Clin. exp. Immunol. (1972) **11,** 89–98.

ACTIVITIES OF IMMUNOSUPPRESSIVE AGENTS *IN VITRO*

II. DIFFERENT TIMING OF AZATHIOPRINE AND METHOTREXATE IN INHIBITION AND STIMULATION OF MIXED LYMPHOCYTE REACTION

MARIE-ANNE BACH AND J.-F. BACH

Clinique néphrologique, Hôpital Necker, Paris

(*Received* 10 *November* 1971)

SUMMARY

Azathioprine (AZ) and Methotrexate (MTX) inhibit thymidine incorporation in mixed lymphocyte reaction (MLR) at non-cytotoxic concentrations. AZ is active only when introduced in the culture during the first 24 hr, whereas MTX is still active when introduced at day 4. These data indicate that AZ might act on antigen recognition by T cells, as previously suggested by its effects on inhibition of rosette formation, and not on cell proliferation, as MTX probably does. Stimulation of thymidine incorporation has been observed at low drug concentrations, especially when the drugs were added more than 24 hr after the beginning of the culture, and most particularly for MTX added 6 days after start of the culture.

INTRODUCTION

Azathioprine (Imuran°) (AZ) and amethopterin (Methotrexate°) (MTX) are among the most widely used immunosuppressive drugs. Numerous studies have provided information on their biochemical impact. However, the mechanism of their immunosuppressive (i.s.) activity is still only partly documented. The fact that a single injection of antimetabolites of that class is most effective when made 2 days after antigen stimulation (Berenbaum, 1962) has suggested that they act by inhibiting cell proliferation, an hypothesis which is supported by their known effect on DNA synthesis. However, in a single injection experiment, drug activity is rapidly reversed due to degradation, whereas antigen catabolism is much slower. In studying this problem further, it was found that better insight could be gained from an *in vitro* system. The mixed lymphocyte reaction seemed particularly suitable since it represents a cell-meditated immune reaction correlated with the intensity of graft rejection (Bach *et al.*, 1970b). Moreover, it is a thymus-dependent (T) reaction (Takiguchi *et al.*, 1971) and thus interesting in terms of the recent demonstration of a selective action of AZ on T rosette-forming cells (RFC) (Bach & Dardenne, 1972b).

Correspondence: Dr M.-A. Bach, Clinique Néphrologique, Hôpital Necker 151, rue de Sèvres, Paris 15ème, France.

MATERIALS AND METHODS

Mixed lymphocyte reaction (MLR)

Lymphocytes are isolated from peripheral blood of normal subject with a Ficoll-Triosil gradient (Harris & Ukaejiofo, 1969). Two allogenic lymphocyte populations are mixed in Eagle's medium with 250 μg/ml streptomycin, 250 U/ml penicillin G, 12·5 U/ml heparin (Roche), and 20% human serum inactivated by heating (30 min, 56°C) taken from a pool of several sera. The lymphocyte concentration is 10^6/ml (5×10^5 for each population). Culture tubes are Nunclon 115 × 13 mm tubes and are filled with 1 ml of the cell suspension, or 2–4 ml in some experiments. Control tubes are studied for each experiment containing the same cell number as mixed cultures. Tubes are put at 37°C in CO_2 enriched (5%) atmosphere for 5 to 7 days. Four hours before the end of the culture, 1 μCi/ml tritiated thymidine (specific activity 1 Ci/mM) is introduced into each tube. Cellular DNA is precipitated and counting in liquid scintillation is performed as described by Bain (1970). Results expressed in dpm are given as the mean of two tubes.

Mitomycin treatment is used as described by Bach & Voynow (1966).

Chemicals

Azathioprine (Burroughs Wellcome) and methotrexate (Specia) are introduced into the mixed lymphocyte culture (MLC) at graded concentration ($\log_2$ ratio) at day 0 or at days 1, 2, 3, 4, 5 or 6 after the beginning of the culture.

