Immunology 1985 **55** 685

Some early events in the primary mitogenic stimulation of lymphocytes differ from later interleukin stimulation and other quiescence to growth activation systems

A. P. JOHNSTONE & D. DARLING *Department of Immunology, St George's Hospital Medical School, London*

Accepted for publication 8 March 1985

Summary. The requirement for activity of the enzyme ADP-ribosyl transferase (ADPRT) and changes in single-strand DNA breaks were assessed during the initial stimulation of quiescent murine splenic lymphocytes with mitogen alone, the stimulation of activated blasts with IL-2-containing medium and, for comparison, the serum stimulation of quiescent fibroblasts and the induction of haemoglobin synthesis in an erythromyeloid cell line K562. Inhibitors of ADPRT, at concentrations previously found to have no effect on the proliferation of lymphoblastoid cell lines, blocked the stimulation of spleen cells by Con A or LPS; non-inhibitory analogues had much less effect. No early increase in ADPRT activity after mitogenic stimulation was detectable. The rejoining of single-strand breaks was observed after stimulation of splenic lymphocytes with Con A, but not consistently with LPS. Conversely, ADPRT inhibitors had only little effect on the IL-2-induced stimulation of Con A blasts, and no effect on the stimulation of fibroblasts or K562. Neither were any changes in strand breaks associated with these systems. These findings implicate ADPRT activity and the rejoining of strand breaks in the early mitogenic response as being distinct from later IL-2 activation and changes from quiescence to growth in other cell types.

Abbreviations: ADPRT, ADP-ribosyl transferase (EC 2.4.2.30); Con A, concanavalin A; IL-2, interleukin-2; LPS, lipopolysaccharide; PHA, *Phaseolus vulgaris* phytohaemagglutinin.

Correspondence: Dr A. P. Johnstone, Dept. Immunology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

INTRODUCTION

The molecular basis of lymphocyte activation has been the subject of considerable study for a long time (reviewed by Ling & Kay, 1975). *In vitro* stimulation with various mitogens is the most common model used, and the transition from quiescent to activated state has been correlated with a host of biochemical changes, ranging from plasma membrane events during the first minutes to DNA synthesis and cell division after several days.

Some of the most recent reports have described the rejoining of single-strand DNA breaks, apparently associated with a requirement for activity of the enzyme ADP-ribosyl transferase (ADPRT), during the first few hours following stimulation of human peripheral blood lymphocytes with PHA and Con A (Johnstone & Williams 1982; Johnstone, 1984), and mouse spleen and thymic lymphocytes with Con A and LPS (Greer & Kaplan, 1983, 1985). Such changes in these and other cell types have led to the suggestion that they play a crucial role in a general mechanism for eukaryotic differentiation (Farzaneh, Shall & Zalin,

1980; Farzaneh *et al.*, 1982; Williams & Johnstone, 1983).

In order to investigate the significance of these observations to the various stimulatory signals now thought to be required for lymphocyte activation, the present study compared changes in DNA breaks and requirement for ADPRT activity during the initial stimulation of quiescent cells with mitogen alone and of activated blasts with IL-2-containing medium. Two other very different cell systems were also studied for comparison—the induction of fibroblast quiescence or growth by manipulation of serum concentration, and the induction of haemoglobin synthesis in an erythromyeloid cell line by haemin.

MATERIALS AND METHODS

Lymphocytes

BALB/c mouse spleens or thymuses were disrupted through a wire mesh, and lymphocytes were isolated by density centrifugation on a mixture containing 8·2% Ficoll and 9·4% sodium metrizoate (Nyegaard, Oslo, Norway).

For mitogen stimulation assessed by DNA synthesis, the cells (10^6/ml) were cultured in a flat-bottomed microtitre plate (200 μl/well) in RPMI-1640 medium containing 5% fetal calf serum 10^{-5} M 2-mercaptoethanol at 37° in an atmosphere of 5% CO_2 in the presence of either 2 μg/ml Con A (Sigma, Poole, Dorset) or 10 μg/ml LPS (*E. coli* 055:B5; Difco, West Molesey, Surrey) and varying concentrations of an inhibitor of ADPRT (3-methoxybenzamide, Aldrich, Gillingham, Dorset). After 48 hr, [^{3}H]thymidine (5 Ci/mmol; Amersham International, Amersham, Bucks; final concentration 2 μCi/ml) was added to the cultures, and 4 hr later the cells were collected, lysed and the radioactivity of the DNA precipitate determined as detailed by Johnstone (1984). For assay of DNA strand breaks by nucleoid analysis, the lymphocytes were cultured under identical conditions, except that 800 μl cultures in sloped 10-ml conical-bottomed tubes were used. After varying times, the cells were washed in PBS containing 25 mM methyl-α-D-mannopyranoside (Koch-Light, Haverhill, Suffolk) and the sedimentation rate of their nucleoids determined (Johnstone & Williams, 1982; Johnstone, 1984).

