Action of chloroquine phosphate in rheumatoid arthritis

I. Immunosuppressive effect

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Controlled clinical trials have shown that the administration of chloroquine to patients with rheumatoid arthritis (RA) improved clinical, functional, and laboratory indices (Freedman and Steinberg, 1960; Popert, Meijers, Sharp, and Bier, 1961); in particular, the titre of rheumatoid factor decreased. This report describes a study of the effects of prolonged chloroquine phosphate therapy on phytohaemagglutinin-induced lymphocyte stimulation.

Materials and methods

(1) Patients

Patients with classical or definite RA (Ropes, Bennett, Cobb, Jacox, and Jessar, 1959) or degenerative joint disease (DJD) were studied. They were in functional grades 1 or 2 (Duthie, Thompson, Weir, and Fletcher, 1959), had not been treated with corticosteroids, phenylbutazone, indomethacin, or immunosuppressive drugs, and had no other complicating disease. The patients were divided into three groups (Table I) matched for age and sex; those with RA had disease of similar duration and severity.

(2) Lymphocyte cultures

Heparinized venous blood (5.0 ml.) was centrifuged at 400 G. for 5 min. and the cells washed three times with sterile phosphate-buffered saline (PBS), pH 7.3. After the final wash the cell pellet was re-suspended to a volume of 5 ml. in PBS. Total leucocyte and differential counts were recorded.

(3) Estimation of thymidine-6-³H uptake

Triplicate cultures of 0.1 ml. washed blood cells in 2 ml. tissue culture medium were prepared. Tissue culture medium (TCM) contained 20 per cent. calf serum in Eagle's minimal essential medium (Burroughs Wellcome) with 100 i.u. penicillin, 10 0µg. streptomycin, and 5 i.u. heparin per ml. ('Pularin', Evans Medical Ltd.). Some cultures contained only TCM and others PHA (Burroughs Wellcome) at a dilution of 1:150. Cultures were incubated at 37°C. in 5 per cent. CO₂:95 per cent. air, and 18 hours before the end of the incubation period 0.5 µCi./ml. of thymidine-6-³H (Radiochemical Centre, Amersham; specific activity 5 Ci./mmol.) was added to each culture. Incubation was terminated at 72 hrs by chilling the cultures to 4°C. They were then washed twice with ice-cold PBS, once with 2 per cent. acetic acid, once with 5 per cent. trichloroacetic acid, and once with methanol. Cellular material was digested with 0.2 ml. hyamine hydroxide (Nuclear Enterprises Ltd., Edinburgh), taken up in 5 ml. of a toluene-based liquid scintillator (NE233, Nuclear Enterprises Ltd.) and incorporated label measured in a Nuclear Enterprises well-type scintillation counter. Correction for counting efficiency was made by an external standard method and results expressed as disintegrations per minute (dpm) per 10⁶ mononuclear cells.

(4) Estimation of mitotic index

Cultures consisting of 0.4 ml. washed blood cells in 10 m.l. TCM and, where appropriate, PHA at a dilution of 1:100 were set up in duplicate and incubated in 5 per cent. CO₂:95 per cent. air at 37°C. for 72 hrs before the addition of 0.2 ml. 0.2 per cent. desacetylmethylcolchicine ('Col-

Table I Clinical details of patients studied

Group	Diagnosis	Treatment	No. of patients		Age (yrs.) M. ± S.E.M.†	Duration of disease (yrs) M. ± S.E.M.	Years on chloroquine M. ± S.E.M.
			Male	Female			
A	RA	Aspirin*	4	8	58.0 ± 2.0	6.8 ± 2.2	—
B	RA	Aspirin + chloroquine†	5	9	51.0 ± 3.0	7.3 ± 1.5	3.6 ± 0.8
C	DJD	Aspirin	3	8	48.0 ± 3.5	8.1 ± 3.4	—

* Soluble aspirin: 3.23–5.2 g. daily.

† Chloroquine phosphate: 250 mg. daily.

‡ M. ± S.E.M. = mean and standard error of the mean.

cemid', Ciba Ltd.). Incubation was continued for a further 2 hrs to allow accumulation of metaphase figures. The cultures were then centrifuged at 1,100 G. for 5 min. and processed according to the method of Hungerford (1965) to obtain cell nuclei suitable for chromosomal analysis. Cultures were incubated at 37°C. for 5 min. in 0.075 M potassium chloride, and the resulting cell nuclei were washed five times in freshly prepared fixative (one part glacial acetic acid: three parts methanol). Cell nuclei were re-suspended in 0.3 ml. fixative. Smears were prepared on glass slides, air-dried, and stained with Giemsa. The number of metaphase figures per 1,000 nuclei was counted. The Mitotic Index was expressed as a percentage.

Lymphocyte responsiveness to PHA was estimated in both systems independently and without prior knowledge of the group to which the patient belonged.