Evaluation of cell viability

In several experiments the number of cells in the culture and their viability (trypan blue test) are evaluated. The trypan blue test is performed as follows: one drop of the cell suspension is added with three drops of trypan blue 2% solution diluted 4/5 in 4·5‰ NaCl immediately before the test. The percentage of blue-stained cells is evaluated after 2 min incubation.

Statistical tests

The Student's *t*-test or the χ^2 test are used in all experiments.

RESULTS

Azathioprine

A. *Inhibition of thymidine incorporation.* When introduced at the beginning of the culture, AZ inhibited thymidine incorporation as measured on day 5 (Fig. 1.) The concentration was 10 μg/ml in most cases (13/15), 1 μg/ml in one case, and 0·01 μg/ml in the last case. Minimal inhibiting concentration (m.i.c.), defined as the minimal drug concentration giving significant inhibition (*t*-test, $P<0{\cdot}01$) in comparison with controls done without AZ, was more variable from one experiment to the other. In 15 experiments, including thirteen different pairs of subjects, the m.i.c. was five times lower or equal to 0·01 μg/ml, it was 0·1 μg/ml in four cases, 1 μg/ml in one case, and 10 μg/ml in five cases. At the concentration of 10 μg/ml, the rate of thymidine incorporation was between 6 and 15% of controls done without AZ, except in four cases where this level was, respectively, 20%, 38%, 42%, and 45%.

No incorporation of thymidine over the background level was detected at the concentration of 100 μg/ml. At concentrations lower or equal to 10 μg/ml, the number and viability of the cells, as evaluated by the trypan blue test, was not different from that of controls without AZ (between 65 and 80% of the original number of cells in culture were still present and viable). Therefore, inhibition obtained at such concentrations was not due to cytotoxicity. Conversely, at concentrations of 100 μg/ml the percentage of dead cells was nearly 100% at day 5, already 90% after the 24th hour.

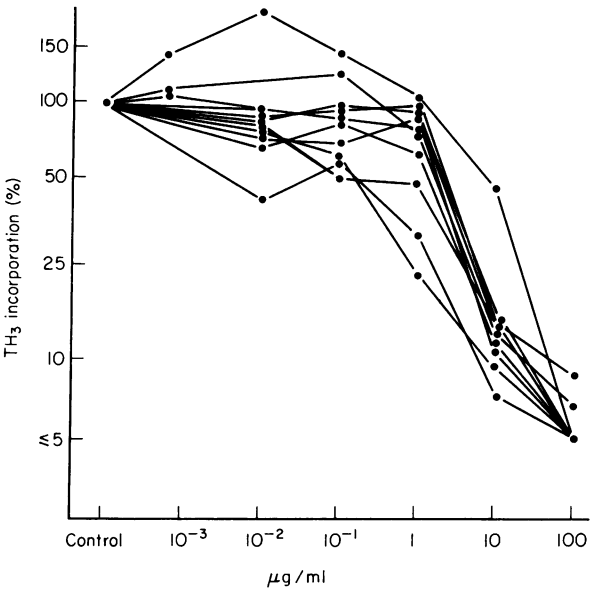

FIG. 1. Inhibition of MLR by AZ at graded concentrations (eleven cultures). Thymidine incorporation is given as percentage of controls, done without AZ.

B. *Reproducibility.* In two cases AZ was tried on the same pair of subjects at several months' interval. The concentration inhibiting 50% of the incorporation, which was 10 μg/ml at the first trial, was the same for the second test in the two pairs studied. Conversely, the m.i.c. was modified in the two cases (0·1 μg/ml instead of 10 μg/ml for the first pair, and 0·1 μg/ml instead of 0·01 μg/ml for the second one).

C. *Influence of the duration of culture.* In two cases AZ action was compared in MLC stopped at day 5 or 7. M.i.c. and concentrations inhibiting 50% of incorporation were identical whether the culture was stopped at day 5 or 7.

