Chromosomal studies in patients taking phenylbutazone

ALAN C. STEVENSON AND JEAN BEDFORD

MRC Population Genetics Unit, Old Road, Headington, Oxford

AND

ALAN G. S. HILL AND HILARY F. H. HILL

Rheumatic Diseases Research Centre, Stoke Mandeville Hospital, Aylesbury, Bucks.

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In course of investigating a family for another purpose, an elderly woman was found to have a high proportion of grossly damaged chromosomes. During the preceding 15 months she had been taking phenylbutazone for osteoarthritis. She had never had any external or internal radiation therapy, and the only other drug she was taking was a 50 mg. nembutal capsule each night. As there was no obvious explanation of her chromosomal damage, and because phenylbutazone has long been suspected of occasionally causing blood dyscrasias, and possibly of being associated with leukaemia (Dougan and Woodliff, 1965), it was decided to examine the chromosomes of a series of patients who had been taking the drug. Findings in these patients and in controls, some preliminary results from testing patients before starting to take phenylbutazone, and at intervals thereafter, and some miscellaneous observations, including experiments *in vitro*, form the subject of this report.

Patients studied

Fifty patients, 32 women and 18 men, who had taken phenylbutazone for at least 3 months, were investigated. All except one patient were still taking the drug, or had been taking it up to a few weeks before being tested. The remaining patient (No. 12 in Appendix A) had taken the drug regularly for 3 years but not for the previous 15 years. There were also 31 control subjects who had identical chromosomal investigations. As will be seen from Appendix B, they consisted of 24 patients with rheumatic disorders who had never had phenylbutazone (with the exception noted below) and these included eight who were subsequently treated with the drug, and also seven normal subjects. No patients or controls were accepted for study if they had any history of internal or external ionizing radiation therapy or diagnostic procedures using radioisotopes. Patient No. 48 (Appendix B), after being accepted as a control, was found to have had phenylbutazone mainly by suppositories, and then only for less than 3 weeks, 2½ years before being tested. However, she was retained as a control.

Cytological techniques used, types of chromosomal damage scored, and rationale of interpretation of findings

Venous blood received into lithium heparin tubes was cultured for 48 hrs, using the technique of Arakaki and Sparkes (1963). After 45 hrs, *i.e.* 3 hrs before harvesting the lymphocytes for chromosomal examination, colchicine was added to the culture. Chromosomes of cells in metaphase were then scored for chromosomal type damage, *i.e.* breaks or the sequelae of breaks.

The rationale of this procedure requires some elucidation for those not conversant with cytogenetics. As is fully discussed in UNSCEAR (1969) and by Evans (1970), it is generally accepted that, if the above type of peripheral blood culture lasting for 48 hrs is followed, chromosome type damage found in all lymphocytes in the metaphase stage of cell division must have occurred *in vivo* when the lymphocytes were in the G1 (pre-DNA synthesis) stage of interphase. The circulating lymphocytes have a long life *in vivo*, probably on average about 3 years. They remain in the G1 phase and very seldom divide. However, when stimulated by phytohaemagglutinins in the culture medium, some at least, probably those which are immunopotent, proceed into a division cycle and many have reached metaphase by 45 hrs in culture. Colchicine added then stops these cells and others which subsequently reach this stage in metaphase, and they proceed no further. Any cells which have passed through metaphase by 45 hrs in culture certainly have not yet reached that stage in a second division cycle.

It follows that chromosome breaks, *i.e.* those affecting equally both arms of chromatids which are held together only at the centromeres in the metaphase stage, must have occurred before DNA synthesis and the formation of chromatids, *i.e.* in the G1 stage of the cell cycle when the chromosomes were long single threads and were closely coiled in the nuclei of the lymphocytes *in vivo*. It follows also that chromosome type breaks and their sequelae are not caused by adverse cultural conditions; a statement which is well supported by abundant experimental evidence. In contrast, damage to chromosomes after the G1 stage in culture predominantly causes chromatid type damage, as can readily be seen in experiments *in vitro*. This is manifest in the first metaphase after the damage by breakage of only one of the two chromatids (See Fig. 1A).

The origins of the types of chromosomal damage originating *in vivo* in G1 which were scored in the cells of patients in this study are set out in diagrammatic form in Fig. 2 (opposite).

Photographs of corresponding types of damage of chromosomes in cells of patients are shown in Fig. 1. Additional evidence that cells have not passed through a complete cell cycle since the damage occurred in G1 is afforded by the presence of acentric fragments in the cells in metaphase. Such fragments are usually lost in anaphase and fail to be incorporated in daughter nuclei. Single breaks of a chromosome will result in a centric and an acentric fragment (Fig. 1A). If two chromosomes are broken there may simply be two centric fragments and two acentric fragments, or the broken ends of the centric fragments may unite leading to a dicentric chromosome, as in Fig. 1B. There may be two acentric fragments, as in the diagram, or the two may unite to form a single larger

fragment. (There are other possible explanations of there being only one fragment which need not concern us here.) As will be seen from Fig. 1C, breaks on either side of the centromere may result in a centric ring and fragments. These three types of cell damage all result in cells which are unstable, are conventionally termed 'Cu', and are so classed in Tables I and II (overleaf). They are unstable essentially because mistakes occur in anaphase and fragments are lost so that daughter cells may have a surplus; or daughter cells have a deficiency of genetic material and so be relatively inviable.

The two remaining sequelae of breaks which we need to consider here are the types whose genesis will be evident from Fig. 1D and E, and it will be noted that both can follow only from two or more breaks in G1. However, the daughter cells from these configurations will have a full chromosome complement, although abnormally arranged, and they will be viable. Such cells are, following convention, called stable and denoted as 'Cs' in Table I.

