Br. J. Pharmac. (1970), **38**, 608–615.

The effects of some drugs which induce agranulocytosis on the metabolism of separated human polymorphonuclear leucocytes and lymphocytes

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Summary

1. Lymphocytes and polymorphonuclear leucocytes were separated from normal human blood by the method of Rabinowitz (1964).

2. The oxygen uptake, lactic acid production and glucose uptake of suspensions of these cells *in vitro* were measured. The values obtained agree with published data using comparable techniques.

3. Amidopyrine, chloramphenicol, chlorpromazine, phenylbutazone and thiouracil, in high concentrations, depressed the oxygen uptake of polymorphs, but not that of lymphocytes. With amidopyrine, chlorpromazine and thiouracil significant inhibition was produced by concentrations equal to therapeutic plasma levels. High concentrations of the drugs, except chlorpromazine, stimulated the glucose uptake of lymphocytes but not that of polymorphs. Tetracycline and sulphisoxazole were without effect on either parameter of leucocyte metabolism.

4. Because of erythrocyte contamination the lactic acid production of lymphocytes could not be determined. Lactate production by polymorphs was not affected by any of the drugs studied.

5. The significance of these results is discussed. It is suggested that there is a relationship between ability to induce agranulocytosis and effect on leucocyte metabolism *in vitro*.

Introduction

Drug-induced blood dyscrasias occur infrequently and at present unpredictably. Agranulocytosis is the most common, constituting 40% of all reported cases (Huguley, 1964). Several general mechanisms by which a drug may cause such a disorder have been proposed (Huguley, Lea & Butts, 1966). However, the precise pathogenesis remains obscure and little is known of the predisposing factors. A heritable defect, significantly altering the effects or the metabolism of the drug in the sensitive patient, might be involved. However, before any such abnormality can be implicated it is necessary to study the actions of drugs associated with agranulocytosis on the metabolism of normal human leucocytes *in vitro*.

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Unlike aplastic anaemia, agranulocytosis involves only the myeloid cells. It is thus important to study the biochemical behaviour of the individual cell types. Techniques for separating polymorphonuclear leucocytes and lymphocytes from normal human blood depend on the ability of the former cells to adhere to siliconed glass surfaces. A method of obtaining lymphocytes was described by Johnson & Garvin (1959) and the factors affecting the adherence of the polymorphs to the glass wool examined by Garvin (1961). Utilizing the same principles, a method for obtaining relatively pure suspensions of both lymphocytes and granulocytes was examined quantitatively by Rabinowitz (1964). This technique was adopted for this work since it allowed a study of the effects of drugs on the individual morphologic types to be made.

Methods

Glassware

Acid-cleaned glassware was coated with M 441 (I.C.I.) silicone. The glass beads, of mean diameter 0·2 mm (Grade 11, Jencons Scientific Ltd.), were cleaned similarly, but for coating water-soluble Siliclad (Clay-Adams Inc., New York) was used.

Materials

All solutions were freshly made from Analar grade chemicals and glass-distilled water.

Phosphate-buffered Hank's solution containing per litre: 8·0 g NaCl, 0·4 g KCl, 0·14 g $CaCl_2 \cdot 6H_2O$, 0·2 g $MgSO_4 \cdot 7H_2O$, 1·78 g KH_2PO_4, 9·55 g Na_2HPO_4, 1·0 g glucose; gum guiacum resin (Brome and Schimmer, Ltd.); Triton-X-100 (Lennig Chemicals, Ltd.); Fermcozyme 653 AM (Hughes and Hughes (Enzymes) Ltd.); peroxidase, activity 60 u./mg (Hughes and Hughes (Enzymes) Ltd.); chlorpromazine hydrochloride (CPZ, May and Baker, Ltd.); amidopyrine (AP) and phenylbutazone (PB) from Geigy (UK) Ltd.; thiouracil (TU, Koch-Light Ltd.); chloramphenicol succinate (CAP, Parke-Davis and Co. Ltd.); tetracycline hydrochloride (TC, Lederle Laboratories); sulphisoxazole (SO, Roche). All drugs were dissolved at known concentration in the incubation medium (50% autologous plasma and 50% phosphate-buffered Hank's solution) immediately before use. The pH was adjusted to $7{\cdot}40 \pm 0{\cdot}02$ at 37° C.

Cell counts

Nucleated and red cell counts were performed by standard haemocytometer methods. Differential cell counts were made on air-dried smears stained with Leishman stain.