D. *Effect of delayed addition of AZ to the culture.* When AZ was added 24 or 48 hr after the beginning of the culture, the inhibiting effect was significantly decreased in comparison to what was observed when the drug was added at day 0. The m.i.c. increased in 4 out of 7 experiments when AZ was introduced at day 1, and became 100 μg/ml in 5 out of 7 experiments when AZ was added at day 2. In fact, in these five experiments no inhibition was observed when AZ was added at non-cytotoxic concentrations at day 2 (Fig. 2). In two experiments, AZ was added at day 0, 1 and 2 and the culture continued until day 7.

The concentration giving 50% inhibition of thymidine incorporation on day 7 was 10 μg/ml when AZ was added at day 0 and 1, and 100 μg/ml when it was added at day 2.

E. *Action of AZ in one-way MLR.* AZ was tried on MLR after treatment of one population with mitomycin. The m.i.c. was not significantly different from that found in the two-way reaction in three different experiments.

F. *Stimulation of thymidine incorporation.* In some experiments AZ stimulated thymidine incorporation when used at low concentrations. Such a stimulating effect was particularly clearcut when the product was added at day 1 or 2 of the culture (Fig. 3). In fact, the

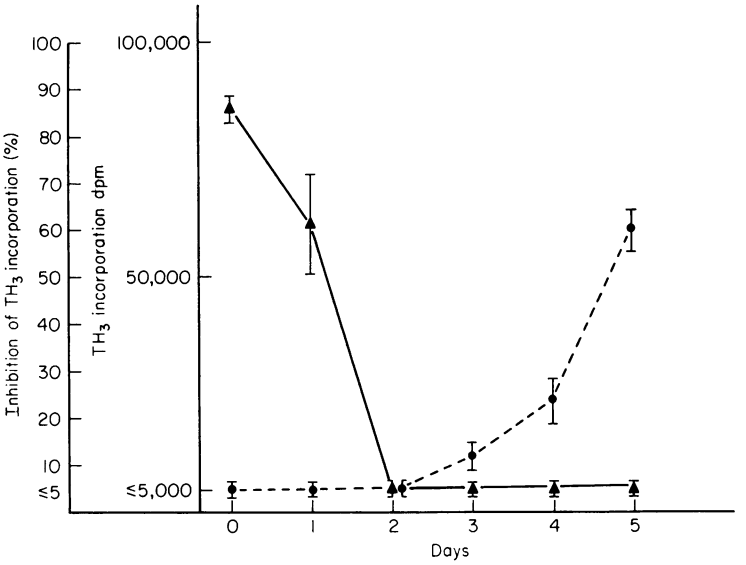

FIG. 2. Kinetics of MLR inhibition (—) by AZ and DNA synthesis (---) in MLR. Inhibition of thymidine incorporation has been evaluated at day 5 after adding 10 μg/ml AZ on different days after beginning of culture. It is given as percentage of incorporation at day 5 in control tubes, done without AZ (means ± S.E.).

stimulation was observed one out of seven times when AZ was added at the beginning of the culture, twice out of seven times when it was added at day 1, and four times out of seven when added at day 2. The concentrations giving the maximum stimulation varied between 0·001 and 0·1 μg/ml in various experiments.

Methotrexate

A. *Inhibition of thymidine incorporation.* MTX inhibited thymidine incorporation in MLR. In five out of six experiments, the MTX m.i.c. was 0·1 μg/ml (Fig. 4). In only one experiment it was 0·01 μg/ml. In all cases the MTX concentration giving inhibition of at least 50% thymidine incorporation was 0·1 μg/ml. The inhibition obtained at such a concentration was more important when the culture was continued until day 7 instead of 5 (the mean percentage of thymidine incorporation compared with controls done without MTX being respectively 16 and 48%). At the concentration of 0·1 μg/ml the cell viability, as evaluated by the trypan blue test, was not different in tubes with or without MTX.

