven males and ten females) who were treated for grand mal epilepsy

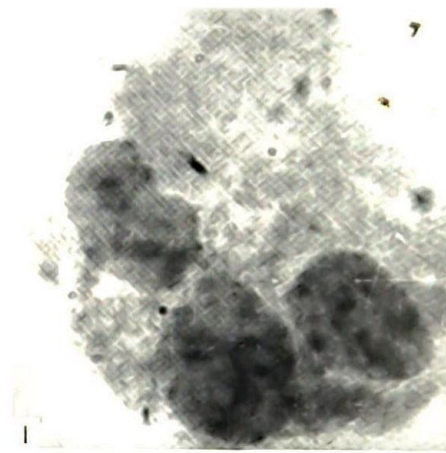
with diphenylhydantoinate (Epanutin-Park Davis Co.) in a dosage of 3–6 mg/kg/day for a duration ranging between 6 months and 5 years. The control study group was comprised of forty-five subjects: thirty patients aged 3–15 years (eighteen males and twelve females) of whom ten patients had bilharziasis, ten had active tuberculosis, ten were epileptics and fifteen were normal subjects aged 3–14 years (nine males and six females). All the thirty control patients were studied prior to therapy with any of the tested drugs. Chromosome studies were performed on peripheral blood lymphocytes (Pls) cultured without and with phytohaemagglutinin (PHA: extracted from native red kidney beans) for 72 h using the modified techniques of Moorhead *et al.*, (1960). Ten slides stained with conventional Giemsa stain were examined for each patient. Lymphocytes cultures grown without PHA were scored for blastogenic index and those grown with PHA were scored for mitotic index as determined per 1000 Pls. All cultures were scanned for any cytonuclear pathology, chromosomal fragility (gaps, breaks, fragmentation or pulverization) and for any numerical or structural chromosomal aberrations. One hundred metaphases were scored for each patient and any aberrations were recorded as percent incidence. All scored indices or aberrations are expressed as mean percent incidence and standard error. Selected figures were photographed by automatic Leitz Microscopic camera using Kodak Contrast Copy Film (M–135–36) and a magnification ×1000.

Analysis of results

Results are illustrated in Table I and Figs 1–10.

In the study group 'A', Pls cultures of the niridazole treated patients, 10·0 ± 0·84% of Pls were spontaneously transformed into lymphoblasts some of which were binucleated, an incidence which is significantly higher than that of all other study groups ($P < 0·001$). In the PHA stimulated cultures, polynucleated giant cells (3–8 nuclei) appeared in 4·3 ± 0·75% of transformed Pls and polyploidy (3n–4n) in 2·7 ± 0·84% of metaphases. Gaps, single and isochromatid breaks occurred in 9·0 ± 0·62, 9·0 ± 0·83 and 4·9 ± 0·62% of metaphases respectively and were randomly distributed among chromosome groups A–E mostly in mid regions of either arms. Cross chromosomal configurations in form of dicentrics, tri and quadriradials and occasional ring forms appeared in 5·1 ± 0·63% of metaphases. Chromosome fragmentation or pulverization was evident among parts of 2·0 ± 0·54% of metaphases (Figs. 1–3).

In Pls cultures of tartar emetic treated patients 4·0 ± 0·59% of Pls were transformed into lymphoblasts. The PHA stimulated Pls cultures revealed a mitotic index which is significantly lower than all other study groups ($P < 0·001$). Fragmented and micronuclei appeared after PHA stimulation in 4·2 ± 0·51% of transformed Pls. Gaps, single and isochromatid breaks appeared in 3·2 ± 0·54, 6·8 ± 0·54, 6·8 ± 0·46 and 3·7 ± 0·57% of metaphases



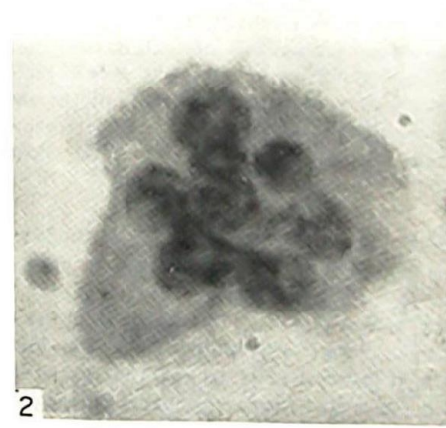


FIG. 1. Trinucleate giant cell (after niridazole).