FIG. 1 Typical damaged cells from subjects in study.

- A Simple break and small paired fragment affecting short arms of a B group chromosome $\uparrow$: 'Cu'. In the chromosome above this is a chromatid gap or possible break of a long arm of a D group (13-15) chromosome $\leftarrow +$. This is not scored as 'chromosome' damage.
- C A ring chromosome $\uparrow$, no acentric fragments. They may have been too small to see. 'Cu' cell.

- B Dicentric chromosome probably derived from two C group chromosomes $\uparrow$. One paired fragment $\uparrow +$ 'Cu' cell. Note that there are 46 centromeres.
- D The chromosome marked $\uparrow$ is presumptively a chromosome No. 2 but a pericentric inversion has occurred, i.e. it is a 'marker' chromosome. Note the more terminal position of the centromere than in its normal homologue. $\uparrow +$ 'Cs' cell marked. The picture shows only 43 chromosomes. The three remaining chromosomes were displaced to a portion off the field.

E Chromosome marked ↑ is of the B group with a portion of the short arms missing. The missing portion is attached to a G (21-22) group chromosome marked X which therefore has much longer arms than the other three G group chromosomes, marked X.

F A cell showing the result of many breaks and rearrangements. There are two dicentrics (D) (one with a terminal centromere), and one tricentric (T). There are only 45 obvious centromeres in the cell. There are five (paired) acentric fragments (F). There is also a fragment which probably is a small centric ring (R), so explaining the missing centromere. Such damage must have involved at least eight but probably more breaks in G1.

- Breaks induced in G1 stage of interphase	Results of breaks (still in G1)	Results of breaks as seen at metaphase	Appearances which would have been seen if breaks had not occurred
A. A single break in chromosome leading to centric and (paired) acentric fragments. ("Cu")			Instead of
B. Breaks in two chromosomes leading to a dicentric chromosome and two (paired) acentric fragments ("Cu")			Instead of
C. Two breaks in one chromosome leading to ring chromosome (duplicating) and two (paired) acentric fragments ("Cu")			Instead of
D. Two breaks in one chromosome leading by pericentric inversion to a "marker" chromosome ("Cs")			Instead of
E. Breaks in two chromosomes leading to somatic reciprocal translocation of centric fragments and two "marker" chromosomes ("Cs")			Instead of

FIG. 2 Diagrammatic representation of end-results of chromosome breaks in G1 stage of interphase as seen in first subsequent metaphase. For explanations see text.

Finally, if a cell has any Cu type chromosomal damage, it is so scored in Tables I and II even if there is also Cs type damage in the same cell.

Findings in patients taking phenylbutazone and in controls

As will be seen from Table I, the frequency of damaged cells in the fifty patients taking phenylbutazone was 100/4,298 (2 per cent.) and in the 31 controls 15/3,102 (0.48 per cent.). The difference is highly significant ($\chi^2 = 31.13$; d.f. 1; $P < 0.001$). The difference between the frequency of cells with at least one dicentric in patients 22/4,928 (0.45 per cent.), and in controls 2/3,102 (0.064 per cent.) is

also impressive ($\chi^2 = 8.08$; d.f. 1, $P < 0.01$). It will be noted from Table I that over 60 per cent. of the patients taking the drug had 2 per cent. or fewer damaged cells. Of the fifty patients 32 were female. It was very difficult to find elderly controls with rheumatic disorders who had not had phenylbutazone. However, there appears to be no relationship between the frequency of damaged cells by sex or by age, either in patient or in controls, as is obvious from Appendices A, B, and D. The frequencies of damaged cells in patients do not give a significant correlation with the duration of treatment, the mean daily dose, or an index of accumulated dose, namely months multiplied by mean daily

Table I *Summary of chromosomal findings in patients taking phenylbutazone and control subjects*

Percentage of damaged cells	Patients having phenylbutazone								Control subjects							
	No. of patients	No. of cells analysed	Types of damaged cells			Multicentric chromosomes			No. of control subjects	No. of cells analysed	Types of damaged cells			Multicentric chromosomes		
			Cu	Cs	T	Cells with one dicentric	Other multicentrics in same cell	Total			Cu	Cs	T	Cells with one dicentric	Other multicentrics in same cell	Total
0-	14	1,345	0	0	0	0	0	0	19	1,868	0	0	0	0	0	0
1-	11	1,100	11	0	11	2	0	2	10	1,034	8	3	11	1	0	1
2-	6	722	14	1	15	2	1	3	2	200	4	0	4	1	0	1
3-	6	600	13	5	18	1	1	2								
4-	8	743	23	6	29	4	2	6								
5-	2	200	10	0	10	6	1	7								
6-	1	100	5	1	6	3	0	3								
7-	1	65	5	1	6	1	0	1								
8-																
9-	1	53	4	1	5	3	0	3								
Total	50	4,928	85	15	100	22	5	27	31	3,102	12	3	15	2	0	2
Observed frequency			2.0 per cent.					0.55 per cent.			0.48 per cent.					0.06 per cent.