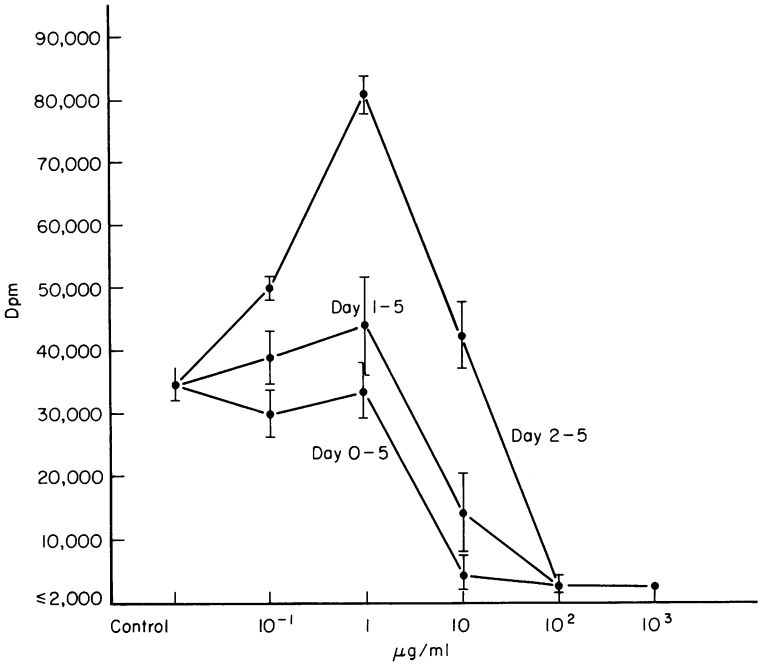

FIG. 3. Effects of AZ on thymidine incorporation (dpm) by lymphocytes in mixed cultures when added at the beginning of the culture (day 0–5), after 24 hr (day 1–5), or after 48 hr (day 2–5). Control represents MLR performed without AZ (means ± S.E.).

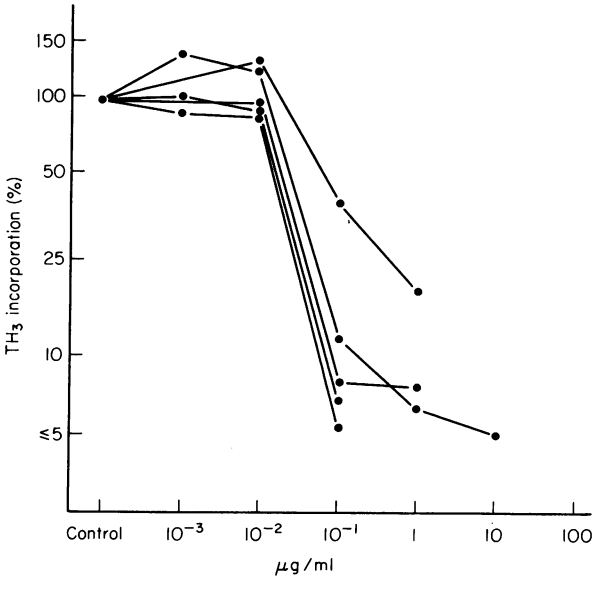

FIG. 4. Inhibition of MLR by MTX at graded concentrations (five cultures). Thymidine incorporation is given as percentage of controls, done without MTX.

