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Inhibition of human lymphocyte transformation by two aryloxyalkylamidines

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The incorporation of [^{3}H]thymidine into human lymphocytes stimulated by phytohaemagglutinin (PHA) was inhibited by anilino-*N*-2-m-chlorophenoxypropylacetamidine (501C) and xylamidine. These amidines antagonize 5-HT, but 5-HT did not alter [^{3}H]thymidine incorporation. 501C inhibited PHA-induced lymphocyte transformation as observed by [^{3}H]thymidine incorporation, [^{3}H]uridine incorporation, [^{3}H]leucine incorporation, DNA content, potassium content, and histological examination. 501C also inhibited increased [^{3}H]thymidine incorporation in human mixed lymphocyte cultures. The IC50 of 501C for inhibition of these processes lay between 4 and 8 μM. When added late in culture (after 6–8 h) 501C was less effective. Possible mechanisms by which 501C inhibits transformation are discussed.

When lymphocytes in culture are exposed to appropriate antigens, or to certain plant lectins, they are activated and transformed into lymphoblasts. The process is probably an essential stage in the development of cell mediated immunity (Elves 1972). RNA, DNA and protein are synthesized, morphological changes occur in cell nuclei and cytoplasmic volume is increased. Incorporation of [^{3}H]thymidine into the cell DNA is a convenient measure of the process. Incorporation is inhibited by various organic bases including colchicine (Wang et al 1975), noradrenaline (Hadden et al 1970), histamine (Plaut et al 1975), histamine analogues (Beets & Dale 1979), chloroquine (Hurvitz & Hirschhorn 1965), chlorpromazine (Ferguson et al 1975) and mepacrine (Trist & Weatherall 1981).

We have now found that certain amidines also inhibit transformation. The amidines tested, xylamidine (Copp et al 1967) and 501C (Mawson & Whittington 1970), are antagonists of 5-hydroxytryptamine (5-HT), but 5-HT itself seems to have little effect on lymphocyte transformation (Schwartz et al 1969).

The experiments reported here are directed to establishing whether xylamidine and 501C act in the same way as any of the other bases mentioned above.

MATERIALS AND METHODS

Preparation and culture of lymphocytes

Lymphocytes were cultured and incorporation of leucine, uridine and thymidine was measured as previously described (Trist & Weatherall 1981).

Measurement of mitosis

Aliquots (3 ml) of cell suspension were distributed into flat-sided culture tubes (Nunc). Drugs and PHA 0·5 ml of each, were added with a randomized allocation of treatments. The tubes were incubated at 37 °C in 5% CO_2 in humidified air for 0, 24, 48 or 72 h. Six hours before harvesting, colchicine (1 μM) was added to the tubes to synchronize the cells and to stop undifferentiated daughter cells being formed (Gunther et al 1974). Lymphocytes were harvested by centrifugation (75*g* for 8 min). The cell pellet was washed in trisodium citrate (0·34 M, pH 7·4) and fixed in absolute methanol–glacial acetic acid (3:1 v/v). The lymphocytes were mounted on glass microscope slides, stained in May-Grunwald's stain followed by Giemsa's stain (pH 6·8, Raymond A. Lamb) and examined with a Leitz Orthoplan microscope at a magnification of ×400. The various stages of lymphocyte transformation were identified and the percentage of normal lymphocytes, lymphoblasts not undergoing mitosis, and lymphoblasts in mitosis (mitotic figures) were recorded.

DNA content of lymphocytes

Aliquots (5 ml) of lymphocytes were cultured as above. The cells were harvested by centrifugation (660 *g* for 30 min) and the DNA content of the lymphocytes was measured by the method of Tedesco & Mellman (1966).

Potassium content of lymphocytes

The potassium content of the lymphocytes was estimated from the perchloric acid hydrolysate used to estimate the DNA content. After suitable dilution the potassium was measured by atomic absorption spectrometry (AAS, Techtron) at 766 nm using an air, natural gas flame.

