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Action of chloroquine phosphate in rheumatoid arthritis

II. Chromosome damaging effect

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Viruses (Nichols, 1970) and mycoplasma (Fogh, Fogh, and Dowling, 1970) can damage chromosomes. Chromosomal abnormalities have been described in scleroderma (Emerit, Housset, Grouchy, and Camus, 1971) and in NZB mice with autoimmune disease (Halpern, Emerit, Housset, and Feingold, 1972). Since viruses and mycoplasma (Duthie, 1971) and autoimmunity (Glynn, 1972) have been postulated as possible aetiological factors in rheumatoid arthritis (RA), it was decided to study the peripheral blood lymphocytes from rheumatoid patients for possible chromosomal aberrations.

Materials and methods

(1) *Patients*
The study group consisted of patients with classical or definite RA (Ropes, Bennett, Cobb, Jacox, and Jessar, 1959) or degenerative joint disease (DJD). Patients who had any complicating disease, or who had been treated with corticosteroids, indomethacin, or immunosuppressive drugs, were excluded. Also excluded were patients who had taken or were taking phenylbutazone, as it has been reported that this drug can cause chromosomal damage (Stevenson, Bedford, Hill, and Hill, 1971).

All patients in the study were taking 3·2 to 5·2 g. soluble aspirin daily. Some of the patients with RA had also been taking 250 mg. chloroquine phosphate daily for up to 6 yrs and, in the subsequent analysis, this group was compared with the RA and DJD groups who were taking aspirin alone. The three patient groups were similar with respect to age, sex, severity and duration of disease, and exposure to diagnostic irradiation.

(2) *Lymphocyte cultures*
Cultures consisting of 0·4 ml. heparinized venous blood in 10 ml. tissue culture medium and phytohaemagglutinin (PHA; Burroughs Wellcome) at a dilution of 1:100 were set up in duplicate. Tissue culture medium contained 20 per cent. calf serum (Flow Laboratories) in Eagle's minimal essential medium (Burroughs Wellcome) with penicillin 100 i.u./ml., streptomycin 100 μg./ml., and heparin 5 i.u./ml. ('Pularin', Evans Medical Ltd.). Cultures were incubated in 5 per cent. CO_2: 95 per cent. air at 37°C. for 48 and 72 hrs.

(3) *Chromosomal preparation*
At the end of the incubation period, 0·2 ml. of 0·2 per cent. desacetylmethyl-colchicine ('Colcemid', Ciba) were added to each culture and incubation was continued for 2 hrs at 37°C. to allow accumulation of metaphase figures. The cultures were then processed to obtain lymphocyte nuclei suitable for chromosomal analysis (Hungerford, 1965). They were centrifuged at 1,100 G for 5 min. the cells were incubated at 37°C. for 5 min. in 0·075 M potassium chloride, and the resulting nuclei were washed five times in freshly prepared fixative (one part glacial acetic acid: three parts methanol) before being re-suspended in 0·3 ml. of fixative. Smears were prepared on glass slides, air-dried, and stained with Giemsa. For each patient, 100 metaphase plates were examined without previous knowledge of the group to which the patient belonged at a magnification of ×1,250, and chromosomal abnormalities scored according to the criteria of Buckton and Pike (1964).

(4) *Statistical analysis*
Previous studies in this field have pooled the information from all the metaphase plates obtained from one group of patients and compared this with similar pooled information from a second group. The resulting contingency tables have then been analysed using the χ^2 test of significance. We believe this approach to be statistically invalid for reasons expressed in the Statistical Appendix.

For any abnormality under study the approach in this paper has been to consider separately the proportion of abnormalities obtained from each individual, and to use this to compare the abnormalities in three groups of patients (DJD, RA + chloroquine phosphate, and RA) by means of non-parametric methods. In particular, Wilcoxon's Rank Sum Test and Fisher's Exact Test have been applied.

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Results

(1) *Kinetics of cell proliferation*
Lymphocytes from patients with RA who had been treated with chloroquine did not produce any metaphase figures at 48 hrs after stimulation with PHA. However, at 72 hrs the mitotic index of this group was similar to that of the other groups at 48 hrs (Panayi, Neill, Duthie, and McCormick, 1973). Hence results for chromosomal analysis are given for 72 hrs of culture for the chloroquine-treated patients and for 48 hrs for the others, so that all lymphocytes were examined during their first division in culture (Buckton and Pike, 1964).