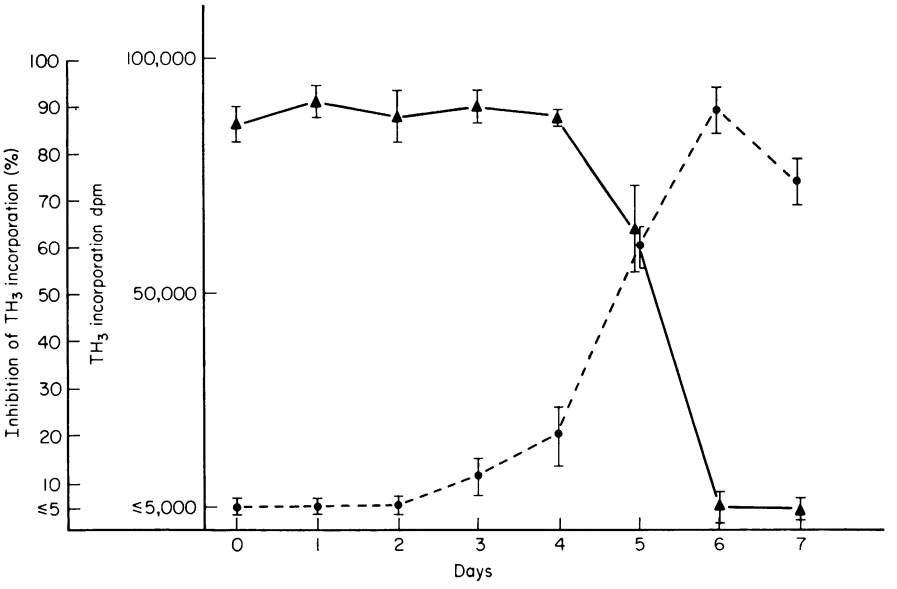

FIG. 5. Kinetics of MLR inhibition (—) by MTX and DNA synthesis (---) in MLR. Inhibition of thymidine incorporation has been evaluated at day 7 after adding 0·1 μg/ml MTX on different days after beginning of culture. It is given as percentage of incorporation at day 7 in control tubes done without MTX (means ± S.E.).

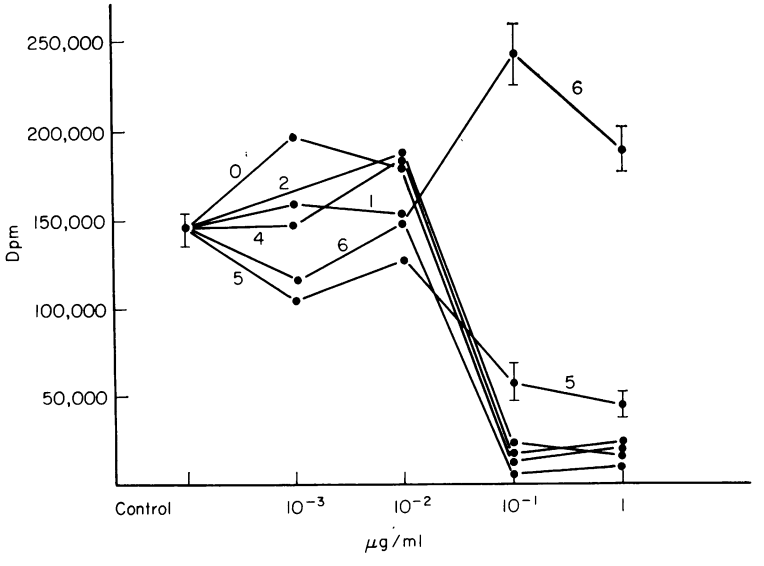

FIG. 6. Effects of MTX on thymidine incorporation (dpm) by lymphocytes in mixed cultures when added at the beginning of the culture (0) or after 1–6 days (0, 1, 2, 3, 4, 5, 6). Control represents MLR performed without MTX (means ± S.E.).

B. *Effect of delayed addition of MTX to the cultures.* Introduction of the drug at day 1, 2, 3 or 4 did not modify the inhibiting activity observed when the product was introduced at the beginning of the culture and stopped at day 7 (Fig. 5). The m.i.c. and the rate of incorporation for different concentrations was not different whether MTX was introduced at day 1 to 4 or at day 0 (16%). This rate of inhibition was only 48% when the drug was added at day 5 and no inhibition was observed at all when it was added at day 6, for concentrations between 0·1 μg/ml and 1 μg/ml (Fig. 6).

C. *Stimulation of thymidine incorporation.* Stimulation of MLR by MTX was observed under certain conditions. The stimulating effect ($P<0·01$) was obtained regularly when MTX was introduced at day 6, i.e. the day before stopping the culture at concentrations between 0·01 and 1 μg/ml. In one out of three experiments the stimulating effect was also noted when the drug was added at the beginning of the culture at concentrations of 0·01 or 0·001 μg/ml. In the two others, this effect was not observed after introducing MTX at day 5 or before. Finally, the stimulating effect is the more clearcut the later the drug is introduced into the culture.