Difference between frequency of damaged cells in patients $\frac{100}{4,928}$ and in controls $\frac{15}{3,102}$: $\chi^2 = 31.13$; d.f. = 1; $P < 0.001$

Difference between frequency of multicentric chromosomes in patients $\frac{27}{4,928}$ and in controls $\frac{2}{3,102}$: $\chi^2 = 8.08$; d.f. = 1; $P < 0.01$

Table II *Findings in patients tested before taking phenylbutazone and at intervals after starting to take Butazolidin 300 mg. daily (100 cells analysed from each specimen) (RA = rheumatoid arthritis; AS = ankylosing spondylitis)*

Patient number	Sex	Disease	Age (yrs)	Numbers of cells with damaged chromosomes															
				Pre-treatment	Time after treatment when specimens taken (wks)														
					2-	4-	6-	8-	10-	12-	14-	16-	18-	20-	22-	24-	26-	28-	30-
3	M	AS	36	1	1	0			2*		1		1					0	
4	F	RA	48	0				1					2*		2				
13	M	AS	49	0		4													
17	F	RA	68	0						3				4***		4		3*	
23	M	AS	80	0			0			0		2		4***		4*			
26	M	AS	19	0		6			3				0		4**				
27	M	AS	29	1			0												
44	M	AS	34	1		0			1*		2*								
43	M	RA	49	0		2			3**		3								
Total cells					1	12	0	5	5	8	1	2	3	8	6	8		3	

* ** *** denotes one, two or three cells with a dicentric chromosome.

dose ($r = -0.18$). The overall picture suggests that, if there is an effect from phenylbutazone, then there must be large variations in responses of patients.

Complete protocols of patients are set out in Appendices A and B, so that readers have available as much information about the patients and controls as the authors.

INTERPRETATION OF THE FREQUENCIES OF DAMAGED CELLS IN PATIENTS AND CONTROLS

There is now a considerable literature on the frequency of cells with chromosomal damage in normal individuals. In published results where culture times of 50 hrs or less were used, and over 2,000 cells were analysed, estimates range from 1.32 per cent. (Court Brown, Jacobs, Buckton, Tough, Kuenssberg, and Knox, 1966) to 0.13 per cent. (Norman, 1967). Our findings (0.48 per cent.) are just below the mean of the range. Court Brown and others (1966) considered that the frequency was significantly higher in females than in males and that it increased with age. However, when their longer (over 50 hr) culture time data is excluded, these effects are not apparent. Adams (1970), Sandburgh, Cohen, Rimm, and Levin (1967), and Bochkov, Koslov, Pilosov, and Sevankaev (1968) could find no such association and as noted above there appears to be none in our data (Appendix D).

The frequency of cells with dicentric chromosomes in normal individuals is usually estimated to be considerably less than 0.1 per cent. The data reviewed in UNSCEAR (1969) ranges from 0.01 to 0.1 per cent.

All the patients with one exception had had diagnostic radiology of their joints, whereas seven of the controls did not have rheumatic disorders, and those who had arthritis or spondylitis were on average younger and so had probably had fewer radiographs. It proved impossible to collect accurate data on diagnostic radiological exposures. Some authors have suggested that after a few radiographs, mostly of the chest, patients show an increase of cells with chromosomal damage, but this has not been found by all authors (UNSCEAR, 1969; Adams, 1970). In our own laboratory, limited investigation of the chromosomes of patients after excretion urography has yielded negative results. In any event, the differences between patients and controls in our series seem too large to be explained on a basis of past joint radiographs. In particular the frequency of dicentric chromosomes is much too high.

Other drugs taken by patients and controls

A code number is given in Appendices A and B for drugs other than phenylbutazone taken by patients and controls in recent years. The key to the code

will be found in Appendix C. Patients with rheumatic disorders inevitably are tried with several preparations until they find something which helps and, indeed, it proved difficult to find control patients who had not taken phenylbutazone. It is impossible adequately to set out all the details of different doses and durations for each patient. The drugs listed in the Appendices had all been taken during the 5 years prior to the test, but each for varying periods. There seems to be no pattern of association with chromosomal findings with any one drug except phenylbutazone. It will be noted that of our control patients (Appendix B) four had had at least one course of mycristin injections, six a course of indomethacin, and four hydroxychloroquine, and three were taking preparations containing paracetamol.

Patients tested before and after taking phenylbutazone

A series of patients who presented at the Rheumatism Research Centre, and for whom phenylbutazone was indicated, was tested and on the day of collection of the specimen began to take 100 mg. phenylbutazone (in the form of Butazolidin) three times a day. The pre-treatment specimens from eight of the patients were used as controls (Appendix B).

The preliminary results are set out in Table III (overleaf).

Clearly it is not possible to conclude much from the data, but there were only three damaged cells in 900 from pre-treatment specimens (0.3 per cent.), but significantly more, 62 in 3,000 (2.1 per cent.) in post-treatment specimens ($\chi^2 = 11.65$; d.f. 1; $P < 0.001$). The latter include several from patients after only a few weeks of treatment. Even more impressive is the finding that there were 16 cells in which the damage included a dicentric chromosome in 3,000 cells from patients after treatment where there were none in 900 cells before treatment ($\chi^2 = 3.603$; d.f. 1; $P < 0.05$).

These 'preliminary findings appear to support those from patients tested after longer periods on the drug.

Haematology

Haematological findings were available for thirty of the patients and nine of the controls. None showed any leucopenia or the presence of abnormal white cells.

Findings in bone marrow in patients taking phenylbutazone

So far four patients taking the drug have had marrow biopsies and chromosomal examination of marrow cells from 3-hr cultures. Patients Nos. 19, 58, and 84 had no abnormal cells in 100 cells analysed; patient No. 59 had one damaged cell.

