Inhibition of DNA synthesis in cultured human lymphocytes by phenylbutazone and oxyphenbutazone

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Inhibition of DNA synthesis in normal human bone marrow cells *in vitro* by short-term exposure to therapeutic concentrations of the analgesic antipyretic drugs phenylbutazone (PBZ) and oxyphenbutazone (OPB) has recently been reported (Dewse & Potter, 1975). This inhibition was variable, sometimes severe, and was moderated by the presence of serum, presumably by protein binding (Prescott, 1968). Depression of DNA synthesis in bone marrow cells *in vivo* would bring about reduced haematopoiesis and this variable response to the drugs could account for the different susceptibilities of patients to deleterious side-effects associated with treatment with these drugs, for instance aplastic anaemia (Bithell & Wintrobe, 1967; Committee on Safety of Medicines, 1973). A small reduction in mean cellular DNA content caused by 48 h continuous exposure to PBZ has been observed in cultured Ehrlich ascites tumour cells by Breull & Karzel (1970). These workers found such treatment also inhibited all phases of mitosis to a comparable degree, and Wissmüller (1971) confirmed inhibition of mitosis by the drug on cultured human lymphocytes, though he found different mitotic phases were affected by different drug concentrations. The effects of short-term exposure to PBZ and OPB on DNA synthesis in cultured human lymphocytes have been investigated to see whether the cytostatic action of therapeutic concentrations of the drugs can be mediated by depression of DNA synthesis, which would bring about a reduction in numbers of dividing cells, as well as by direct inhibition of mitosis.

White blood cells, separated from 10–15 ml fresh peripheral blood by dextran sedimentation, were washed with hypotonic ammonium chloride (3 parts 0·84% NH_4Cl plus 1 part Eagle's MEM) to lyse contaminant erythrocytes and were suspended in Eagle's MEM supplemented with 20% pooled AB serum. Purified phytohaemagglutinin (1 μg ml^{-1}) and pokeweed mitogen (0·05%) were added to transform the lymphocytes to the proliferating blastic form, and the cultures were incubated at 37° for 3 days, when DNA synthesis is proceeding at almost maximal rate (Salzman, Pellegrino & Franceschini, 1966). At harvesting, the cells were washed with MEM to remove residual serum from the culture medium. Replicate suspension of 10^5–10^6 cultured lymphocytes in 1 ml MEM containing 5 μCi tritiated thymidine (^{3}H-TdR; spec. act. 46 Ci mM^{-1}) were incubated at 37° for 1 h. Final concentrations of PBZ and OPB of 10, 40, 100 and 150 μg ml^{-1} (approximately 0·03, 0·15, 0·3 and 0·5 mM respectively) were used in the suspensions to represent the range of serum drug concentrations in patients undergoing long-term therapy (Walker, Price Evans & others, 1975). The effects of incubation of the lymphocyte/drug suspensions without and with serum (20%, from a single unmedicated person for uniformity) were compared because of the strong protein binding properties of the drugs (Burns, Rose & others, 1953; Prescott, 1968). The incorporation of ^{3}H-TdR was estimated by TCA precipitation of DNA on fibre glass discs and liquid scintillation counting as described previously (Dewse & Potter, 1975). The amount of ^{3}H-TdR uptake was represented as percentage of uptake in parallel cultures with no drug (controls), and all values following are expressed as percent (mean ± standard error of the mean) control ^{3}H-TdR uptake.

Experiments were performed in duplicate with cultured lymphocytes from 9 healthy subjects of either sex and different ages.

Increasing inhibition of ^{3}H-TdR uptake with increasing drug concentrations was found with PBZ without serum. The degree of this inhibition was variable, particularly at the lowest PBZ concentration used (10 μg ml^{-1}) where the 8 subjects tested showed inhibition of ^{3}H-TdR uptake ranging from 92 to only 19%, the mean value being 60·0 ± 5·66% control. At a PBZ concentration of 150 μg ml^{-1} all 5 samples tested showed less than 10% control ^{3}H-TdR incorporation (mean value 6·7 ± 1·2), whilst 2 of 3 samples tested only to 100 μg ml^{-1} showed over 90% inhibition and the final subject showed 74% inhibition at this concentration of PBZ. The response of the lymphocytes was not evidently governed by the sex or age of the subject. The presence of serum in each instance moderated the inhibition of ^{3}H-TdR uptake, to the extent that in only 1 case (AF, male) out of 8 was there any appreciable inhibitory effect (66% control uptake of thymidine with PBZ at 100 μg ml^{-1}).