DISCUSSION

The inhibition of MLR by AZ and MTX has already been reported (Vas & Lowenstein, 1965). AZ probably does not act in its original chemical form, since it is broken down by erythrocytes and white cells to its active form, as suggested by biochemical data (Elion, 1967) and demonstrated for rosette inhibition (Bach & Dardenne, 1970a and 1971). *In vitro* metabolism, which is not necessarily identical to *in vivo* metabolism, makes difficult the comparison of m.i.c. of AZ and MTX, particularly regarding duration of effect. However, for both products, m.i.c. as well as the concentrations used for the timing studies, are not cytotoxic since the trypan blue test does not show any difference with control tubes and cultures continued until day 7, when AZ is added at day 2 (10 μg/ml), give normal stimulation after 5 day exposure.

Our results on the timing of AZ in inhibiting the MLR *in vitro* are in contradiction with results reported by Berenbaum (1962) for *in vivo* timing of other thiopurines, showing that the optimal moment for a single i.s. injection is day 2 after antigen stimulation; Berenbaum interpreted this fact as being in favour of an antiproliferative action of these drugs. Our results show *in vitro* timing of AZ to be quite distinct from the *in vivo* timing. AZ inhibited the response if added at day 0 or 1 but not at day 2 or after. This is not due to the fact that an exposure of 5 days is highly inhibitory, whereas an exposure of 3 days is not, since addition of AZ at day 2 is not suppressive even when the culture is continued for 5 days until day 7. A purine-degrading system may develop in MLR cultures after two days *in vitro*, which would explain the absence of AZ activity after day 2, but the inhibition observed when AZ is added at day 0 still indicates that the drug is active during the first 24 hr. Finally, this hypothesis is the most likely interpretation of our findings. Such *in vitro* timing is well compatible with action on early stages of the MLR: macrophage processing, lymphocyte recruitment, and especially antigen recognition, as suggested by rosette studies (Bach & Dardenne, 1971). Another interpretation is that AZ is acting by impairing the turnover of cell surface antigen. There are no data supporting this hypothesis and rosette studies rather suggest that AZ is acting primarily on receptor availability. It may be reconciled with Berenbaum's data if one considers (1) that AZ has a rapid metabolism with

a serum half-life of four hours (Bach *et al.*, 1969; 1971) (2) that its action is reversible both *in vivo* and *in vitro*, as we have shown on RFC (Bach & Dardenne, 1971) and MLR (Bach, unpublished results); long-term *in vivo* treatments do not prevent antibody production if stopped the same day as antigen stimulation (Hersh *et al.*, 1966). If AZ acts in antigen recognition, it is possible that if given to a mouse at day 0 or before antigen stimulation, it will have lost its activity on day 2 because of rapid metabolism and reversibility of action, whereas the antigen is still at an immunogenic level and will induce an immune response. Conversely, given at day 2, AZ will be able to prevent antigen recognition at a stage when antigen recognition still occurs (as proven by immunosuppression performed by passive antibody injected 2 days after the antigen (Wigzell, 1969)), but immunogenic level is already significantly decreased and not sufficient to stimulate lymphocytes when the AZ action has disappeared. *In vitro* timing of action for AZ is more compatible with an action on antigen recognition than on proliferation since DNA synthesis cannot be detected on day 2 when the product is no longer suppressive, whereas *in vivo* timing data do not select action on antigen recognition or on proliferation. Conversely, for MTX, both *in vivo* and *in vitro* data suggest an action of proliferation. The fact that MTX does not suppress any more DNA synthesis when added at day 6, that is at its maximum rate, is probably due to an insufficient exposure time of the high rate proliferating cells to the drug.