(2) *Types of chromosome damage*
Several types of damaged chromosomes were seen: *B Cells* were those with chromatid gaps or breaks; *Cu cells* were cells with unstable chromosome abnormalities such as fragments and dicentrics; *Cs Cells* were cells with stable chromosome abnormalities.

(3) *Incidence of chromosome damage*
The results were analysed according to the diagnosis and therapy received by each patient and are shown in the Table. None of the groups differed significantly at the 5 per cent level from any other group with respect to B-cell abnormalities. However, both of the rheumatoid arthritis groups had a higher incidence of B-cell abnormalities than the patients with degenerative joint disease, these differences reaching significance at the 10 per cent level.

The chloroquine-phosphate treated group of rheumatoid patients showed a significantly larger number of Cu abnormalities than either of the other two groups ($P < 0.01$).

The other abnormalities occurred less commonly and, although the chloroquine-phosphate group showed more dicentric chromosome abnormalities, the numbers were insufficient to demonstrate a significant difference between the groups at the 5 per cent level.

Table *Results of chromosome analysis in three groups of patients*

Group description		*Number of patients with given number of B-cell abnormalities in 100 metaphase figures*																*Overall percentage of abnormal B-cells*
Disease	*Drugs*	0	1	2	3	4	5	6	7	8	9	10	11	14	18	23	*Total*	
(1) Rheumatoid arthritis	Aspirin Chloroquine	1	3	3	2	2	3	2	2	2	2	2	1	0	1	1	27	6·30
(2) Rheumatoid arthritis	Aspirin	0	1	1	1	2	2	2	1	1	0	0	1	1	0	0	13	5·85
(3) Degenerative joint disease	Aspirin	3	1	11	6	4	3	2	1	4	2	1	0	0	0	0	38	3·97

Comparisons of groups using Wilcoxon's rank sum test: 1 *v.* 2, not sig.: 1 *v.* 3, $0.05 < P < 0.1$; 2 *v.* 3, $0.05 < P < 0.1$

Group description		*Number of patients with given number of abnormalities in 100 metaphase figures*											
Disease	*Drugs*	*Cu (including dicentrics)* 0	1	2	3	4	*Percentage abnormal*	*Cs.* 0	1	*Percentage abnormal*	*Dicentrics* 0	1	2	*Percentage abnormal*
(1) Rheumatoid arthritis	Aspirin Chloroquine	7	14	3	3	0	1·07	24	3	0·11	22	4	1	0·22
(2) Rheumatoid arthritis	Aspirin	11	0	1	1	0	0·38	13	0	0·00	13	0	0	0·00
(3) Degenerative joint disease	Aspirin	27	7	3	0	1	0·45	33	5	0·13	37	1	0	0·03
Comparison of groups	1 *v.* 2	$P < 0.01$						Not sig.			Not sig.			
	1 *v.* 3	$P < 0.1$						Not sig.			$0.05 < P < 0.1$			
	2 *v.* 3	Not sig.						Not sig.			Not sig.			
Test applied		Wilcoxon						Fisher's exact test			Fisher's exact test			

Discussion

Lymphocytes from patients with RA and DJD were analysed for the presence of damaged chromosomes after stimulation with PHA. Lymphocytes from rheumatoid patients who were being treated with soluble aspirin had an increased incidence of B-cell abnormalities when compared with the lymphocytes of patients with DJD who were also treated with aspirin. If B-cell abnormalities were an *in vitro* phenomenon (Evans, 1970), this may mean that lymphocytes from rheumatoid patients are more susceptible to adverse cultural conditions and this may be an explanation for the reduced responsiveness to PHA stimulation shown by lymphocytes (Panayi and others, 1973). Lymphocytes from patients with RA during treatment with 250 mg. chloroquine phosphate in addition to aspirin showed a significant increase in Cu-cell abnormalities and took longer to produce metaphase figures.