The lymphocytes of 5 of the 7 subjects tested (there were too few cells for a complete assay in the other 2 cases) showed very severe inhibition of ^{3}H-TdR incorporation, even at the lowest concentration of OPB without serum (mean values 5·6 ± 0·83% control at 10 μg ml^{-1} and 3·14 ± 0·37% control at 150 μg ml^{-1} OPB). One subject showed lesser response at 10 μg ml^{-1} OPB (18% control ^{3}H-TdR uptake), but showed the severe inhibition at higher drug concentrations. The cells of the seventh subject (again AF) showed relatively less sensitivity to OPB without serum, ^{3}H-TdR incorporation being reduced to some 62% at the lowest drug concentration and to 6% at the highest concentration used, though of course this still represents severe depression of thymidine uptake. Again, in each instance, the presence of serum moderated the inhibition caused by the drugs. The lymphocytes of one of the 7 subjects

showed no inhibition, whilst those of 4 subjects showed a definite inhibition ranging from 26–42% (mean 35·25 ± 2·26% control) at the highest drug concentration used. The cells of subject AF were least sensitive to OBP without serum, but the presence of serum moderated the inhibition caused by OPB on his lymphocytes and on those of another subject (PC, female) to a comparable extent, so that incorporation of ^{3}H-TdR was reduced to 24 and 18% of control levels respectively with OPB at 150 μg ml^{-1}. Without serum the lymphocytes of AF were less sensitive than the others to OPB and were in the middle of the range of sensitivity to PBZ. However, the presence of 20% serum moderated the inhibitory action of both drugs on these cells less than in other cases. The lymphocytes of subject PC were among the group most sensitive to OPB without serum, but in this case also, 20% serum reduced the depression of ^{3}H-TdR uptake incompletely and there was still inhibition by some 82% with OPB at 150 μg ml^{-1}.

The effects of increasing the serum concentration from 20 to 40% were investigated in these cases where there was still appreciable depression of ^{3}H-TdR uptake in the presence of 20% serum. In these assays the lymphocytes of subject AF showed greater sensitivity to OPB than was shown previously but there was still severe inhibition of ^{3}H-TdR uptake when 20% serum was added, simulating better the *in vivo* condition. In each instance there was further moderation of the inhibitory action of the drugs with the higher serum concentration, but even so, the inhibitory effect of OPB was not completely removed with either subject's lymphocytes. However, the increased serum concentration did fully negate the inhibition caused by PBZ on AF's lymphocytes.

The possibility that the reductions in ^{3}H-TdR uptake shown above could be caused by lethal toxic effects of the drugs on the lymphocytes was eliminated by doing cell counts and trypan blue exclusion tests on parallel cultures. All these counts showed no statistically significant changes after treatment with the drugs, except that the series of cultures with OPB and with serum showed a statistically significant reduction in cell viability with increased concentrations of the drug ($t = 2{\cdot}86$; $0{\cdot}10 > P > 0{\cdot}05$), where viability declined by between mean values of 7 and 13% after incubation with the drug. However, this degree of cell loss alone could not account for reductions in ^{3}H-TdR uptake of the order described. The depression in ^{3}H-TdR incorporation illustrated above must therefore reflect a real inhibition of DNA synthesis in the lymphocytes caused by the drugs.

These results show that DNA synthesis in cultured normal human lymphocytes is variably inhibited by short-term exposure to PBZ and OPB. This inhibitory response is in all cases moderated by the presence of serum and generally, and in each individual instance, more severe depression of DNA synthesis is caused by OPB than by PBZ. Thus the inhibitory effects of the drugs on cultured human lymphocytes are similar to those observed previously on normal human bone marrow cells (Dewse & Potter, 1975).

Graphs of the complete data from these experiments showed a non-linear non-dose response in the lymphocytes in most cases with PBZ and serum, with thymidine incorporation values rather higher than control. The case of one subject, AF, has been dealt with above. Mean values for the other 7 of the 8 individuals tested were: 10 μg ml^{-1} PBZ-106·08 ± 2·29; 40 μg ml^{-1} PBZ-100·86 ± 2·32; 100 μg ml^{-1} PBZ-109·00 ± 2·33 and 150 μg ml^{-1} PBZ-107·75 ± 2·85% control. The following equation was used to assess the degree of variation in the assays shown on the graphs:

$$S^2 = \frac{\sum_{i=1}^{n} ({}^{x}1_i - {}^{x}2_i)^2}{2n}$$

where S^2 is the variance, ${}^{x}1_i$ and ${}^{x}2_i$ are the duplicate values for each individual point on the graphs, and n is the number of points on the graphs, and where there are n degrees of freedom. From these calculations, the variances of all the assays involving each drug with and without serum and of all controls with and without serum were grouped, and are tabulated below:

With serum			Without serum		
Controls	PBZ	OPB	Controls	PBZ	OPB
7·42	32·92	36·95	12·28	22·28	17·05

Thus the variability in the assay results when the drugs were incubated with serum is greater than when there was no serum present, and there was least variability in the series of controls.

