Inhibition of DNA Synthesis by Guanidine Compounds in Uraemia

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Introduction

Uraemic patients are known to have impaired cellular immunity (Hume et al, 1955; Damin et al, 1957; Kirkpatrick et al, 1964; Huber et al, 1969) and lymphocytes from these patients may exhibit decreased in-vitro reactivity to mitogens (Kasakura and Lowenstein, 1967; Szewczk and Melcer, 1970; Nakhla and Goggin, 1973). Uraemic plasma has also an inhibitory influence on the transformation of lymphocytes in vitro (Silk, 1967). The mechanisms of these in-vitro phenomena are not known. Methylguanidine has been shown to cause uraemic manifestations when administered to dogs (Giovanetti et al, 1969), and it is known to accumulate with other guanidine compounds in the plasma and tissues of uraemic patients. However, the effects of guanidine hydrochloride (GH), methylguanidine (MG), guanidinosuccinic acid (GSA) and guanidinoacetic acid (GAA) on in-vitro lymphocyte reactivity have not been fully investigated. We therefore studied these compounds in relation to the degree of $^3$H-thymidine incorporation of phytohemagglutinin (PHA)-stimulated blood lymphocytes from healthy volunteers as an index of in-vitro DNA synthesis. The distribution of methylguanidine between plasma and lymphocytes was compared for normal and uraemic subjects. The transport of methylguanidine from a tissue-culture medium containing methylguanidine into lymphocytes was also investigated.

METHOD

Venous blood from normal subjects drawn into heparinised syringes was sedimented at room temperature. The lymphocyte content of the leucocyte-rich plasma (LRP) was determined by means of a total and differential white-cell count. A cell suspension was set up using LRP and bicarbonate-buffered TC 199 (Wellcome)
with 10% autologous plasma. Sufficient LRP was used to give $10^6$ lymphocytes/3 ml of the suspension. PHA-P (Difco) was added in a concentration of 0.001 ml of reconstituted material per millilitre of lymphocyte suspension. No antibiotics were used. The cultures were grown in triplicate in Nunc tissue culture tubes, each containing 3 ml cell suspension and $10^6$ lymphocytes. MG hydrochloride (Sigma) was reconstituted with distilled water and added to the cultures, the final concentrations of MG in each tube of a triplicate set varying from one to 25 mg/100 ml. Control cultures with PHA only were included in each experiment; all the cultures were incubated for 70 h at 37°C. Two hours before harvesting 3 μCi of $^3$H thymidine was added to each tube. At harvesting, the cells were washed and the DNA prepared by precipitation with 5% trichloroacetic acid in the cold. Radioactivity was measured by liquid scintillation counting using the method of Moorhead and McFarland (1966). The mean of triplicate culture counts was taken as the counts per minute (CPM) in each case. The test (T) to control (C) ratio x 100% was calculated by dividing the CPM of the test culture by that of the control. The resultant figure depends on the degree of $^3$H-thymidine uptake by the lymphocytes and represents the percentage of DNA synthesis in the test culture compared with the control. Similar studies were also carried out by adding GSA, GH and GAA (all from Sigma) separately to the lymphocyte cultures. The concentrations of each of these varied from one to 10 mg/100 ml. Trypan-blue exclusion tests were performed after the addition of each of the four test compounds separately to the lymphocyte suspensions and incubating for two hours.

Plasma concentration of MG was estimated from seven uraemic subjects (plasma creatinine 7.8 to 18.2 mg/100 ml) and from eight normal subjects. The method is based on separating MG through a cation-exchange resin column (Dowex-50 W 12% crosslinked, 50–100 mesh) and then using the Sakaguchi reaction. The method employed is similar to the one described by Menichini et al (1971).

Intracellular MG concentration of lymphocyte was also measured for the above subjects, after separating the lymphocytes on a Ficol-Triosil System (Varghese et al, to be published). In order to study the distribution ratio of MG between the tissue-culture medium and the intracellular fluid, a series of lymphocyte cultures from a lymphocyte cell line were set up in 3 ml of TC 199 medium containing two different concentrations of MG (150 and 300 μg/3 ml). Tissue-culture tubes containing 3 ml TC 199 medium and a $10^6$ viable lymphocyte were incubated for 0 hr, 6 hr, and 24 hr. At the end of the incubation period cells were harvested and washed four times in normal saline. The intracellular concentration of MG was estimated as before.

Plasma concentration of creatinine was measured by an autoanalyser method.

RESULTS

The pooled results of 21 experiments were analysed for DNA synthesis. The
percentage of DNA synthesis (mean ± 2 SEM) for various concentrations of the four guanidine compounds was plotted in Figure 1. MG and GH caused reductions of DNA synthesis which were dose dependent. The effect of MG appeared to be more marked than that of GH, but the difference was not statistically significant. At doses of up to 10 mg/100 ml, GSA and GAA had little inhibitory effect, and a dose-dependent relationship was absent.

![Graphs showing DNA synthesis vs. guanidine concentration](image)

Figure 1. Percentage of DNA synthesis of normal human lymphocyte cultures containing Mg, GH, GSA and GAA.