Thus chloroquine can be added to the long list of drugs and chemicals causing damage to chromosomes (Shaw, 1970). The incidence of Cu-cell abnormalities in chloroquine-treated patients is similar to that of men employed at United Kingdom Atomic Energy Authority establishments who have received an average accumulated dose of 3·8 rads (Buckton, Dolphin, and McLean, 1967). The mechanism whereby chloroquine produced this damage is not clear. It has many biological and biochemical properties (Sams, 1967) and three of these properties may be pertinent to chromosome damage:

(1) It binds to deoxyribonucleic acid (Cohen and Yielding, 1965);

(2) It inhibits nucleic acid repair mechanisms (Gaudin, Yielding, Stabler, and Brown, 1971);

(3) It accumulates in the lysosomes of lymphocytes (Fedorko, 1967).

The demonstration of induced chromosomal damage is of importance since such damage may be associated with genetic change (Evans, 1970) or with neoplasia (Nowell and Hungerford, 1961). From both these points of view, the aberrations noted in Type B cells are of no significance since they are believed to result from damage during culture and do not reflect damage inflicted to chromosomes *in vivo* (Evans, 1970). Cu-cells contain unstable chromosomal abnormalities which will lead to death of those cells at a subsequent mitosis. Cs-cells contain stable chromosomal abnormalities and are, therefore, of great relevance when considering the possible genetic and/or neoplastic consequence of any drug. There is, however, no significant increase in the incidence of such abnormalities in patients treated with chloroquine.

From the differences which have been demonstrated it is concluded that, although chloroquine phosphate can produce damage to human chromosomes, the nature of this damage is such that it does not warrant discontinuing the use of this drug in the treatment of rheumatoid arthritis. Although the chromosomal damage produced by chloroquine does not appear to be of long-term significance, it should not be used in pregnancy, since it is selectively concentrated in the foetal uveal tract (Ullberg, Lindquist, and Sjöstrand, 1970) and can cause retinopathy in the new-born infant (Paufique and Magnard, 1969).

Summary

Phytohaemagglutinin-stimulated lymphocytes from patients with RA and DJD were examined for chromosome abnormalities. Patients with RA treated with 250 mg. chloroquine phosphate daily show a significantly increased incidence of Cu-cell abnormalities and a higher incidence of dicentric chromosomes. The nature of such abnormalities should not lead to abandonment of the drug, but its use in pregnancy is contraindicated.

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STATISTICAL APPENDIX

As described under Methods, other studies have used a χ^2 test of significance for the comparison of chromosome abnormalities in two groups. This test is based on the assumption that every metaphase figure contributing to the Table is independent of all others. When the Tables are formed by the amalgamation of many results from each of a number of individuals this is not the case. The assumption would hold only if it were true that there was no variation between individuals in a treatment group with respect to chromosome abnormalities. This is highly unrealistic. Indeed, if the χ^2 approach were valid, there would be no need to consider more than one individual from each group and to produce from these individuals a large number of metaphase figures, sufficient for a 'powerful' test. The folly of such an experiment is obvious. When χ^2 is incorrectly applied to these experiments the levels of significance obtained will tend to be too optimistic (*i.e.* 'significant' results occur too frequently).

Indeed a χ^2 analysis of the B-cell abnormalities would have shown a significant difference between the degenerative joint disease group and the rheumatoid patients treated with chloroquine phosphate ($P < 0{\cdot}0005$) and between patients with degenerative joint disease and rheumatoid patients treated with aspirin ($P < 0{\cdot}01$). Using other methods, we found neither comparison yielded a significant difference at the 5 per cent level.

An alternative procedure to the χ^2 test is to apply

non-parametric methods, taking as the basic measurement the proportion of chromosome abnormalities found in individual patients. The Kolmogorov–Smirnov Test or the Wilcoxon Test are perhaps most suitable for many applications, with Fisher's Exact Test the method of choice when the total number of abnormalities is small. Parametric methods (*e.g. t*-test) have not been used because the assumption of Normality appears to be unsatisfied, even after the use of transformations.

Although these non-parametric tests of significance appear to be more appropriate than the χ^2 test, care must be taken in drawing inferences from the results obtained in this study. The treatment given to each patient in this study was that considered by his physician to be the most appropriate for the care of the patient. Hence, this study cannot have the power of a controlled clinical trial, and there is no method of ensuring that biases are absent from group comparisons. However, there is no indication of differences between the rheumatoid groups with respect to age, sex, or severity of disease.

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