The response to IL-2 was assessed on blasts prepared from large-scale cultures (2×10^6/ml; 10–20 ml in culture flasks) of cells from two spleens stimulated with 3 μg/ml Con A for 2 days. The cells were washed three times by the addition of RPMI-1640 and centrifugation, and then resuspended at $2{\cdot}5\times10^5$/ml in RPMI-1640 containing 5% fetal calf serum, 12·5 mM methyl-α-D-mannopyranoside, varying amounts of 3-methoxybenzamide and 10–25% (v/v) conditioned medium containing IL-2. The IL-2 preparations were from various sources; they showed no detectable stimulation of resting cells and all gave similar results. Human conditioned medium (a gift from Robin Thorpe, National Institute for Biological Standards and Control, London) was obtained after stimulation of peripheral blood lymphocytes with phorbol myristate acetate for 16 hr and PHA for 6 hr, followed by washing and collection of the supernatant for the next 44 hr. Rat conditioned medium (a gift from Veronica Britten of this department) was obtained after stimulation of splenic lymphocytes with Con A for 2 hr, followed by washing and collection of the supernatant for the next 24 hr. The blasts plus IL-2 were cultured for 16 hr at 37°. For assay of DNA synthesis, the cultures in microtitre plates (100 μl/well) were pulsed with [^{3}H]thymidine for 2 hr, and then harvested as described above for mitogen stimulation; the addition of 1–3 μg/ml Con A to the blasts caused no increased incorporation. For assay of strand breaks, the cultures were in sloped 10-ml conical-bottomed tubes (2 ml/tube), and these were washed and analysed as described above for mitogen stimulation.

Fibroblasts

Swiss 3T3 cells (Flow, Irvine, Ayrshire) were harvested by trypsin/EDTA treatment, counted and plated at $2{\cdot}5\times10^4$/ml in Dulbecco's modified Eagle's medium containing 10% fetal calf serum into Costar 24-well clusters (1 ml/well) or 35 mm petri-dishes (4·5 ml/dish). 210C cells (Winterbourne & Mora, 1978) were obtained from Colin McInnes of this Medical School, and treated as above except the plating concentration was reduced to 10^4/ml. Inhibitors of ADPRT, 5 mM 3-aminobenzamide or 2·5 mM 3-methoxybenzamide were included in some cultures. The cultures were incubated at 37° in an atmosphere of 5% CO_2, and their growth was followed under an inverted microscope and assessed at various times by a protein dye-binding assay (developed by Dr D. Winterbourne, St George's Hospital Medical School). Briefly, the supernatant medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) and then fixed with 3% gluteraldehyde in PBS for 20 min. The fixative was removed and the cells stained with 0·04% Coomassie Brilliant Blue R in 25% (v/v) ethanol, 12% (v/v) acetic acid for 30 min with gentle

agitation. After destaining in three washes with 10% ethanol, 5% acetic acid, the bound dye was desorbed in 1 M potassium acetate in 70% (v/v) ethanol (twice the original culture volume) and its absorbance at 590 nm was determined. A cell concentration of 5×10^4/ml gave an absorbance of about 0·75 with a background (no cells) of 0·1.

In order to investigate the cessation and initiation of growth, the medium in some cultures was removed and replaced with medium containing no serum 16–24 hr after plating. When the cells had stopped growing (usually after about 2 days), the medium was removed and replaced with medium containing 10% serum and, in some cultures, inhibitors of ADPRT as above. The cultures were followed visually and by dye-binding as described above. They were also assessed for the presence of strand breaks at various times: cultures were removed by treatment with trypsin/EDTA, which was terminated after 2 min by dilution with PBS containing trypsin inhibitor, counted and then subjected to nucleoid sedimentation analysis (2×10^5 cells/tube) as described for lymphocytes, except that the speed was reduced to 13,000 r.p.m.