Differences existed between the effects of MG and GH on the one hand and GAA and GSA on the other which became statistically significant when the concentrations of these compounds rose above 5 mg/100 ml (p < 0.05). The possibility that MG was lymphocytotoxic was tested by adding it at a concentration of 10 mg/100 ml to the cultures at the time of addition of 3H thymidine and incubating for two hours. The uptake of thymidine was not significantly different from that of the controls. Trypan-blue exclusion tests also confirmed that none of the guanidine compounds were lymphocytotoxic at concentrations up to 10 mg/100 ml.
Figure 2. A scattergram showing plasma and lymphocyte methylguanidine concentration in uraemic and normal subjects.

Figure 3. Shows the intracellular concentration of methylguanidine after incubating one million lymphocytes with two different concentrations of methylguanidine.

The results obtained for both plasma and lymphocyte MG concentration in uraemic and normal subjects are shown in Figure 2. The mean plasma concentration of MG was significantly higher (p < 0.001) in uraemic subjects than in normal subjects. Lymphocyte MG concentration also showed a similar trend.
(p < 0.005). However, there was no significant difference between the mean ratio of plasma and lymphocyte MG concentration in uraemic and normal subjects. The concentration of MG in plasma was two to 10 times higher than that of lymphocytes. Because of the small number of patients studied (seven), no significant correlation was apparent between plasma creatinine and MG concentration. There was a significant correlation between plasma and lymphocyte MG concentration in uraemic subjects (r = 0.9; p < 0.01).

The in-vitro incubation studies with normal lymphocyte cell lines indicated an increase in intracellular concentration after a lag period of six hours (Figure 3). The distribution ratio of MG obtained between the tissue-culture medium and lymphocytes was related to the duration of incubation, but independent of the concentration of MG in the medium. The ratios obtained for 150 and 300 \(\mu\)g MG in the medium after equivalent periods of time (9.5 and 9.6) were within the range of values found in uraemic subjects.

**DISCUSSION**

Chronic intoxication of dogs with MG was found to cause a decrease in body weight, anaemia, increased serum triglycerides, neurological and gastrointestinal disturbances by Giovanetti et al (1973). MG and GH were found to inhibit oxygen consumption and several of the enzymes related to respiration (Lascelles and Taylor, 1966). In the present study, these compounds showed a dose-dependent inhibitory effect on DNA synthesis in vitro (Figure 1). GSA was found to inhibit platelet factor 3 (Stein et al, 1968) and to depress transketolase levels in-vitro (Lonergan et al, 1970), whilst GAA appears to be an innocuous compound. Neither of these substances was found to have a significant dose-related effect on DNA synthesis in our experiments.

The maximal plasma concentrations of MG, GSA, and GAA found in uraemic patients are in the region of 0.6 mg/100 ml (Giovanetti et al, 1973), 5.41 mg/100 ml (Stein et al, 1969) and 1.06 mg/100 ml (Cohen et al, 1968) respectively, whereas the highest whole blood level of guanidine in normal subjects is less than 0.4 mg/100 ml (van Pilsum et al, 1956). The values found for MG in the present study were similar to those of Giovanetti et al (1973).

All of these in-vitro concentrations, with the exception of GSA, are at least 10 times lower than the highest in-vitro concentrations used in this study. Additional experiments have shown that a concentration of GSA up to 50 mg/100 ml produces no inhibitory effect. Tissue concentrations of MG are higher than those found in plasma, up to 1.36 mg/100 g in skeletal muscle in uraemic dogs (Giovanetti et al, 1973). Our in-vitro data suggests that MG at this concentration would not have much effect on DNA synthesis.

Our studies indicate that intracellular concentration of MG in lymphocyte is related to the plasma concentration, and that the prolonged contact of guanidine
compounds and lymphocytes would result in an increased intracellular concentration affecting lymphocyte function. Further experiments have suggested that the combined effect of several guanidine compounds may be more marked in vitro than that of individual compounds.

This preliminary study suggests that high concentrations of some guanidine compounds play a part in altering the in-vitro reactivity of lymphocytes. However lymphocyte toxic concentrations of these substances are rarely attained in uraemia.

References

Cohen, B D, Stein, I M and Bonas, J E (1968) American Journal of Medicine, 45, 63
Giovannetti, S, Balestri, P L and Barsotti, G (1973) Archives of Internal Medicine, 131, 709
Huber, H, Pastner, D, Kittrich, P and Braunsteiner, H (1969) Clinical and Experimental Immunology, 5, 75
Kasakura, S and Lowenstein, L (1967) Transplantation, 5, 283
Kirkpatrick, C H, Wilson, W E C and Talmage, D W (1964) Journal of Experimental Medicine, 119, 727
Menichini, G C, Gonella, M, Barsotti, G and Giovannetti, S (1971) Separatum Experientia, 27, Birkhäuser Verlag, Basel, Page 1157
Nakhla, L S and Goggin, M J (1973) Immunology, 24, 229
Silk, M S (1967) Investigative Urology, 5, 195
Stein, I M, Cohen, B D and Horowitz, H I (1968) Clinical Research, 16, 397
Szewczyk, Z and Melcer, H (1970) Archivum Immunologiae et Therapiae Experimentalis, 18, 460

Open Discussion

D COHEN (Israel) Did you find an increase in the intracellular concentration of guanidine compounds apart from MG?

VARGHESE We studied guanidinosuccinic acid up to a concentration of 25 mg/ml and we didn’t find any significant effect. However, we haven’t estimated the inter-cellular distribution of that compound.