The MLR may be considered as a thymus-dependent reaction since it is depressed by neonatal thymectomy in the mouse (Takiguchi *et al.*, 1971) and therefore, the suppressive action of AZ on MLR is not surprising in view of the known action of AZ on T cells: (1) AZ inhibits spontaneous theta-positive RFC (Bach & Dardenne, 1972b) (2) neonatal thymectomy suppresses the sensitivity of RFC to AZ (Bach & Dardenne, 1972b) and, conversely (3) thymosin, a cell-free extract, gives sensitivity to bone marrow B RFC after 90 min *in vitro* incubation (Bach *et al.*, 1971) (4) AZ-sensitive RFC are antigen-sensitive T cells since their depletion leads to immuno-incompetence in the spleen cells which is corrected by injection of thymus cells (Bach *et al.*, 1970c, 1972a,b). (5) AZ selectively suppresses homing of chromium-labelled lymph node T cells to lymph nodes (Fournier & Bach, in preparation). (6) Lastly, there seems to be a preferential action of AZ on cell-mediated immunity since AZ suppresses kidney-graft-rejection in man without altering the production of blocking factors (Quadracci *et al.*, 1971) or antibody production against influenza, tetanus toxoid (Denman *et al.*, 1970) or flagellin (Rowley *et al.*, 1969). It is realized that most data reported above have been obtained in mice and than extrapolation to man is hazardous. However, immunological features in immune deficiencies suggest that B-T cellular cooperation takes place in man (Good & Finstad, 1971).

None of these facts have been demonstrated for MTX which acts both on cell-mediated immunity and humoral responses, and is probably active both on B and T cells. The stimulation of thymidine incorporation observed for both products studied is not understood. It is known that folic acid antagonists interrupt the folic acid cycle and thus inhibit the conversion of DUMP into TMP, a step requiring N^5, N^{10}-Met-THFA. Therefore, at MTX doses that do not kill cells, the uptake of exogenous thymidine may be increased. Such an increase due to MTX has already been reported in lymphocytes stimulated by phytohaemagglutinin (Pegoraro & Benzio, 1971; Caron, 1969). AZ, through its active derivative 6-MP, inhibits NAD synthesis, and folic reductase activity requires NADPH2; therefore, increased thymidine uptake might again be observed at low AZ doses, especially

when the drug is added at day 2 when no suppressive effect is obtained. It is not known whether such stimulation may have an *in vivo* significance. This might be possible since it has also been found with AZ metabolites present in the serum of patients having been treated with AZ (Laborde & Bach, 1971). Inhibition of MLR by AZ can be applied to the study of the individual reactivity to AZ which is known to vary from one subject to another (Bach & Dardenne, 1970a). It will be particularly interesting to examine such inhibition in enzyme defiiency such as present Lesch-Nyan syndromes. Another application of the MLR inhibition is the detection of serum metabolites: we have shown that serum of AZ-treated subjects inhibited MLR at low concentrations (Laborde & Bach, 1971).

REFERENCES

BACH, F.H. & VOYNOW, N.K. (1966) One-way stimulation in mixed leukocyte cultures. *Science*, **153**, 545.

BACH, J.F., DARDENNE, M. & FOURNIER, C. (1969) *In vitro* evaluation of immunosuppressive drugs. *Nature* (*Lond.*), **222**, 998.

BACH, J.F. & DARDENNE, M. (1970a) Etudes sur le métabolisme de l'azathioprine. *C.R. Acad. Sci.* (*Paris*), **271**, 453.

BACH, J.F., DEBRAY-SACHS, M., CROSNIER, J., KREIS, H. & DORMONT, J. (1970b). Correlation between mixed lymphocyte culture performed before renal transplantation and kidney function. *Clin. exp. Immunol.* **6**, 821.

BACH, J.F., DARDENNE, M. & MULLER, J.Y. (1970c). *In vivo* specific antigen recognition by rosette-forming cells. *Nature* (*Lond.*), **227**, 1251.

BACH, J.F., DARDENNE, M., GOLDSTEIN, A., GUHA, A. & WHITE, A. (1971). Appearance of T cell markers in bone marrow rosette-forming cells after incubation with purified thymosin, a thymic hormone. *Proc. nat. Acad. Sci.* (*Wash.*), **68**, 27.