FIG. 2. Octanucleate giant lymphoblast (after niridazole).

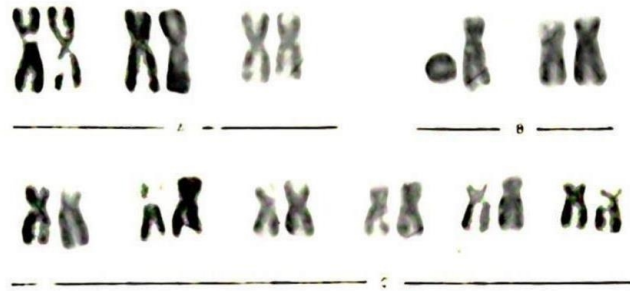


FIG. 3. Isochromatid break of one chromosome (A1) and ring chromosome of one chromosome B4 (after niridazole).

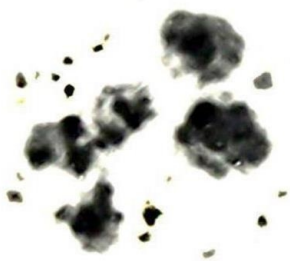


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FIG. 4. Micronuclei (after tartar emetic).

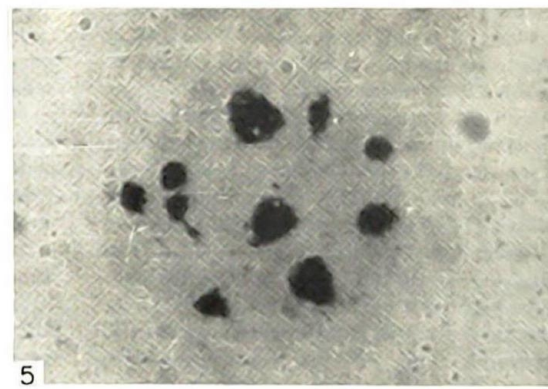

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FIG. 5. Nuclear fragmentation (after tartar emetic).

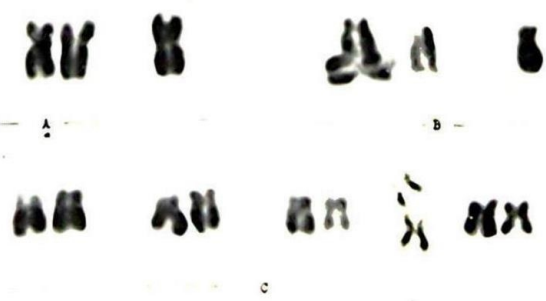

FIG. 6. Tri- and bi-radial chromosome configurations amidst chromosome groups A, B and C (after tartar emetic).

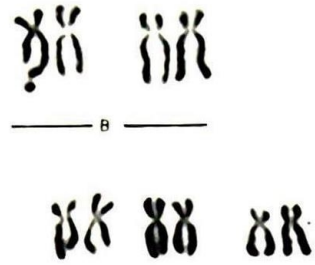

FIG. 7. Single chromatid break of one chromosome B4 (after isoniazed).

respectively and were randomly distributed among chromosome groups A–D and G mostly in mid regions of either arms. Gross chromosomal configurations in form of bi- and tri-radials were noted in 4·0 ± 0·59% of metaphases. Also 3·0 ± 0·52% of metaphases revealed fragmentation of parts of its chromosome complement (Figs 4–6).