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^{51}Cr release

Lymphocytes suspended at $3{\cdot}0 \times 10^6$ ml^{-1} were loaded with ^{51}Cr from medium containing $Na_2{}^{51}CrO_4$ (80 μCi ml^{-1}, The Radiochemical Centre, Amersham), at 37 °C for 30 min. Cells were harvested by centrifugation (180 *g* for 10 min), washed three times and resuspended at $1{\cdot}0 \times 10^6$ cells ml^{-1} in RPMI medium containing 10% human AB serum. Aliquots (1 ml) were incubated at 37 °C with PHA and drugs in flat-sided tubes for 24, 48 and 72 h. After this time the medium was removed and the cells lysed with 0·1 ml 2% Triton X-100 by incubation at 37 °C for 50 min. ^{51}Cr in 0·5 ml each of the medium and lysate was determined by liquid scintillation spectrometry.

Drugs

All drugs were made up in RPMI 1640 medium containing 10% human AB serum. Stock solutions (400 μM) of freshly prepared colchicine (Koch-Light Laboratories Ltd), (±)-mepacrine hydrochloride (K & K Laboratories Inc.), 5-hydroxytryptamine creatine phosphate (Sigma), xylamidine tosylate and 501C HCl (supplied by Dr H. F. Hodson, Chemical Research Laboratories, Wellcome) were serially diluted to the appropriate concentrations and added to the cell cultures. PHA (8 or 20 μg ml^{-1}) was added in RPMI 1640 containing AB serum.

RESULTS

Effect of 501C and xylamidine on [^3H]thymidine incorporation in PHA-stimulated lymphocytes

The addition of a suitable dose of PHA to the cell cultures increased the incorporation of [^{3}H]thymidine by one or two orders of magnitude. The effect depended on the concentration of mitogen added, and, as is well known, high concentrations of PHA (above 10 μg ml^{-1}) reduced the response. Therefore, PHA was used at 2 or 5 μg ml^{-1}, concentrations which gave near maximal incorporation. Xylamidine was inhibitory in concentrations above 3·2 μM with an IC50 of 10·6 ± 1·1 μM (s.e., n = 3). 501C acted similarly and was slightly more potent with an IC50 of 6·2 ± 0·5 μM (s.e., n = 21) (Fig. 1). Above the IC50 the dose-response curves dropped steeply to complete or nearly complete inhibition. They closely resembled those seen with mepacrine and chloroquine and were quite different from the effect of colchicine, prostaglandin E_1, and isoprenaline (see Fig. 1, Trist & Weatherall 1981). The inhibition by 501C was accompanied by suppression of the histological changes produced by PHA (Fig. 2). 501C did not alter the morphology of cells undergoing transformation but, at 24, 48 and 72 h after exposure to PHA, the proportion showing each phase of transformation was reduced. In contrast, the antimitotic drug colchicine at the same concentration inhibited fewer cells, but prevented the appearance of mitosis throughout. Mitotic figures were seen after 501C, indicating that the block to transformation might be transitory.

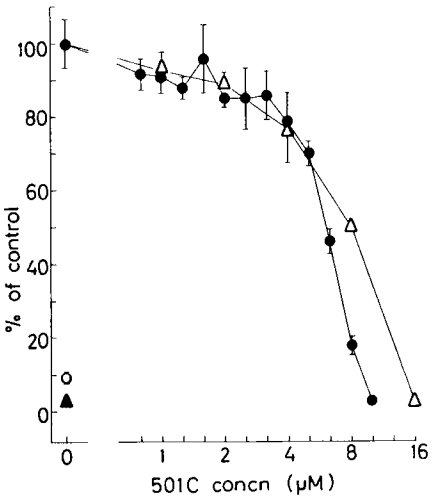

FIG. 1. [^{3}H]Thymidine incorporation induced in human lymphocytes by phytohaemagglutinin (PHA) and by mixing lymphocyte cultures from two donors (MLC): inhibition by 501C. Ordinate, % of control incorporation; abscissa, 501C concentration (μM). △, PHA 2 μg ml^{-1}; ●, MLC; ▲, ○ appropriate background incorporation. Error bars are 1 s.e. (n = 4), where no error bars s.e. < 1·5%. Control uptake (100%) = 10 700 counts min^{-1} per 10^5 cells for PHA and 7400 counts min^{-1} per 10^5 cells for MLC.