K562

This cell line was obtained from Robin Thorpe (National Institute for Biological Standards and Control, London) and grown in RPMI-1640 medium containing 10% fetal calf serum at an initial concentration of 10^5/ml in Costar culture flasks (10 ml/25 cm^2 flask). 3-methoxybenzamide was added to some flasks (final concentration 2·5 mM or 5 mM), and haemoglobin synthesis was induced by the addition of 20 μM haemin (Rutherford, Clegg & Weatherall, 1979; Dean *et al.*, 1983) to some cultures. Their growth was followed by counting in a haemocytometer each day, and the haemoglobin content of each flask was measured spectrophotometrically after detergent lysis (Rutherford *et al.*, 1979).

ADPRT assay

Human lymphocytes were obtained and cultured in the presence or absence of PHA (Johnstone, 1984). The ADPRT activity of the cells was assessed at various times using the buffers of Halldorsson, Gray & Shall (1978) with modification of the washing procedure. For each time point, 10^7 cells were washed twice with RPMI-1640 medium and permeabilized in 380 μl of hypotonic buffer at 0° for 30 min. As judged by inclusion of eosin, the permeabilization reaches a plateau between 20 and 30 min, with 80% of cells permeable. The permeabilized cells were diluted 10-fold by the addition of isotonic buffer and warmed to 25° for 2 min before adding, in triplicate, 300 μl to 50 μl of [^{3}H]NAD$^+$ (obtained from Dr W. J. D. Whish of Bath University; final specific activity 140 mCi/mmol, 11 μM). A further triplicate was set up containing, in addition, 0·05% Triton X-100 and 0·1 mg/ml freshly dissolved DNAase (this gives the maximal activity inducible by damaging DNA). After 10 min at 25°, 1 ml of 10% trichloracetic acid containing 1% nicotinamide, 2% tetrasodium pyrophosphate and 5 mM NAD was added and the tubes left on ice for 10 min. The precipitate was collected by filtration onto Whatman GF/C discs which had been soaked in the acid/NAD solution overnight at 4°. The discs were washed with ice-cold acid solution containing 1 mM NAD, and then with ice-cold ethanol:diethyl ether (1:1 v/v), dried and their radioactive content determined by scintillation counting (efficiency 15%).

RESULTS

When added at the same time as or slightly before mitogen, the competitive inhibitors of ADPRT inhibited the proliferative response of fresh mouse spleen lymphocytes to an optimal dose of both LPS and Con A in a dose-dependent manner (Fig. 1). The curves are very similar to those obtained previously for PHA and Con A stimulation of human peripheral blood lymphocytes (Johnstone, 1984), with 2·5 mM 3-methoxybenzamide giving about 90% inhibition and having no toxic effect demonstrable by exclusion of eosin. 3-methoxybenzoic acid, an analogue which is much less inhibitory for ADPRT (Purnell & Whish, 1980), had considerably less effect (only 30–40% inhibition at 2·5 mM; data not shown; this chemical exhibits some toxicity at 2·5 mM and above), indicating the specificity of the action of the amide.

By contrast with its effect on stimulation of quiescent lymphocytes by mitogen alone, 3-methoxybenzamide did not prevent the continued stimulation of activated blast cells by conditioned medium containing IL-2 (Fig. 1); 2·5 mM gave only 30% inhibition.

Changes in single-strand breaks in DNA were followed in cells during stimulation with mitogen or IL-2 using nucleoid sedimentation analysis. Nucleoids are released from cells by lysis in 0·5% Triton X-100, 2 M NaCl, 10 mM EDTA and 10 mM Tris pH 8·0 (Cook & Brazell, 1976a, b). They consist of supercoiled DNA from which most of the protein has dissociated; any