In the study group 'B' of isoniazid treated patients, Pls cultures with no PHA revealed 4·4 ± 0·60% transformed lymphoblasts. In PHA stimulated Pls cultures, 2·3 ± 0·51% of transformed cells were polynucleated (2–4 nuclei) and 1·20 ± 0·23% of the metaphases were polyploid (3n–4n). Cross chromosome configurations in form of biradials were discerned in 2·9 ± 0·55% of metaphases. Gaps, single and isochromatic breaks were noted in 3·5 ± 0·55, 3·7 ± 0·80 and 3·5 ± 0·55% of metaphases respectively and were randomly distributed among chromosome groups A–C and E (Figs 7–8).

In the study group 'C' of epanutin treated patients, Pls cultures with no PHA revealed 5·3 ± 0·61% transformed lymphoblasts. PHA stimulated Pls cultures revealed 3·4 ± 0·20% polynucleated (3–5 nuclei) lymphoblasts and 2·80 ± 0·35% polyploid (3n–4n) metaphases. Gaps, single and isochromatid breaks occurred in 5·2 ± 0·31, 6·8 ± 0·87 and 5·6 ± 0·21% of metaphases respectively and were randomly distributed in terminal regions of either arms of the X chromosome and those of groups A, B and C. Chromosome pulverization was noted in 1·8 ± 0·67% of metaphases (Figs 9 and 10).

In Pls cultures of the normal controls, 5·0 ± 0·31% of Pls underwent spontaneous lymphoblastoid transformation. This latter incidence is not significantly different from that noted in Pls cultures of untreated bilharzial, tuberculous and epileptic patients ($P > 0·05$). It also matches the rate of spontaneous lymphoblastoid transformation of Pls cultures of patients receiving tartar emetic, isoniazid and epanutin ($P > 0·05$) but is significantly below that noted in Pls cultures of the niridazole treated bilharzial patients ($P < 0·001$) (Table 1).

None of the PHA stimulated Pls cultures of the normal controls or those of the three study groups prior to therapy revealed any polynucleated transformed giant cells, chromosomal pulverization or cross chromosomal configuration figures. The PHA stimulated Pls cultures of normal controls as well as those of the bilharzial, tuberculous and epileptic patients prior to therapy revealed single and occasional iso-chromatid breaks in 2·5 ± 0·25, 2·8 ± 0·50,

FIG. 8. Polypoid partially fragmented metaphase (after isoniazed).

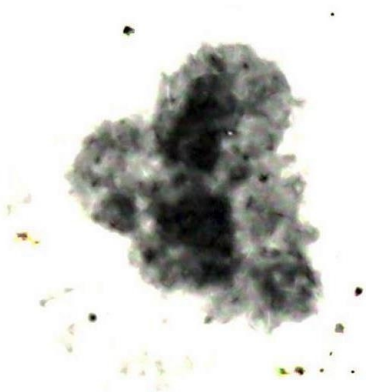

FIG. 9. Polynucleate giant lymphoblast (after epanutin).

3·8 ± 0·61 and 3·0 ± 0·20% of metaphases respectively. Also 0·2, 0·3 and 0·2% polyploid (3n–4n) metaphases were noted in PHA stimulated Pls cultures of the normal controls and those of the untreated epileptic and bilharzial patients respectively. Either incidences of chromatid breaks and/or polyploidy noted in PHA stimulated Pls cultures of the normal controls or those of bilharzial, epileptic and tuberculous patients prior to therapy are significantly below their corresponding rates in the three study groups of patients after specific therapy ($P < 0{\cdot}001$).