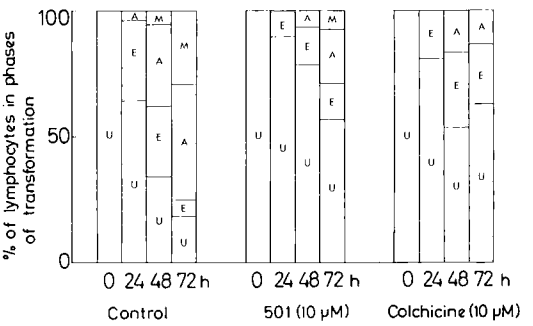

FIG. 2. Suppression of PHA-induced transformation by 501C and colchicine. Each block shows the proportion of human lymphocytes in phases of transformation at 0, 24, 48 and 72 h after stimulation with PHA (2 μg ml^{-1}). U = unchanged; E = Early changes (reduced heterochromatin and enlarged nucleoli); A = Advanced changes (nuclear changes as before with increased cytoplasmic volume); M = cells undergoing mitosis. Note that 501C reduces the number of cells at all stages of transformation, whereas colchicine mainly reduces the number of cells at advanced stages and completely reduces mitosis.

Effect of 501C on mixed lymphocyte cultures

The effects of natural antigens were examined in the mixed lymphocyte response, in which the immunoglobulins of donors stimulate transformation in each other's lymphocytes (Bach & Bach 1972). The incorporation of thymidine is optimal 5 days after the cells are mixed and is much smaller than in the PHA response, because the proportion of cells which respond to the stimulus is much less. 501C inhibited thymidine incorporation to the same extent that it inhibited PHA-stimulated incorporation (Fig. 1), with an IC50 from four experiments of 5·5 ± 0·5 μM (s.e.).

Time at which 501C acts

Stimulation of lymphocytes by PHA initiates a long sequence of events which progress even if PHA is removed or neutralized with antiserum (Kay 1969; Younkin 1972). If 501C blocked PHA receptors, it would be ineffective after combination had taken place and triggered the subsequent stages. This was not the case: 501C was as effective an inhibitor 6 h after PHA as at the same time, and still partially effective at 18–30 h (Fig. 3). This time course is quite different from the inhibitory action of corticosteroids, which become ineffective within 2–4 h (Caron 1967; Bach et al 1975). It resembles the effect of chloroquine (Gery & Eidinger 1977; Trist & Weatherall 1981), but not of mepacrine which persists for 30–40 h after PHA (Fig. 3).

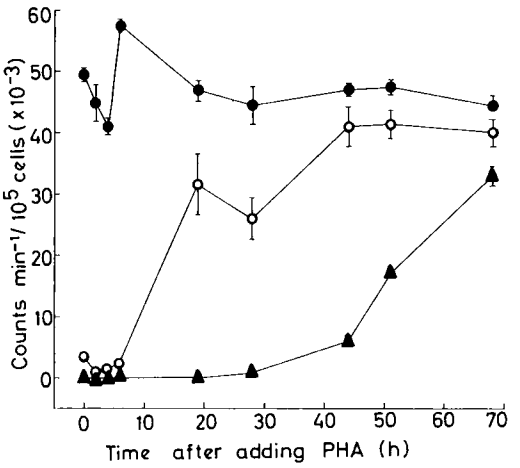

FIG. 3. [³H]Thymidine incorporation by human lymphocytes: inhibition by agents applied at various times after PHA (2 μg ml⁻¹). Ordinate, thymidine incorporation, counts min⁻¹ per 10⁵ cells; abscissa, time in hours at which compound was added after addition of PHA. Controls received addition of medium only at times shown. ●, control; ○, 501C (10 μM, 1·6 × IC50); ▲, mepacrine (3·2 μM, 1·4 × IC50). Error bars are 1 s.e. (n = 4). Where no error bars are shown s.e. <250 counts min⁻¹ per 10⁵ cells.