The greater variability demonstrated in the results of assays when serum was present in the incubates can not alone explain the magnitude of the non-dose-related response of lymphocytes to PBZ in the presence of serum mentioned above especially when the response to OPB was generally dose-related. In other, unpublished assays analogous to those reported here, a variety of drugs including PBZ and aspirin (but not OPB) in the presence of serum appear to cause a mild increase in ^{3}H-TdR uptake, particularly at lower concentrations. These two factors, together with any inherent variation in the response to drugs *in vitro* in lymphocytes from the same source could account for the non-dose-related response of the lymphocytes to PBZ with serum.

If the variability in response of bone marrow and lymphocytes does reflect the differential susceptibility of subjects to the blood dyscrasias which are side effects of the drugs, the responses of the lymphocytes of subject AF to both drugs and of subject PC to OPB are the

most interesting of those illustrated here. In these instances, the lymphocytes are of intermediate or lesser sensitivity than others to the drugs without serum, but are considerably inhibited by the drugs with 20% serum and this inhibition is only partly reduced by doubling the serum concentration to 40%. Cell proliferation could therefore be severely depressed by exposure to these drugs over a period of time. Because the serum used in all these experiments was from the same source, no great variation in the binding of the drugs to the serum proteins in experiments using cells from different subjects is likely, so the difference in response would seem to be confirmed as a property of the lymphocytes themselves. It may be that the serum protein binding sites are saturated with only 20% serum and further binding occurs when the serum concentration is raised to 40%. This is strongly supported by the results of Burns & others (1953) showing that 98% of PBZ at concentrations of 50–150 $\mu g\ ml^{-1}$ bound to protein in whole plasma whereas at a concentration of 250 $\mu g\ ml^{-1}$ protein binding of the drug declined to 88%. These views are also supported by the experiments reported here using concentrations of serum increased from 20 to 40%, where there was further moderation of the inhibition but there still remained appreciable inhibition of DNA synthesis caused by OPB.

The small reduction in mean cellular DNA content of Ehrlich ascites cells was estimated by Breull & Karzel (1970) by the microchemical method of Ceriotti (1952) based on the DNA-indole reaction. This technique gives a mean cellular value which will be dependent on the proportions of cells in the different compartments of the cell cycle at the time of assay. G_1 (presynthetic) cells have half the DNA content of G_2 and M (postsynthetic and mitotic) cells and cells in S period (the DNA synthetic phase) have an intermediate value, so that an actively proliferating cell population will generally have a higher mean cellular DNA content than a static cell population. Inhibition of DNA synthesis such as is reported here would cause a reduction in S period cells and, consequently, in G_2 and M cells which would produce a lower mean cellular DNA content, and is, therefore, consistent with the report by Breull & Karzel (1960) of their treated cell populations.

This demonstration of drug-induced inhibition of ^{3}H-TdR uptake in already transformed lymphocytes may bear on the interpretation of results in experiments designed to show immunosuppression by drugs. This is often done by estimation of the effect of drugs on the rate of ^{3}H-TdR uptake in lymphocytes *in vitro*, using this parameter as an index of the degree of lymphocyte transformation in response to challenge with PHA or other antigens. For instance, immunosuppression by aspirin and related drugs (Opelz, Terasaki & Hirata, 1973), rifampicin (Nillson, 1971) and other antibiotics (Dam, Malkinson & Gewurz, 1975) have been investigated. Whereas blastogenesis is a prerequisite of DNA synthetic activity in normal lymphocytes, reduction in the rate of ^{3}H-TdR uptake may, at least in part, reflect direct pharmacological action of a drug on DNA synthesis, as with PBZ and OPB in the experiments reported here. Salzman & others (1966) showed no difference in the amount and quality of morphological transformation of human lymphocytes in response to PHA in untreated controls and when DNA synthesis was almost completely inhibited by 5-fluorodeoxyuridine. Thus reduced proliferation of lymphocytes in response to PHA or other antigens, which would produce suppression of overall immunological response may be pharmacologically superimposed on a system where lymphocyte transformation has already occurred.

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