Preparation of cell suspensions

Blood, 400 ml, was freshly collected and mixed with 20 ml physiological saline containing 4,000 units of preservative-free heparin. Sedimentation of the erythrocytes was achieved by the addition of high molecular weight dextran (Pharmacia, mol. wt. 250,000). The leucocyte-rich plasma was removed and reduced in volume (to 45 ml) before application to the column of glass beads. The dimensions of the column were 2·5 cm diameter and 35 cm height. The polymorphonuclear leucocytes

and lymphocytes were separated by the method of Rabinowitz (1964) using the column wash-out solutions he describes. The collected cells were finally suspended in autologous plasma and the leucocyte count adjusted to $30–50 \times 10^6$ cells/ml.

Oxygen consumption

The oxygen consumption of separated leucocytes was measured using standard Warburg manometric techniques (Umbreit, Burris & Stauffer, 1957). Various incubation media, including Hank's solution, autologous plasma, medium 199 and phosphate-buffered Hank's solution, were tested. The medium in which the maximum rate of oxygen uptake was observed was 50% autologous plasma and 50% phosphate-buffered Hank's solution. This medium was used for all metabolic studies and the oxygen consumption was measured at 37° C for 2 h after allowing an equilibrium period of 15 min. The rate of oxygen uptake was constant after 30 min, but a higher rate was found in the 0–30 min period. For these experiments the rate of uptake was calculated over the 30–90 min period and expressed as

$$\Delta O_2 = \mu l\ O_2 \text{ consumed/h per } 10^{10} \text{ leucocytes.}$$

Lactic acid production

L-Lactate was estimated in deproteinized aliquots of the incubation medium by the method of Hohorst (1965) using materials supplied in kit form (Biochemica Test Combination, Boehringer Ltd.). The change in optical density at 340 nm due to the accumulation of NADH was measured on a Unicam SP 500 spectrophotometer. Lactic acid production was measured over a two hour incubation period at 37° C and expressed as

$$\Delta LA = \mu\text{mol lactic acid produced/hour per } 10^{10} \text{ leucocytes.}$$

Glucose uptake

The glucose content of the incubation medium was measured before and after incubation for 2 h at 37° C. An automated assay method based on the glucose oxidase-peroxidase system (Hill & Kessler, 1961) with gum guiacum resin as the reducing agent (Hill, 1965) was used. The hydrogen peroxide resulting from the catalytic action of glucose oxidase on β-D-glucose was measured by the oxidation of gum guiacum in the presence of horseradish peroxidase. The blue colour of the oxidized dye was estimated spectrophotometrically at 625 nm. Preparation of the reagents in 1·5 M Tris-HCl buffer at pH 7·0 suppressed any carbohydrase activity in the enzyme preparation (Hill & Kessler, 1961 ; Johnson & Fusaro, 1965) and allowed specific estimation of glucose. Glucose standards of concentrations 5, 10, 15 and 20 μg/ml were prepared in glass-distilled water and kept at 37° C for 2 h to ensure mutarotation. Samples were deproteinized with equal volumes of 10% (w/v) trichloroacetic acid and appropriate dilution of the protein-free supernatant made with distilled water. Samples were run on the Autoanalyzer (Technicon Instruments, Ltd.) bracketed by standards run first in ascending and then in descending order of concentration. Glucose concentrations were calculated from the average of the two sets of standards. This procedure minimized any error due to deviations in the position of the standard curve. Provided the number of samples inserted between each set of standards did not exceed six, this method enabled statistical

procedures to be used for the calculation of glucose concentration. The error in estimating glucose standards did not exceed $\pm 0{\cdot}5\%$; in six determinations the mean percentage recovery ($\pm$ S.D.) of varying amounts of glucose added to human plasma was $99{\cdot}9\% \pm 0{\cdot}3\%$.

The initial glucose concentrations at which uptake was measured ($5 \pm 0{\cdot}5$ mM) were all within the range quoted by Munroe & Shipp (1965) and by Rauch, Loomis, Johnson & Favour (1961) as being optimal for the glucose uptake of peripheral leucocytes. Although the glucose uptake of both cell types was found to be linear over a period of 4 h, incubations were restricted to 2 h so that conditions for measuring the different parameters of leucocyte metabolism might be comparable. Uptake was expressed as

$$\Delta\text{Gluc.} = \mu\text{mol glucose/hour per } 10^{10} \text{ leucocytes.}$$

Estimation of drug effect

In any one experiment duplicate aliquots of the leucocyte suspensions were incubated either with the drug or in the medium alone. The results obtained for the various metabolic parameters were then compared and the drug effect was expressed as a percentage of the control value—for example, percentage inhibition of oxygen uptake. Each drug concentration was tested in two experiments.