Other processes inhibited by 501C

RNA synthesis occurring in the first 24 h after PHA is measured by the [³H]uridine incorporation into acid-insoluble material (Kay & Korner 1966). 501C inhibited [³H]uridine incorporation with dose response curves closely similar to those for inhibition of incorporation of thymidine. There was no significant difference in IC50 for the two processes (Table 1), and high concentrations reduced the uridine incorporation to a level below that of cells not exposed to mitogen. However, the inhibition by high doses in each of four experiments was less complete (89–96%) than the inhibition of thymidine incorporation (95–99%).

Table 1. Inhibition by 501C of [³H]leucine, [³H]uridine and [³H]thymidine incorporation into human lymphocytes stimulated by PHA.

IC50 (μM), mean ± s.e. (n)		
[³H]leu	[³H]tdr	[³H]ur
7·3 ± 1·2 (3)	6·2 ± 0·5 (21)	5·1 ± 1·3 (4)

t-tests show differences between incorporations are insignificant ($P > 0·25$) for each comparison.

Synthesis of protein, measured by the incorporation of [³H]leucine was, like DNA synthesis, insignificant at 24 h. The significant incorporation at 72 h was inhibited by 501C with a dose-response curve similar to the curve for inhibition of thymidine incorporation (Table 1).

The lymphocyte DNA approximately doubled in 72 h after PHA. The increase was accompanied by an uptake of potassium of similar proportion (Fig. 4). Inhibition by 501C reduced or abolished both increments. Concentrations of 501C (8 μM or above) which reduced the [³H]thymidine incorporation below the level of unstimulated cells also caused the cell K to fall, over 72 h incubation, to 66·1 ± 5·1% (s.e., $n = 3$) of that of resting lymphocytes. The effect was observed in the first 24 h after PHA and thereafter the loss of K was trivial and statistically insignificant ($P > 0·2$) (Fig. 4). 501C (10 μM) had no significant effect on the total number of cells which deteriorated in culture sufficiently to release ^{51}Cr with which they had previously been labelled (Fig. 4).

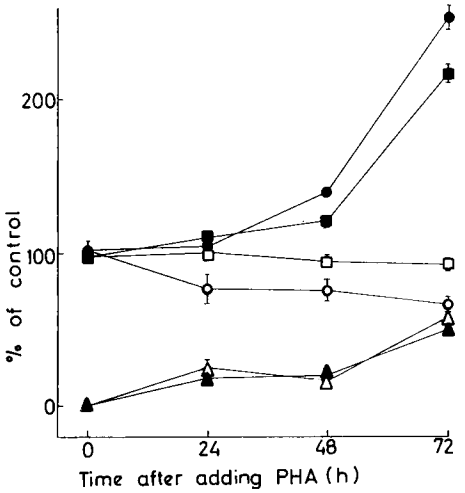

FIG. 4. Effect of 501C on cell potassium, cell DNA, and ^{51}Cr released by human lymphocytes stimulated with PHA (2 μg ml^{-1}). Ordinate, percentage of control; abscissa, time after PHA (h). 100% values: cell K = 35·2 ± 2·4 nmol per 10^6 cells; cell DNA = 4·9 ± 0·1 μg per 10^6 cells; ^{51}Cr release = 529·9 ± 270 counts min^{-1} per 10^6 cells. ●, cell K, no 501C; ○, cell K with 501C (8 μM); ■, cell DNA, no 501C; □, cell DNA with 501C (8 μM); ▲, ^{51}Cr released, no 501C, △, ^{51}Cr release with 501C (10 μM). Errors bars are 1 s.e. (n = 3)

Effect of 5-HT on [^3H]thymidine incorporation

Apart from a statistically insignificant reduction at the highest concentration tested (100 μM) ($P > 0·05$), 5-HT had no effect on thymidine incorporation (Table 2). This confirms the observation of Schwartz et al (1969).