Table III *Second experiment in vitro*

Culture		Cells scored	Findings in culture harvested at:					
			72 hrs			96 hrs		
			Chromatid		Chromosome breaks	Chromatid		Chromosome breaks or sequelae
			Gaps	Breaks		Gaps	Breaks	
Control	1	100	3	2	0	—	—	—
	2		—	—	—	3	2	4 (including 1 dicentric)
Treated	1		6	1	0	—	—	—
	2		4	1	1 (marker)	—	—	—
	3		—	—	—	3	1	1
	4		—	—	—	2	0	1 (tetraploid cell with pair of dicentrics)

Notes (1) Treated cultures had 100 mg. per cent. phenylbutazone sodium added at 56 hrs.

(2) Chromatid or isochromatid gaps occur where there is an interruption of staining on one or both chromatids respectively, but the distal fragments were not re-aligned; probably these are not breaks and that under most circumstances spontaneous restitution occurs.

Clearly there is no evidence of damage in marrow cells in these patients. However, it would not be expected that marrow damage was identifiable even if it occurred. The turnover of marrow cells is very rapid; probably each cell divides at least once in 72 hrs, so that probably most Cu cells would be eliminated by that time. Damage to cells from phenylbutazone must occur at a very low rate and is only detectable in lymphocytes because, as already explained, the damage accumulates because they do not divide. However, the only Cu cells which would be detected in marrow would be those which had been induced in the preceding few days at an exceedingly low frequency. As will have been noted, Cs cells which might accumulate in marrow stem cells represent only about 15 per cent. of all damaged cells, and it would be unlikely that an increase in cells would be detectable. Even after deep x-ray therapy involving marrow, damaged cells can be detected for only a few days after treatment.

Experiments *in vitro*

It is not easy to design realistic experiments involving the exposure to phenylbutazone of cells growing in culture. The pure drug has a very low solubility and the sodium salt determines difficulties with the electrolytic buffering of the medium which is critical to the growth of cells.

The mean steady-state plasma concentration of phenylbutazone in patients taking 300 mg. per day is mainly between 60 and 130 mg. per cent., and any damage to lymphocytes in G1 must occur only at very low rates, as the build-up of damaged cells to a detectable level takes at least several weeks. It follows that, even if lymphocytes are exposed to

equivalent concentrations *in vitro* while in G₁, and the cultures are harvested after they have gone through a division, *i.e.* on long culture, it is unlikely that a detectable proportion of cells would be damaged. By analogy with the action of other drugs and ionizing radiation, it would be expected that any damage occurring after exposure in culture would be of the chromatid type. However, most other drugs known to damage chromosomes and ionizing radiation act directly on DNA, and there must be considerable doubt as to the mode of action of phenylbutazone. In a preliminary experiment three cultures were set up from bloods of each of three subjects; one culture from each subject was untreated and two were cultured in the same medium having a concentration of 20 mg. per cent. pure phenylbutazone. The cultures were harvested after 48 hrs. In 100 cells analysed from each culture there was no evidence that chromatid (or chromosome) damage had been inflicted. In the 300 cells from controls there was one chromatid break, and in the 600 cells from those cultures with phenylbutazone there were two. There was one Cu cell in the 300 control cells, and one in the 600 exposed cells.

In a second experiment, two blood samples from one subject were cultured in the usual medium and harvested at 72 and at 96 hrs. The remaining four samples from the same subject were cultured in normal medium for 56 hrs when sodium phenylbutazone was added, giving a concentration of 100 mg. per cent., and two were harvested at 72 hrs. and two at 96 hrs. The results are set out in Table III. As will be seen, there is nothing to suggest that the phenylbutazone caused chromosome or chromatid damage.

There have been a few previous publications on

effects of phenylbutazone *in vitro*. Heres (1969) examined cells from the meristems of *Allium cepa* grown in culture medium containing (a) phenazone, (b) dimethylphenazone (DMAP), (c) phenylbutazone, and (d) irgapyrin (a mixture of DMAP and phenylbutazone). He concluded that in varying degree all had harmful effects. Phenylbutazone appeared to reduce the frequency of mitosis and to interfere with accurate spindle formation in metaphase and consequently with anaphase. In addition, DMAP appeared to determine an increase in chromosome breaks, but no such effect was found with phenylbutazone.

Wismuller and Gebhart (1970) treated cultures of human lymphocytes with phenylbutazone. After culture for 48 hrs, concentrations of phenylbutazone of 67, 36, and 19 mg. per cent. were added; in one experiment the cultures were harvested after 6 hrs and in the other after 18 hrs. In each experiment there was an increase in frequency of chromatid breaks in the two higher concentrations; where the exposure was for 18 hrs the increase associated with a 67 mg. per cent. concentration of phenylbutazone was significant at the 1 per cent. level. In the other three instances the differences were significant at the 5 per cent. level.

Discussion

The information assembled strongly suggests that chromosomal damage occurred in the lymphocytes of the patients taking phenylbutazone. The main evidence comes from the significantly higher frequencies of cells with chromosomal damage, particularly dicentric chromosomes, in patients who have taken the drug for 3 months or more than in controls. Data so far accumulated by the follow-up of patients starting to take the drug support and probably strengthen the other observations. As is well known, phenylbutazone (1, 2-diphenol-3, 5-diketo-4-n-butyl pyrazolidine) is one of a series of substances synthesized from pyrazolone and pyrazolidine over the past 70 years. Only a limited number of these drugs has been found to be useful in therapy and free from too frequent serious side-effects. Phenylbutazone itself probably occasionally causes agranulocytosis, aplastic anaemia, or thrombocytopenia. However, the true size of the risk is difficult to estimate in view of the very large number of people who take the drug over substantial periods, so that some cases of blood dyscrasia would be expected to occur by chance. The evidence that phenylbutazone causes leukaemia, reviewed by Dougan and Woodliff (1965), is not very impressive.