Results

Cell separation

On average (forty-three determinations) 30% of the polymorphs and 36% of the lymphocytes were recovered from whole blood. Rabinowitz (1964) obtained recoveries of 22% and 32% respectively. The purity of our suspensions was not as high as that of Rabinowitz (97·7% lymphocytes and 99·99% polymorphs). The final lymphocyte suspensions had an average composition of 9 polymorphs : 91 lymphocytes : 890 erythrocytes, the corresponding figures for the polymorph suspensions being 93 : 7 : 24.

Oxygen consumption

The erythrocytes contaminating the two leucocyte suspensions were found to exert a negligible effect on the ΔO_2 values (McCurrach, 1968), and the following results were obtained (mean $\pm$ S.D., $n=43$)

For polymorphs, $\Delta O_2 = 1{,}488 \pm 472$

For lymphocytes, $\Delta O_2 = 2{,}726 \pm 524$

These values are somewhat lower than those of Rabinowitz (1964) and Hedeskov & Esmann (1966). The difference may reflect variations in the time period over which oxygen uptake was measured. Thus in our experiments if oxygen consumption is calculated over the 0–60 min period mean figures of 3,053 and 4,546 are obtained. These agree with those quoted by Rabinowitz (3,600 and 4,100 respectively). A " crowding effect " of cell number on ΔO_2 was evident for polymorphs but not for lymphocytes. But because in any one experiment the same number of cells was incubated with and without the drug this effect could not influence the results as expressed. The effects of seven drugs on leucocyte oxygen uptake were examined

and the results are shown in Fig. 1. In both cell types the change in ΔO_2 is statistically significant ($P<0.05$) only if it exceeded $\pm 10\%$. Amidopyrine, chlorpromazine, phenylbutazone, thiouracil and, to a much lesser extent, chloramphenicol inhibited polymorph oxygen consumption at concentrations which did not affect the oxygen uptake of lymphocytes. In fact, only 10 mM amidopyrine had any depressant effect on lymphocytes. Sulphisoxazole and tetracycline were without effect on either cell type.

Lactic acid production

Measurement of lymphocyte lactic acid production was not possible because of interference by erythrocytes. Attempts to correct for this by measuring the lactic acid production of pure suspensions of erythrocytes were unsuccessful. Erythrocyte contamination exerted a negligible effect on polymorph lactic acid production. In thirty-six determinations a mean value ($\pm$S.D.) of 918 ± 180 μmol lactic acid produced/hour per 10^{10} polymorphs was obtained. Hedeskov & Esmann (1966) quote a lower figure for ΔLA of 640. As with oxygen consumption a significant ($P<0.01$) crowding effect was observed; changes in ΔLA were significant ($P<0.05$) only if they were greater than $\pm 10\%$, and by this criterion none of the drugs studied affected polymorph lactic acid production, even at dose levels which produced a profound inhibition of oxygen uptake. This suggests that a marked Pasteur effect is not operating.

Glucose uptake

Erythrocytes in the numbers contaminating the leucocyte suspensions did not interfere with the estimations of leucocyte glucose uptake. Sixteen determinations of glucose uptake yielded the following results for ΔGluc. (mean $\pm$ S.D.):

For polymorphs, $331{\cdot}4 \pm 62{\cdot}4$

For lymphocytes, $64{\cdot}8 \pm 31{\cdot}9$

These results are in agreement with published values (Esmann, 1962; Hedeskov & Esmann, 1966; McKinney, Martin, Rundles & Green, 1952; Rauch *et al.*, 1961).