Results were analysed for significance by the Student's *t*-test.

Results

(1) Thymidine incorporation

The results of stimulating lymphocytes with PHA are given in Table II. Incorporation of thymidine-6-³H by lymphocytes in tissue culture medium alone was greater in Group C than in Groups A and B. The differences were statistically significant (Group C *v.* Group A, *P* < 0.05; Group C *v.* Group B, *P* < 0.05). The resting level of incorporation of thymidine by Groups A and B was not significantly different. After stimulation with PHA Group B showed significantly less response when compared with Group A (*P* < 0.001) and with Group C (*P* < 0.001).

Table II Responses of peripheral blood lymphocytes to stimulation by phytohaemagglutinin

Group	No. of patients	Thymidine-6- ³ H Incorporation (d.p.m. per 10 ⁶ mononuclear cells)	
		Lymphocytes cultured with TCM	TCM + PHA
A	12	2,947 (3,477 – 2,497)	276,648 (310,395 – 246,570)
B	14	2,940 (3,510 – 2,462)	108,456 (130,340 – 90,246)
C	11	6,204 (7,988 – 4,818)	370,905 (469,348 – 293,109)

Results expressed as geometric mean $\pm$ standard error of the mean.

(2) Mitotic index

When PHA responsiveness was measured by the mitotic index (Table III), Group B had significantly fewer lymphocytes arrested in metaphase as com-

Table III Mitotic index of lymphocytes stimulated with phytohaemagglutinin

Group	No. of patients	Mitotic index
A	12	2.3 $\pm$ 0.8
B	14	0.9 $\pm$ 0.3
C	11	2.7 $\pm$ 0.4

pared with Group A (*P* < 0.025) and with Group C (*P* < 0.025). There was no significant difference in the mitotic index between Groups A and C.

(3) Time course of PHA stimulation

Lymphocytes were harvested at 48, 72, 96, and 120 hrs of culture after labelling for 18 hrs with thymidine-6-³H. The peak response in the chloroquine and soluble aspirin treated groups occurred at the same time, *i.e.* at 72 hrs (Figure). Furthermore, the reduced responsiveness to PHA of patients treated with chloroquine was consistent over all four harvesting periods.

FIGURE Consecutive harvesting of PHA stimulated lymphocytes.

●—● PHA response of lymphocytes from five patients with RA treated with soluble aspirin

×—× PHA response of lymphocytes from six patients treated with chloroquine.

Vertical bar represents standard error of the mean

(4) Total leucocyte and mononuclear cell counts

These were similar in the three groups of patients (Table IV, overleaf).

Discussion

Peripheral blood lymphocytes from patients with RA treated with chloroquine phosphate exhibit reduced responsiveness to phytohaemagglutinin when compared with peripheral blood lymphocytes of patients with RA and of patients with DJD treated with soluble aspirin. If responsiveness to PHA is an indicator of thymus-dependent cell-mediated immunity (Gotoff, 1958), these results indicate that chloroquine may have an immunosuppressive effect. This effect was not due to the production of leucopenia or lymphocytopenia by the drug or to alteration in the kinetics of lymphocyte proliferation after PHA stimulation. PHA unresponsiveness was unlikely to be due to debility of the patients, as they were all in

Table IV *Leucocyte and mononuclear cell counts of patients studied*

Group	No. of patients	Leucocytes ($\times 10^{-6}$ /ml. peripheral blood)	Mononuclear cells ($\times 10^{-6}$ /ml. peripheral blood)
A	12	5.0 ± 1.5	1.1 ± 0.4
B	14	5.6 ± 1.5	1.6 ± 0.5
C	11	4.8 ± 1.3	1.4 ± 0.7

Results expressed as mean $\pm$ standard error of the mean.

functional Grade 1 and 2 whereas non-specific impairment of cell-mediated immunity occurred in patients with RA who were in functional Grades 4 and 5 (Hayes, Ward, and Jennings, 1970).

The mechanism whereby chloroquine produces decreased responsiveness to PHA is not clear. The drug has many biological and biochemical effects (Sams, 1967) and it accumulates in lymphocytes (Fedorko, 1967). Chloroquine does not attach onto PHA receptors present on the surface of lymphocytes (Hurvitz and Hirschhorn, 1965). Hence, although PHA would bind onto the surface of lymphocytes as the first step in stimulation (Greaves and Bauminger, 1972), the subsequent metabolic events which lead to

lymphocyte transformation could be inhibited by chloroquine present inside the cell.

Summary

Lymphocytes of patients with RA treated with 250 mg. chloroquine phosphate daily for 1 to 10 years show reduced responsiveness to PHA when compared with lymphocytes from patients treated with aspirin. The results indicate that chloroquine may be exerting an immunosuppressive effect in RA.

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