Drugs based on pyrazole and pyrazolidine have not previously been incriminated as mutagenic and are not of a structure suspected on chemical grounds of damaging DNA or RNA directly. There are no

published reports of damage to chromosomes of patients taking any of this group of drugs.

As might be expected, the proportions of controls with 0 per cent., 1 per cent., and 2 per cent. of damaged cells with a mean of 0.48 per cent. is very closely compatible with a Poisson distribution. However, in patients taking the drug, the proportions with 0 to 9 per cent. of damaged cells with a mean of 2 per cent. is not compatible with a Poisson distribution ($\chi^2 = 16.46$; d.f. = 5; $P < 0.01$). The reason for this is that, relative to a Poisson expectation, there is an excess of patients with less than and with more than 2 per cent. of damaged cells.

As noted earlier, the lack of correlation between the frequencies of damaged cells in patients and the accumulated dose of the drug could be interpreted as indicating that the risks of chromosomal damage had no relationship to accumulated dose. Two factors could contribute to the lack of correlation. First, the daily dose figures are probably very misleading in that patients do not follow instructions, and undoubtedly many take the drug very irregularly, and almost invariably in smaller doses than prescribed. The second possibility, not exclusive of the first, is that there is a considerable variation in different individuals of the response of their lymphocytes to a given dosage. The latter could also explain the lack of fit with a Poisson distribution.

Whittaker and Price Evans (1970) have shown that, for a given dose of phenylbutazone by body weight, the steady-state plasma level is normally distributed. The half life of phenylbutazone in plasma after the exhibition of barbiturates is also normally distributed and is highly correlated with the steady-state plasma level. These observations and the appropriate correlations of the two variables in first-degree relatives indicate that speed of metabolism of the drug is under genetic control and is multifactorial.

However, inherited variation in speed of metabolism of the drug need not necessarily bear any close relationship to the incidence of lymphocyte damage. Such cell damage could be determined by some breakdown product which has a higher concentration in some individuals than in others, or by some other genetic mechanism than that associated with speed of metabolism. However, nothing is known of differing breakdown pathways of the drug. There are only two metabolites of phenylbutazone to be found in the urine and they account for only some 8 per cent. of the drug ingested; 3 per cent. is contributed by a phenolic metabolite (oxyphenbutazone or 'Tanderil') and 5 per cent. by a relatively unstable alcoholic metabolite (Burns, Rose, Goodwin, Reichenthal, Horning, and Brodie, 1955).

A high proportion of all investigations of the

effects of other drugs and chemicals on human chromosomes has been by experiments *in vitro* and evidence that damage is caused *in vivo* is convincing only in respect of very few substances. This is partly because few observations have been made, and partly because the use of long culture times and other factors make it difficult to assess the work of certain observers who suggest that damage has occurred *in vivo*.

Of the alkylating agents, nitrogen mustard has definitely been shown to cause chromosomal damage *in vivo* (Conen and Lansky, 1961). High chromatid damage rates and some chromosome damage have been reported in patients treated with other alkylating agents, such as piperazine (Nasjleti and Spencer, 1966) and with cyclophosphamide (Hampel, Kober, Rösch, Gerhartz, and Meinig, 1966), but the culture times were over 48 hrs and the evidence from experiments *in vitro* suggests that all the agents act mainly on the 'S' or 'G2' stages of interphase, *i.e.* during and after DNA synthesis. Other evidence for production of chromosomal damage *in vivo* comes from the work of Tough and Court Brown (1965), who found a significantly higher frequency of chromosomally damaged cells in workers exposed to ambient benzene than in controls.

Drugs which can provide base analogues for DNA, such as 5-fluorodeoxyuridine (Hampel and others, 1966), 6-mercaptopurine (Nasjleti and Spencer, 1966), and 6-azauridine (Elves, Buttoo, Israels, and Wilkinson, 1963), have been shown to cause chromosomal damage *in vivo*. The evidence that lysergic acid dimethylamide (LSD-25) causes chromosomal damage (Irwin and Egozcue, 1967; Cohen, Hirschhorn, and Frosch, 1967) is at present not entirely convincing. There are many other substances whose effects *in vivo* are not known but they have been shown to cause chromatid damage *in vitro*.

There are several points which may be noted concerning the drugs which are known as or suspected of causing chromosomal damage *in vivo*. Most are used in the treatment of neoplasms for short periods and in near toxic doses. All also appear to cause chromatid damage *in vitro*. Further, when blood from patients taking the drug is cultured, not only chromosome but chromatid damage is found to be present. This is usually considered to be caused by traces of the drug in the serum affecting the 'S' or later stages in the culture medium. The second is that (with the exception of lysergic acid dimethylamide) all the cytotoxic substances have been shown to induce gene or point mutations in bacteria and other simple organisms. It should be remembered that viruses which attack DNA always cause chromatid damage *in vivo* and *in vitro*.

Chromatid damage is not increased in cultures of

cells taken after patients have had external ionizing radiation. We have observed the same situation in the cells of patients taking phenylbutazone relative to our specific controls. Chromatid breaks per 100 cells were 0.85 in patients and 0.91 in controls. These are within the range of levels normally observed in our laboratory. As already noticed, our findings *in vitro* have been entirely negative. We cannot explain the difference in our findings and those of Wismuller and Gebhart (1970).