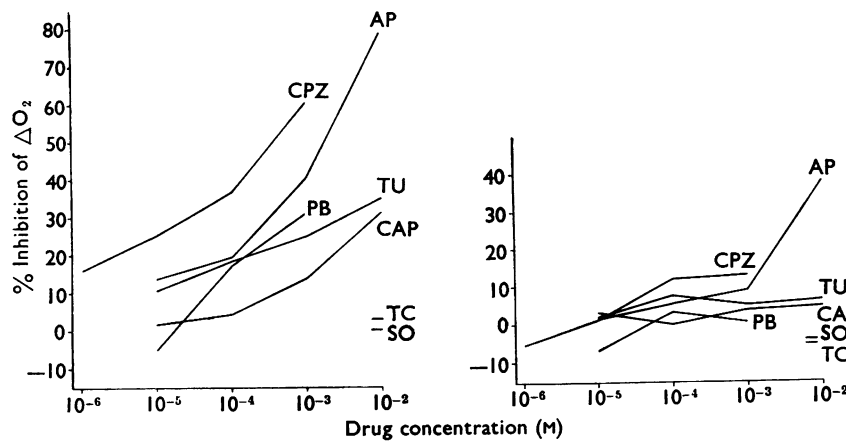

FIG. 1. % inhibition by amidopyrine (AP), chloramphenicol (CAP), chlorpromazine (CPZ), phenylbutazone (PB), sulphisoxazole (SO), tetracycline (TC) and thiouracil (TU) of the oxygen consumption of polymorph leucocytes (on the left) and of lymphocytes (on the right).

Any differences are probably due to variations in the separation techniques and in the incubation media, particularly glucose content, used. There was no evidence of a crowding effect of cell number on glucose uptake over the concentration range examined (40–60 million lymphocytes/ml, 20–30 million polymorphs/ml). Because of the low glucose uptake of lymphocytes the effect of a drug on ΔGluc. of these cells is significant ($P<0.05$) only if the difference is of the order of ±50%. However, for polymorphs a 10% change is significant.

The effects of drugs on the glucose uptake of polymorphs and of lymphocytes are shown in Fig. 2. The effects of chlorpromazine on both cell types are interesting. At low dose (0·01–0·1 mM) it stimulated glucose uptake, but at 1 mM a pronounced inhibition was noted. Chlorpromazine was the only drug which had a significant effect on the glucose uptake of polymorphs. Tetracycline and sulphisoxazole were without effect on either cell type and have not been included in the graphs. Amidopyrine, phenylbutazone and thiouracil, at concentrations between 0·1 and 10 mM, stimulated the glucose uptake of lymphocytes, but chloramphenicol, at 10 mM, had no significant effect.

Discussion

The experiments reported in this paper indicate that certain drugs known to cause agranulocytosis can affect the metabolism of normal mature leucocytes *in vitro*. Chloramphenicol, which has often been associated with aplastic anaemia, but rarely with agranulocytosis, had little effect. Tetracycline and sulphisoxazole, generally considered not to be myelotoxic, had no effect on leucocytes.

Although much is known about the individual drugs studied, their effects on leucocytes cannot be readily related to established pharmacological actions.

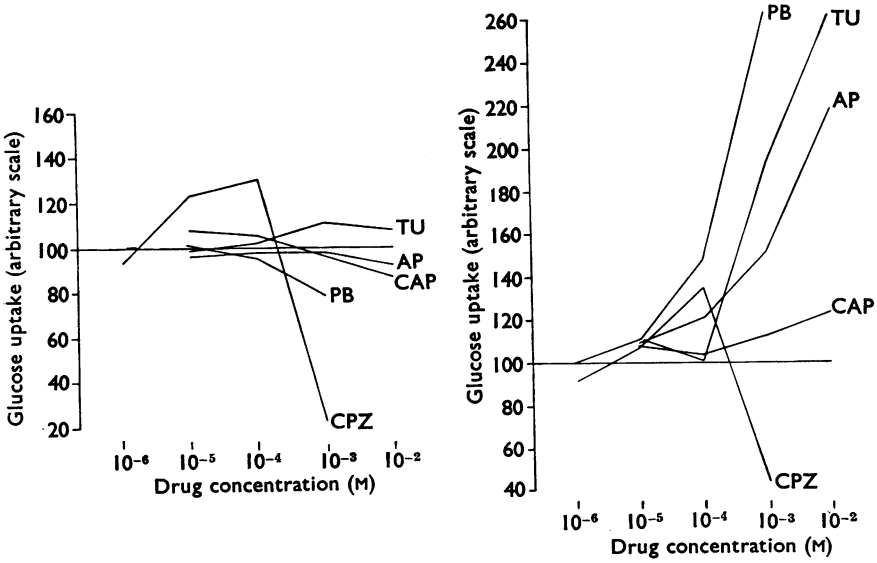

FIG. 2. Effects of amidopyrine (AP), chloramphenicol (CAP), chlorpromazine (CPZ), phenylbutazone (PB), and thiouracil (TU) on the glucose uptake of polymorph leucocytes (on the left) and of lymphocytes (on the right). △ Gluc. (no drug)=100.