Table 2. Effect of 5-HT on human lymphocytes stimulated by PHA (5 μg ml^{-1}).

5-HT (μM)	[^{3}H]tdr incorporation per 10^5 cells (ct min^{-1}) m ± s.e., n = 4
0	11 931 ± 545
0·01	12 945 ± 668
0·1	11 710 ± 580
1·0	12 918 ± 371
10·0	11 811 ± 1200
100·0	9 477 ± 723

Background incorporation in the absence of mitogen and 5-HT was 131 ± 20 ct min^{-1} per 10^5 cells. Analysis of variance shows that no difference between groups is significant at the 5% level.

DISCUSSION

These experiments showed that the amidines xylamidine and 501C inhibit lymphocyte transformation induced by mitogen or lymphocytes from different donors.

The mechanism by which they act is not established. It is not due to competition with receptors for the stimulant to transformation because 501C continues to inhibit with undiminished efficacy several hours after combination with receptors is complete. The process or processes inhibited affect the incorporation of radiotracers used to measure the synthesis of protein, RNA and DNA, and all three syntheses are equally sensitive. Measurements of DNA content and the number of mitotic figures confirmed that 501C was inhibiting transformation and not simply inhibiting the translocation of radioactive tracers into the cell.

The loss of inhibitory activity of 501C when added 6–8 h after PHA is also observed with chloroquine (Hurvitz & Hirschhorn 1965; Gery & Eidinger 1977; Trist & Weatherall 1981). It is within these first few hours of transformation that RNA synthesis is initiated (Darzynkiewicz et al 1965). It has been suggested that chloroquine acts by interfering with RNA synthesis (Hurvitz & Hirschhorn 1965). This concept was supported by the finding that chloroquine inhibits the incorporation of [^{3}H]uridine into human lymphocytes within the first 24 h (Trist & Weatherall 1981). 501C also inhibits [^{3}H]uridine incorporation in the early stages of lymphocyte activation and so is acting like chloroquine. Mechanisms by which chloroquine might be acting have already been discussed (Trist & Weatherall 1981).

Shortly after PHA is applied to lymphocyte cultures a change in membrane permeability to small ions, such as Ca^{2+} and K^+ is seen (Allwood et al 1971; Segel et al 1975). Increases in cell Ca^{2+} and cell K^+ concentrations can initiate cellular processes by activating key enzymes such as phosphorylase kinase (Cheung 1980) and pyruvate kinase (Kachmar & Boyer 1953). Agents which lower intracellular K concentrations such as ouabain (Quastel & Kaplan 1970) and valinomycin (Daniele & Holian 1976) inhibit lymphocyte transformation. It has been suggested that a number of bases such as chlorpromazine (Ferguson et al 1975, 1976), propranolol and alprenolol (Anderton et al 1981) inhibit lymphocyte transformation by stabilizing the cell membrane which would also reduce the permeability of the membrane to small ions. The reduction of cell K by high concentrations of 501C, without affecting ^{51}Cr retention, suggests that this amidine might also act on the cell membrane. This may be the mechanism by which it acts in reducing RNA and DNA synthesis.

The results of this study suggest that an aryloxyalkylamidine (501C) inhibits lymphocyte trans-

formation in a similar way to chloroquine, but not to mepacrine. Observations with high concentrations of drug suggest that effects on cell K^+ may be a mechanism by which inhibition is accomplished. The effects of chloroquine and other bases on cell K and K^+ transport in lymphocytes stimulated with PHA also require investigation.

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