It seems unlikely for a number of reasons that phenylbutazone, like the cytotoxic substances discussed above, acts directly on DNA. It is difficult to see how this drug could attach to or damage directly a DNA molecule, or could provide a base analogue. It may, however, act as a metabolic antagonist like methotrexate (folic acid antagonist) or azaserine (glutamine antagonist). Unlike other drugs known to damage DNA *in vivo*, most phenylbutazone is protein-bound in the serum and the drug is known to have a depressant effect on cell activity and to inhibit or promote a variety of enzyme reactions, as discussed by von Rechenberg (1962). Our findings of an increase in chromosomal damage in treated patients but no increase in chromatid damage in their cells, and our negative findings *in vitro*, seem to support the hypothesis that there is no direct action of phenylbutazone on DNA.

It seems necessary to consider the implications if, as suggested by our findings, phenylbutazone in therapeutic doses over several months causes chromosomal damage to lymphocytes. It cannot be supposed that damage is confined to lymphocytes and *a priori* it has to be assumed that it occurs in cells in all tissues and organs. Probably, in the main, this is not very important. If the data are taken at their face value the frequency of damaged cells in patients is increased by a factor of four, but most of the damage is of the unstable cell type, and many such cell losses must simply be replaced without known human detriment in all individuals.

Nevertheless, most agents of chemical and ionizing radiation which break chromosomes are also carcinogenic, although very few observers believe that this is a direct result of chromosomal breakage, thinking that it is rather the consequence of direct less well understood action on DNA. In the light of the preceding discussion, the risks of leukaemia or other neoplasia are probably not as great when phenylbutazone is the chromosome-breaking agent as with cytotoxic drugs which damage chromosomes by direct action on DNA. Possibly, as such a very large number of people take the drug in this and other countries, if the drug had definitely been leukaemogenic the phenomenon would by now be an established fact. In the United Kingdom there are at least 3,500,000 National Health Service

prescriptions for phenylbutazone each year (Dunlop, 1969).

However, although the main incidence of induced chromosomal damage determines Cu cells, there must be an increase in the frequency of Cs cells as there are so many damaged cells in which more than one break occurred. In our patients the frequency of Cs cells was 15/4,298 (0.34 per cent.), which, although not significant ($\chi^2 = 2.80$; $P > 0.1$), is about three times as great as in controls where the frequency was 3 in 3,102 (0.096 per cent.).

Cs damage is probably of little if any importance in somatic cells and it may be suspected from the evidence presented above that phenylbutazone would not damage chromosomes of oocytes, which are in the special stage of the first meiotic division where they have passed the DNA synthesis stage. However, in the male, there is a constant pool of type A spermatogonia where chromosomal damage of both the Cu and Cs type could accumulate. These could give rise to viable sperm which were unbalanced in chromosome constitution and cause peri-implantation losses of zygotes or surviving deformed foetuses. Spermatogonia with Cs reciprocal translocations would be expected to permit a higher proportion of zygotes to survive to become mal-developed foetuses than viable Cu cells with small deletions.

The overall frequency of children recognized as suffering from the effect of an unbalanced translocation is very low, perhaps not more than 1 in 5,000, but in view of the difficulty in identifying small differences and sizes of chromosomes, unidentified but biologically important translocations must be several times as frequent as those recognized. Nevertheless, the risk to the offspring of a male taking phenylbutazone, although still low, is probably several times above the mean in the population. The amount of chromosome damage is less by an order of magnitude in patients with ankylosing spondylitis taking phenylbutazone than in those treated by deep x-ray therapy of the spine. Nevertheless, very large numbers of patients take the drug and if another can be employed which will keep young patients, particularly males, comfortable, it would seem wise to use it. In the light of the suggestive evidence of individual variation, it would probably be wise, in young patients, to examine their chromosomes after 3 months of treatment.

There are a number of indications for further work, and some of these are being followed in various laboratories. Our findings *in vitro* are not conclusive and the situation *in vitro* must be clarified. This sounds simple, but there are many snags in the planning, control, and interpretation of experiments *in vitro*. The search for the induction of chromosomal abnormalities in spermatogonia of experimental

animals should be pursued, using the 'dominant lethals' technique. There are problems to be overcome in such experiments. The half-life of the drug in mice and other small animals is a matter of hours and it is difficult to arrange for high plasma levels to be maintained over the considerable period of time which, by analogy with our findings, would be necessary if many chromosome breaks were to be induced. Then, tests of induction of mutations in spermatogonia are very time-consuming. It is necessary to wait for at least 70 days after plasma levels have been maintained for a considerable time before the mice are mated and the appropriate tests carried out in the pregnant females. It seems important to test oxyphenbutazone and sulphinyprazole in the same way. In view of the findings with phenylbutazone, chromosome analyses should also be carried out on patients taking oxyphenbutazone and sulphinyprazole. Phenylbutazone and allied substances should also be tested for induction of point mutations in bacteria or other simple organisms.

Summary

Findings in fifty patients who have been taking phenylbutazone for at least 3 months and in 31 controls are presented. Neither patients nor controls had had any radiation therapy. The frequency of chromosomally damaged cells in the patients (2 per cent.) was significantly higher than that in controls (0.48 per cent.) ($P < 0.001$). The frequency of dicentric chromosomes in patients was similarly very much higher than in controls. Preliminary results from the follow-up of nine patients tested before treatment and after taking phenylbutazone for varying periods (Table II) are compatible with and strengthen the other evidence which suggests that chromosome damage is occurring in patients taking the drug. The techniques used ensure as far as possible that the cell damage observed occurs *in vivo* and not in culture.

Evidence is discussed which suggests that there are individual variations and susceptibilities to chromosome damage in lymphocytes and that the drug does not act directly on chromosomal DNA. The problems of assessment of effect on individuals, possibly in the next generation, are discussed, as are some indications for further research.