Information about their effects on blood cells is, in most cases, scanty and not really relevant to the observations we have made.

Perhaps the most interesting feature of our results is the depressant effect of some of the drugs studied on the oxygen uptake of polymorphonuclear leucocytes. With amidopyrine, chlorpromazine and thiouracil a significant ($P<0.05$) inhibition was produced by concentrations equivalent to the therapeutic plasma levels of "free" drug (which generally lie between 1 μM and 0·1 mM). Although phenylbutazone also depressed polymorph oxygen consumption it had no significant effect at 1·2 μM, the plasma level reached under therapeutic conditions. One might expect then that amidopyrine, chlorpromazine and thiouracil could impair polymorph metabolism *in vivo* during therapy.

In contrast, none of the drugs studied had a marked effect on the oxygen uptake of lymphocytes (see Fig. 1). In fact only 10 mM amidopyrine had any depressant action. The differences between the two cell types are further illustrated in Fig. 2, where it can be seen that only chlorpromazine influenced the glucose uptake of polymorph leucocytes. In lymphocytes glucose uptake was stimulated by amidopyrine, phenylbutazone and thiouracil at concentrations higher than those which depress oxygen uptake by polymorph leucocytes. At therapeutic plasma levels the effects of these drugs on the glucose uptake of lymphocytes are not significant.

The fact that at concentrations attainable therapeutically only polymorph leucocytes are affected by drugs which induce agranulocytosis is of particular importance. It is these cells which are especially involved in agranulocytosis and this suggests that there may be some relationship between the ability to induce agranulocytosis and the ability to depress the oxygen uptake of polymorph leucocytes. Thus drugs which induce agranulocytosis but not aplastic anaemia, namely amidopyrine, chlorpromazine and thiouracil, inhibit oxygen uptake by polymorph leucocytes ; phenylbutazone, which induces both dyscrasias, exerts a lesser inhibitory effect while chloramphenicol, which causes aplastic anaemia, has no effect on the oxygen consumption. Although these findings suggest a common factor among drugs liable to induce agranulocytosis, caution must be exercised in interpreting the results. The respiratory process involves a multitude of enzyme systems and the drugs which depress oxygen uptake may be acting in quite different ways.

Although the effect of the drug on polymorph leucocytes *in vivo* may normally be insufficient to be manifest clinically, agranulocytosis might occur in one of two circumstances. First, the circulating leucocytes may be especially susceptible to the drug, thus imposing an excessive demand on bone marrow cell production. This would occur if the leucocytes were subjected to an abnormally high drug concentration ; or if the pathway on which the drug acts is vital only in sensitive subjects, perhaps because an alternative pathway fails. If the abnormality lies in the leucocyte it should be possible to demonstrate increased sensitivity *in vitro*. Secondly, the mature leucocytes may be normally sensitive to the drug to which the patient adversely reacts, but the demand upon granulopoiesis becomes excessive because of a deficiency in the bone marrow itself. Agranulocytosis may thus be the expression of inadequate granulopoiesis in response to a demand for more cells.

Previous workers have found it difficult to conceive of a common pathogenesis in this blood dyscrasia because the drugs involved differ so much in chemical structure, pharmacological action and therapeutic application. Even if the drugs

impair leucocyte function in quite different ways, however, their chief effect is to increase the demand on bone marrow cell production. The exact mechanism of action of drugs which can produce this blood dyscrasia in a small proportion of the population is still far from clear. It will only be elucidated when more information on factors controlling granulopoiesis and on the precise biochemical actions of the drugs concerned becomes available.

The authors thank Professor R. B. Fisher, Dr. S. H. Davies, Dr. R. A. Cumming and Dr. E. A. Robertson for invaluable assistance at various times throughout this study. This work was carried out in the Pharmacology Department of Edinburgh University during the tenure of Graduate Research Scholarships from the Faculty of Medicine (to P.H.McC. and J.K.P.) and with the financial assistance of the Distillers Company Ltd. The help and support of all these organizations are gratefully acknowledged.

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(*Received August* 18, 1969)