Discussion

Peripheral lymphocytes cultures of niridazole treated bilharzial patients depicted *in-vitro* findings which differed significantly in magnitude and type from those of all other study groups. The incidence of spontaneous lymphoblastoid transformation and of binucleate cells in Pls cultures set without PHA and the incidence of polynucleated giant cells and metaphases with chromosome gaps, chromatid breaks and cross configurations in PHA stimulated cultures were significantly higher than in Pls cultures of patients receiving other drugs. The spontaneously forming binucleate cells gave rise to tetraploid and octaploid giant cells after PHA stimulation and repeated nuclear division without cell wall division. With enhanced nuclear replication, sister chromatids failing to separate could yield the different sets of diplochromosomes with the variable grades of chromosome fragility. Pls cultures of bilharzial patients studied prior to therapy and those of normal control subjects revealed a significantly low incidence of spontaneous lymphoblastoid transformation and/or occasional chromatid breaks or polyploid metaphases. Hence niridazole has to be incriminated either in the *de-novo* genesis of the noted nucleochromosomal aberrations or else its effects are synergized by hitherto un-identified factor/s operating in bilharziasis but by itself cannot induce the same degree or type of nucleochromosomal pathology. The enhanced *in-vitro* spontaneous lymphoblastoid transformation and the genesis of binucleate viable cells are indices of a primary proliferative stimulus of niridazole on Pls. The significance of the relatively high incidence of those spontaneous changes together with the appearance of chromosome fragility, polyploidy and giant polynucleate cells after PHA stimulation could endow niridazole with an oncogenic potential. There is hitherto no available reports on carcinogenicity tests for niridazole. Its chemical structure (1-(5 nitro-2 thiazolyl)-2-imidazolidinone) which simulates nitroimidazole and nitrofuran derivatives; two proven carcinogenic chemicals in animals, gives cause for concern about the present niridazole induced cytogenetic findings. The 5-nitrofuran group has been shown to import carcinogenic activity to a number of compounds (Cohen, Erturk & Bryan, 1970). The 5-nitro group of niridazole which might be converted in the stomach to hydroxylamine and other N-Nitrose compounds could be a potential carcinogen for man. Whether the niridazole induced cellular and chromosomal effects are primarily exerted at the DNA level and/or on some cytoplasmic organelles or

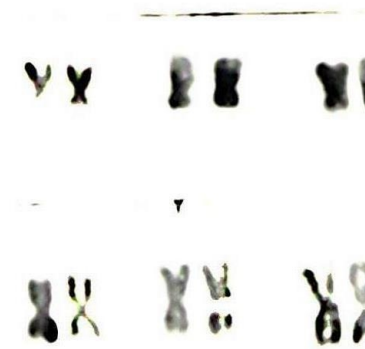

FIG. 10. Isochromatid break of one chromosome A2 (after epanutin).

TABLE 1. Nucleo-chromosomal aberrations in peripheral lymphocyte cultures

Study category and number of subjects	Drugs tested	Peripheral lymphocytes cultures without haemagglutinin		Peripheral lymphocytes cultures + phytohaemagglutinin Mean % incidence ±SE of nucleochromosomal aberrations							
				Nuclear		Chromosomal					
		Blastogenic index %	Mitotic index %	Polynuclei (3–8)	Nuclear fragmentation and micronuclei	Gaps	Chromatid Breaks		Cross chromosomal configurations	Fragmentation and/or pulverization	Polyploidy
							Single	Isochromatid			
A: Bilharziasis											
20	Niridazole	10·0±0·84	11·0±0·30	4·3±0·75	—	9·0±0·62	9·0±0·83	4·9±0·62	5·1±0·63	2·0±0·54	2·7±0·84
15	Tartar Emetic	4·0±0·59	4·0±0·68	—	4·2±0·51	3·2±0·54	6·8±0·46	3·7±0·57	4·0±0·59	3·0±0·52	—
10	—	4·9±0·48	9·5±0·20	—	—	—	2·8±0·50		—	—	0·2%
B: Tuberculosis											
20	Isoniazid	4·4±0·60	8·4±0·76	2·3±0·51	—	3·5±0·55	3·7±0·80	3·5±0·55	2·9±0·55	—	1·2±0·23
10	—	4·3±0·37	19·0±0·19	—	—	—	3·8±0·61		—	—	—
C: Epilepsy											
21	Epanutin	5·3±0·61	10·6±0·10	3·4±0·20	—	5·2±0·31	6·8±0·87	5·6±0·21	—	1·8±0·67	2·8±0·35
10	—	5·2±0·20	13·3±0·50	—	—	—	3·0±0·2	—	—	—	0·3%
Normal 15	—	5·0±0·31	11·8±0·80	—	—	—	2·5±0·25	—	—	—	0·2%

are enhancing to the effects of other environmental clastogens remain un-clarified.