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APPENDIX A

Chromosomal findings and characteristics of fifty patients who had taken phenylbutazone for at least 3 mths

Case no.	Sex	Age (yrs.)	Disease	Phenylbutazone		Cytological findings					Other drugs taken and comment	
				Daily dose (mg.)	Duration of treatment (mths)	Total cells analysed	'Cu' cells	'Cs' cells	Percentage Abnormal cells (Cu + Cs)	Cells with at least one dicentric		Other multi-centrics
2	F	70	RA	200	24	100	0	0	0.0	0	0	7, 18
3	F	65	RA	200	30	100	0	0	0.0	0	0	17
4	F	66	RA	200	24	100	0	0	0.0	0	0	7, 21
16	F	84	OA	300	48	95	0	0	0.0	0	0	19
17	F	67	OA	300	66	50	0	0	0.0	0	0	3, 17, 19
18	M	48	RA	300	78	100	0	0	0.0	0	0	3, 21
29	M	50	AS	450	110	100	0	0	0.0	0	0	17
35	F	76	OA	100	36	100	0	0	0.0	0	0	1, 3, 21
38	M	38	AS	300	6	100	0	0	0.0	0	0	None
39	M	53	Gout	300	36	100	0	0	0.0	0	0	19
40	F	27	RA	300	6	100	0	0	0.0	0	0	17, 21
45	M	55	P	300	4	100	0	0	0.0	0	0	3
61	M	66	OA	300	10	100	0	0	0.0	0	0	24
62	M	48	AS	300	48	100	0	0	0.0	0	0	None
20	F	62	RA	300	12	100	1	0	1.0	0	0	12, 19
21	M	61	RA	300	24	100	1	0	1.0	0	0	3
33	F	82	RA	300	54	100	1	0	1.0	0	0	8
34	F	66	OA	300	72	100	1	0	1.0	0	0	14, 19
36	F	49	RA	300	36	100	1	0	1.0	0	0	
56	F	72	OA	300	36	100	1	0	1.0	0	0	6
58	F	78	RA	300	36	100	1	0	1.0	1	0	17, 23
68	F	39	RA	300	24	100	1	0	1.0	1	0	13, 15, 16, 21
80	M	75	OA	300	36	100	1	0	1.0	0	0	8, 17, 27
85	F	58	RA	300	43	100	1	0	1.0	0	0	15, 19
91	M	22	AS	300	3	100	1	0	1.0	0	0	
10	F	66	RA	200	48	230	5	0	2.2	2	1	19
15	F	70	RA	150	66	92	1	1	2.2	0	0	
32	M	64	CTS	250	36	100	2	0	2.0	0	0	3, 5, 17
59	F	59	RA	400	84	100	2	0	2.0	0	0	3, 21
66	F	31	RA	300	4	100	2	0	2.0	0	0	3, 21
75	M	50	AS	300	7	100	2	0	2.0	0	0	None
26	F	64	RA	300	23	100	3	0	3.0	0	0	13
37	F	62	RA	300	3	100	2	1	3.0	1	1	14, 17
41	F	57	RA	300	3	100	1	2	3.0	0	0	21
44	F	57	Gout OA	300	7	100	3	0	3.0	0	0	17, 19, 21
67	F	74	OA	300	40	100	2	1	3.0	0	0	17, 19
72	F	50	RA	250	9	100	2	1	3.0	0	0	17, 19, 21
19	F	32	Uncertain	300	16	100	3	1	4.0	1	0	17

continued

Case no.	Sex	Age (yrs.)	Disease	Phenylbutazone		Cytological findings					Other drugs taken and comment	
				Daily dose (mg.)	Duration of treatment (mths)	Total cells analysed	'Cu' cells	'Cs' cells	Percentage Abnormal cells (Cu + Cs)	Cells with at least one dicentric		Other multi-centrics
30	F	86	OA	100	48	100	4	0	4.0	0	0	5, 17, 22
31	M	64	AS	500	6	75	3	0	4.0	1	2	17
42	F	68	OA	300	5	68	2	1	4.0	0	0	17, 26 (phenylbutazone-alkabutazolidin)
60	M	63	RA	300	70	100	4	0	4.0	1	0	
63	M	60	RA	300	3	100	3	1	4.0	1	0	17
81	F	44	OA	300	72	100	2	2	4.0	0	0	6, 9, 19
84	M	57	RA	300	20	100	2	2	4.0	0	0	21
11	F	62	RA	300	36	100	5	0	5.0	2	0	19, 21
22	M	48	RA	300	12	100	5	0	5.0	4	1	13
12	F	60	RA	300	36	100	5	1	6.0	3	0	15, 21
7	F	59	RA	300	36	65	4	1	7.7	3	0	5
5	M	69	OA	200	15	53	5	0	9.0	1	0	8, 19
Total						4,928	85	15		22		

RA = rheumatoid arthritis; AS = ankylosing spondylitis; OA = osteoarthritis; P = psoriasis and arthropathy; CTS = carpal tunnel syndrome; 'Cu' and 'Cs' mean unstable or stable cells as explained in the text. For coding of other drugs taken by patient see code in Appendix C.