BACH, J.F. & DARDENNE, M. (1971). Activities of immunosuppressive agents *in vitro*. I. Rosette inhibition by Azathioprine. *Eur. J. Biol. clin. Res.* **16**, 770.

BACH, J.F. & DARDENNE, N. (1972a). Antigen recognition by T lymphocytes. I. Thymus and bone marrow dependence of spontaneous rosette-forming cells in the normal mouse. *Cell. Immunol.* (in press).

BACH, J.F. & DARDENNE, M. (1972b). Antigen recognition by T lymphocytes. II. Similar effects of Azathioprine, antilymphocyte serum, and anti-theta serum on rosette-forming lymphocytes in normal and neonatally thymectomized mice. *Cell. Immunol.* (in press).

BAIN, B. (1970) Tritiated thymidine uptake in mixed leucoyte cultures: effect of specific activity and exposure time. *Clin. exp. Immunol.* **6**, 255.

BERENBAUM, M.C. (1962) The effect of cytotoxic agents on the production of antibody to T.A.B. vaccine in the mouse. *Biochem. Pharmacol.* **11**, 29.

CARON, R.A. (1969). The effect of antimetabolites and corticosteriods on lymphocyte transformation *in vitro*. In *Proc. Third Annual Leucocyte culture Conference*. (Ed. by W. O. Rieke), p. 287. Appleton-Century-Crofts, 1969, New York.

DENMAN, E.J., DENMAN, A.M., GREENWOOD, B.M., GALL, D. & HEATH, R.B. (1970). Failure of cytotoxic drugs to suppress immune responses of patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **29**, 220.

ELION, G.B. (1967) Biochemistry and pharmacology of purine analogues. *Fed. Proc.* **26**, 898.

GOOD, R.A. & FINSTAD, J. (1971). Experimental and clinical model of immune deficiency and reconstitution of immunological capacity. In *Cell interactions and receptors antibodies in immune responses*: p. 27. Ed. by (O. Mäkela, A. Cross and T. U. Kosunen) Acad. Press, 1971, London.

HARRIS, R. & UKAEJIOFO, E.O. (1969). Rapid preparation of lymphocytes for tissue typing. *Lancet.* **ii**, 327.

HERSH, E.M., CARBONNE, P.P. & FREIREICH, E.J. (1966) Recovery of immune responsiveness after drug suppression in man. *J. Lab. clin. Med.* **67**, 568.

LABORDE, M.A. & BACH, J.F. (1971) Inhibition et stimulation par l'azathioprine de l'incorporation de thymidine par les lymphocytes en culture mixte. *C.R. Acad. Sci.* (*Paris*), **272**, 509.

PEGORARO, L. & BENZIO, C. (1971). Effect of methotrexate on DNA synthesis and thymidine kinase activity of human lymphocytes stimulated with phytohemagglutinin. *Experientia*, **27**, 33.

QUADRACCI, L.J., HELLSTROM, I.E., STRIKER, G.E., MARCHIORO, T.L. & HELLSTROM, K.E. (1970) Immune mechanisms in human recipients of renal allografts. *Cell. Immunol.*, **1,** 561.

ROWLEY, J.M., MACKAY, I.R. & MCKENZIE, I.F.C. (1969) Antibody production in immunosuppressed recipients of renal allografts. *Lancet*, **ii,** 708.

TAKIGUCHI, T., ADLER, W.H. & SMITH,, R.T. (1971) Cellular recognition in vitro by mouse lymphocytes. Effects of neonatal thymectomy and thymus graft restoration on allo-antigenic and PHA stimulation of whole and gradient-separated sub-populations of spleen cells. *J. exp. Med.* **132,** 63.

VAS, M. & LOWENSTEIN, L. (1965) The effect of prednisone and azathioprine in mixed leucocyte cultures. In: *Histocompatibility Testing*. p. 213. Williams and Wilkins, Baltimore.

WIGZELL, H. (1969) Use of humoral and cell-bound antibody to achieve depression of immunological reactivity. *Antibiot et Chemother (Basel)*, **15,** 82.