In Pls cultures of tartar emetic treated bilharzial patients, no significant effects on cell growth rates was noted in cultures set without PHA. In PHA stimulated Pls cultures, a significant reduction in mitotic index and an increase in incidence of chromosome breaks and nuclear and chromosome fragmentation occurred. Since Pls cultures of bilharzial patients prior to tartar emetic therapy did not reveal any significant reduction of mitotic index or increase of chromosome fragility, the latter drug might be incriminated in the observed mitotic and nucleochromosomolytic aberrations. Tartar emetic contains 36·5% trivalent antimony and potassium ion is incorporated in its structure. Such an ionic content could be a potential hazard to the proliferating lymphocyte; its cell membrane, spindle protein, DNA phosphorus and may be magnesium content both at the nuclear and mitochondrial levels. Besides potassium ion was shown by Margery (1970) to cause attenuation of certain heterochromatic chromosome regions thereby predisposing them to breakage. No direct deranging effect of tartar emetic on growing cells could however be demonstrated experimentally; pregnant ewes fed sublethal doses of tartar emetic gave birth to normal full term lambs (Lynn, Victor & Wayne, 1966). The Pls chromosome fragility noted in present study could however stem from a potentiating effect of tartar emetic to some specific nutritional deficiencies known to prevail in bilharzial patients where mixed intestinal parasitization frequently co-exist. Freed & Schatz (1969) could induce chromosome damage in cultured chinese hamster cells by depleting the growth medium of specific amino acids, thereby proposing that errors of DNA replication and the consequent chromosome aberrations are the result of transient inhibition of protein synthesis.

PHA stimulated Pls cultures of isoniazid treated patients revealed low incidence of binucleate and tetraploid cells and a significantly high incidence of chromosome gaps, breaks and cross chromosome configurations. Pls cultures of tuberculous patients prior to isoniazid therapy did not reveal comparable cellular or chromosomal derangement. That those effects could be directly related to isoniazid is substantiated by the experiment of Cirnu-Georgian & Lenghel (1971) who demonstrated a high incidence of cells with structural chromosomal abnormalities in bone marrow cultures of Wistar rats after intravenous injection of isoniazid. Also Biancifiori & Sever (1966) could demonstrate the oncogenic potential of isoniazid by experimental induction of leukemia and solid tumours in mice after oral and/or parental administration of large doses of isoniazid. Besides accumulating epidemiologic evidence is incriminating isoniazid in teratogenesis. Errki (1964) reported a one two-fold increase in incidence of malformed fetuses in pregnant females under isoniazid therapy. Isoniazid is a known inhibitor of pyridoxal phosphate which is a coenzyme essential for metabolic bioenergetics of specific aminoacids transformations and nucleo-protein synthesis. Hence isoniazid induced pyridoxal phosphate deficiency could be promoting to the noted cellular and chromosomal aberrations with their onco-teratogenic potentials probably by direct impairment of DNA synthesis.