APPENDIX B
Findings in 31 persons who serve as controls in that they have not taken phenylbutazone (Abbreviations and explanations as for Appendix A)

Control no.	Sex	Age (yrs)	Cytological findings				Drugs taken other than phenylbutazone	Category of control		
			Total cells analysed	'Cu' cells	'Cs' cells	Percentage Abnormal cells (Cu + Cs)		Rheumatic disorders (24)	Patients tested before phenylbutazone therapy (8)	Normal subjects (7)
9	F	37	100	0	0	0.0	—	—	—	+
14	F	48	100	0	0	0.0	1, 11	RA	+	—
23	M	49	100	0	0	0.0	6, 17, 9	AS	+	—
27	F	68	100	0	0	0.0	1, 9, 26	RA	+	—
43	F	80	100	0	0	0.0	13, 19	RA	+	—
46	M	19	100	0	0	0.0	17	AS	+	—
48	F	38	100	0	0	0.0	1, 15, 19, 21	RA	—	—
50	M	47	100	0	0	0.0	3, 17	RA	—	—
52	F	55	100	0	0	0.0	3, 13, 17, 21	RA	—	—
70	F	52	100	0	0	0.0	3, 11, 13	RA	—	—
74	F	56	100	0	0	0.0	21	RA	—	—
77	F	30	100	0	0	0.0	17, 21, 23	RA	—	—
78	F	74	100	0	0	0.0	21, 23	RA	—	—
83	M	49	100	0	0	0.0	4	RA	—	—
A	M	32	100	0	0	0.0	—	—	—	+
B	M	31	100	0	0	0.0	—	—	—	+
D	M	28	100	0	0	0.0	—	—	—	+
E	M	67	68	0	0	0.0	—	—	—	+
F	F	20	100	0	0	0.0	—	—	—	+

continued

Control no.	Sex	Age (yrs)	Cytological findings				Drugs taken other than phenylbutazone	Category of control		
			Total cells analysed	'Cu' cells	'Cs' cells	Percentage Abnormal cells (Cu + Cs)		Rheumatic disorders (24)	Patients tested before phenylbutazone therapy (8)	Normal subjects (7)
13	M	36	100	1	0	1.0	3	AS	+	—
51	M	39	100	1	0	1.0	Uncertain	RA	—	—
54	F	56	134	2	0	1.5	15	RA	—	—
55	M	60	100	1	0	1.0	13, 15	RA	—	—
57	M	29	100	1	0	1.0	Uncertain	AS	+	—
64	M	34	100	1	0	1.0*	17	AS	+	—
65	F	53	100	0	1	1.0	15	RA	—	—
71	F	42	100	1	0	1.0	3, 6, 19	RA	—	—
73	F	41	100	0	1	1.0	Uncertain	RA	—	—
C	F	53	100	0	1	1.0	—	—	—	+
49	M	51	100	2	0	2.0*	19, 21	RA	—	—
76	F	51	100	2	0	2.0	3, 21	RA	—	—

* Includes one cell with a dicentric chromosome

APPENDIX C

Particulars of drugs (code for Appendices A and B)

Code no.	Drug	Trade names	Code no.	Drug	Trade names
1	Aloxiprin	Palaprin Forte (Schering)	14	Guanethidine sulph.	Ismelin (Ciba)
2	Amphetamine (laevo and dextro)	Durophet (Riker): Edrisal (SK and F)	15	Hydroxychloroquine (sulphate)	Plaquenil (Bayer)
3	Aspirin	Solprin (Reckitts) Paynocil (with glycine) (Bencard)	16	Ibuprofen	Brufen (Boots)
4	Betamethasone (di-sodium phosphate)	Betnesol (Glaxo)	17	Indomethacin	Indocid (MS and D)
5	Butobarbitone, codeine, phenacetin	Sonalgin (M and B)	18	Nitrazepam	Mogadon (Roche)
6	Chlordiazepoxide	Librium (Roche)	19	Paracetamol	Solpadeine (Bayer): Panadeine Co (Bayer): Panadol (Bayer): Paracodol (Fisons) (i.e. most include codeine and/or caffeine)
7	Codeine co		20	Polythiazide	Nephрил (Pfizer)
8	Cyclopenthiiazide	Navridex (Ciba)	21	Prednisolone	Prednesol (Glaxo): Prednelan (Glaxo)
9	Dextropropoxyphene hydrochloride	Doloxene (Lilly): Distalgic with paracetamol (Dista products)	22	B-pyridyl carbinol	Ronicol (Roche)
10	Diazepam	Valim (Roche)	23	Salicylates	
11	Dihydrocodeine tartrate	DF 118 (BDH)	24	Thyroid	
12	Dihydroxypropyl-theophylline	Sibephylline (Berk)	25	Tranlycypromine sulphate	Parstelin (SK and F)
13	Gold (colloidal suspension) Sod. aurothiomalate	Myocrysin (M and B)	26	Steroids—not specified	
			27	Methyldopa	Aldomet (MS and D)

APPENDIX D

Frequencies of damaged cells by sex and age

Sex	Male	Female	
Patients	$\frac{32}{1,728}$ (1.85 per cent.)	$\frac{68}{3,200}$ (2.12 per cent.)	$\chi^2 = 0.2949$; d.f. 1; $P > 0.98$
Controls	$\frac{7}{1,368}$ (0.51 per cent.)	$\frac{8}{1,734}$ (0.46 per cent.)	$\chi^2 = 0.0035$; d.f. 1; $P > 0.99$
Age (yrs)	50 or over	Under 50	
Patients	$\frac{72}{3,628}$ (1.98 per cent.)	$\frac{28}{1,300}$ (2.15 per cent.)	$\chi^2 = 0.0659$; d.f. 1; $P > 0.99$
Controls	$\frac{11}{1,902}$ (0.58 per cent.)	$\frac{4}{1,200}$ (0.33 per cent.)	$\chi^2 = 0.4792$; d.f. 1; $P > 0.5$

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