In the group of epanutin treated epileptic patients, PHA stimulated cultures revealed significantly high incidence of Pls multinucleation, polyploidy and chromosomal fragility. The incrimination of epanutin in the genesis of those cytochromosomal findings is enforced by their absence from Pls cultures of epileptic patients prior to therapy. Similar findings are reported by De Toni *et al.* (1966), Ayraud, Kermarec & Martinon (1968) and Marquez-Monter, Ruiz Fragosco & Velasco (1970). Alexander & Roman (1971) also noted chromosome pulverization of bone-marrow cells of albino rats treated with epanutin. The mechanism of induction of such cytochromosomal findings is currently speculative. Epanutin is known to lower serum folate level (Klipstein, 1944). Folic acid being essential at various metabolic levels for DNA synthesis, its deficiency could be implicated in the induction of such nucleochromosomal pathology. Besides those epanutin induced cytogenetic effects, clinical evidence is directly endowing it with oncogenic and teratogenic potentials. Hyman & Sommers (1966) reported the occurrence of malignant lymphadenopathy, Michael, John & Ariz (1968) reported the development of pseudolyphoma syndrome in patients receiving epanutin therapy. Bernard (1971) reported an increased incidence of birth defects among offspring of mothers receiving epanutin therapy during pregnancy. In the present study one epileptic patient, while under epanutin therapy gave birth on two successive pregnancies to anomalous stillbirths depicting absent kidneys, cleft lip and cleft palate. Hence the occurrence of the reported epanutin induced cytogenetic aberrations could be hazardous

to replicating cells of mature tissues with constant turnover rates and to embryonal cells during critical phases of ontogenesis.

The significance of the present *in-vitro* cellular and chromosomal aberrations attributed to the four tested drugs should be critically evaluated. With the exception of the high incidence of the niridazole induced lymphoblastoid transformation and enhanced nuclear replication and the tartar emetic induced nuclear fragmentation and diminished peripheral lymphocytes mitotic response to PHA; the four drugs revealed variable degrees of the same cytochromosomal pathology. Binucleation and polyploidy consequent on nonsynchrony of nuclear and cytoplasmic division and failure of sister chromatid separation during enhanced DNA replication with devitalization of specific chromosome segments, were common to the four drugs, though are most probably invoked differently in each drug. A similar risk of the same cytochromosomal aberrations detected in Pls as one type of somatic cell could stand for other somatic cells and may be for germinal cells as well. However the fact that those changes are demonstrated *in-vitro* after one or two Pls replication cycles and mostly under an artificial mitogenic stimulus with PHA and a mitotic arresting stimulus with colcemid, should not be extrapolated as of carrying an equal *in-vivo* hazard. A peculiar sensitivity to those drugs may be imported on Pls by the set-up of *in-vitro* culturing. Kato & Sandberg (1967) noted that colcemid potentiated the *in-vitro* appearance of chromosome pulverization in binucleate or multinucleate cells. Besides *in-vivo* repair of single chromatid breaks is probably a common event and failure of survival of cells carrying more complex chromosome rearrangements might be the rule. Direct evidence is however accumulating that cells carrying specific chromosomal abnormalities and rearrangements may be the fore-runner of certain types of neoplasia. Recently (Hecht, McCow & Koler, 1973) demonstrated that a tumour originated in a cytogenetically abnormal (14/14 t) lymphocyte clone in a case of ataxia telangiectasia. Also clonal proliferation was shown to arise in cytogenetically abnormal cells in a G6PD heterozygote patient having chronic myelogenous leukemia in whom the Ph positive leukemic cells were the only cells revealing the G6PD-B activity (Barr & Fialkow, 1973). However there is yet no direct evidence as to the oncogenic, mutagenic or teratogenic potentialities of those tested drugs in Man. Also there is no direct evidence that human cells carrying cytogenetic errors might depict an enhanced sensitivity to the transforming effects of known oncogenic viruses, specific chemicals and natural mitogens or mutagens although the probability is currently explored. However epidemiologic and clinical evidence indicate that individuals subjected to agents which induce such cyto-chromosome aberrations during pregnancy have an increased risk of teratogenicity and may be genic mutations in their foetuses. Hence drugs whose chemical structure is likely to promote such risk should be ensured biologic safety prior to their human use. *In-vitro* culturing of peripheral human lymphocytes and may be other relevant somatic cells is one biologic test system which could exhibit cytochromosomal effects of drugs having the potential to interact with the genomic material of somatic cells and may be of germinal cells. Occurrence of chromosome breakage and/or spontaneous polynucleation or replication in any type of human cells during or shortly after therapy by any drug should be considered one biologic indicator negating its safety.

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