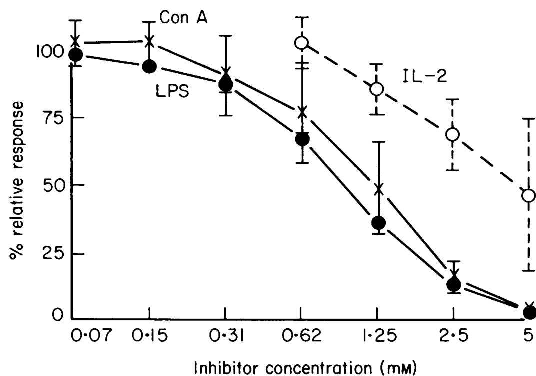

Figure 1. Dose effect of 3-methoxybenzamide. Splenic lymphocytes were incubated with the indicated concentration of 3-methoxybenzamide for 30 min before stimulation with either Con A (×——×) or LPS (●——●). The incorporation of [^{3}H]thymidine was measured after 48 hr, and is presented as a percentage of the response in the absence of inhibitor (routinely 40,000–70,000 c.p.m.); no correction for background (800–1400 c.p.m.) was made. Con A-induced blasts were further stimulated with IL-2-containing culture supernatants in the presence of the indicated concentration of 3-methoxybenzamide (○– – –○) and the proliferative response assessed after 16 hr. The incorporation is presented as a percentage of the response in the absence of inhibitor (6000–10,000 c.p.m.) after subtracting the value for unstimulated cultures (2000–2500 c.p.m.). The mean ± SD is presented of five, two, and four independent experiments, respectively.

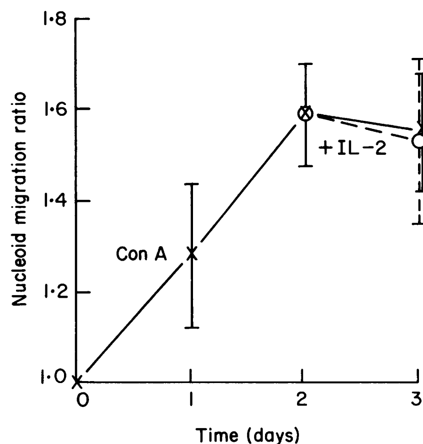

Figure 2. Nucleoid sedimentation changes after Con A and IL-2 stimulation. Splenic lymphocytes were incubated in the presence or absence of Con A for the indicated time and the sedimentation of their nucleoids measured. After 2 days, blasts were stimulated further with IL-2-containing culture supernatants (○– – –○). The incorporation of [^{3}H]thymidine into parallel cultures was monitored to check that stimulation had occurred. The nucleoid migration (mean ± SD of four separate experiments) is expressed as a ratio of that of unstimulated lymphocytes.

breaks in the DNA allow the supercoiled structure to relax and the nucleoid expands. Hence, the rate of sedimentation of nucleoids through a sucrose gradient is a very sensitive means for detecting DNA breaks (Cook & Brazell, 1976b; Durkacz, Irwin & Shall, 1981). Nucleoids sedimented faster after Con A stimulation (Fig. 2), as found for PHA and Con A stimulation of human peripheral blood lymphocytes (Johnstone, 1984). Conversely, the stimulation of 2-day Con A blast cells with conditioned medium containing IL-2 did not have any detectable effect on nucleoid sedimentation rate (Fig. 2).

In order to investigate whether the requirement for ADPRT during mitogen stimulation reflects a need for increased enzyme activity, ADPRT was assayed in lymphocytes at various times up to 54 hr after mitogen stimulation. Both the intrinsic activity being expressed and the maximal activity capable of activation by fragmented DNA were measured (Fig. 3). By 48 hr, a 1·5–2-fold increase in the maximal activity was observed. Occasionally, early transient increases were measured (e.g. 2 hr in Fig. 3a), but these could not be detected reproducibly (Fig. 3b–d).

In order to allow the lymphocyte data in this and earlier papers (Johnstone & Williams, 1982; Johnstone, 1984; Greer & Kaplan, 1983, 1985) to be viewed in the context of changes from quiescence to growth in eukaryotic cells in general, two fibroblast cell lines were also studied. These cells can be forced into quiescence by depriving them of serum, and then induced to grow again by its re-addition. 3-methoxybenzamide had no effect at 2·5 mM on the proliferation of fibroblasts in serum-containing medium (data not shown); this agrees with its lack of action on the proliferation of many other types of growing cells (Farzaneh *et al.*, 1982; Johnstone, 1984). Moreover, the inhibitors of ADPRT did not have any detectable effect on the serum-induced switch back to proliferation again of cells from either fibroblast line that had become quiescent following serum starvation (Fig. 4). The cells retained their normal spread morphology throughout these manipulations and their density was well below confluence.

The nucleoid sedimentation rate was used to analyse any changes in DNA breaks during these growth manipulations. No changes were detected, either upon

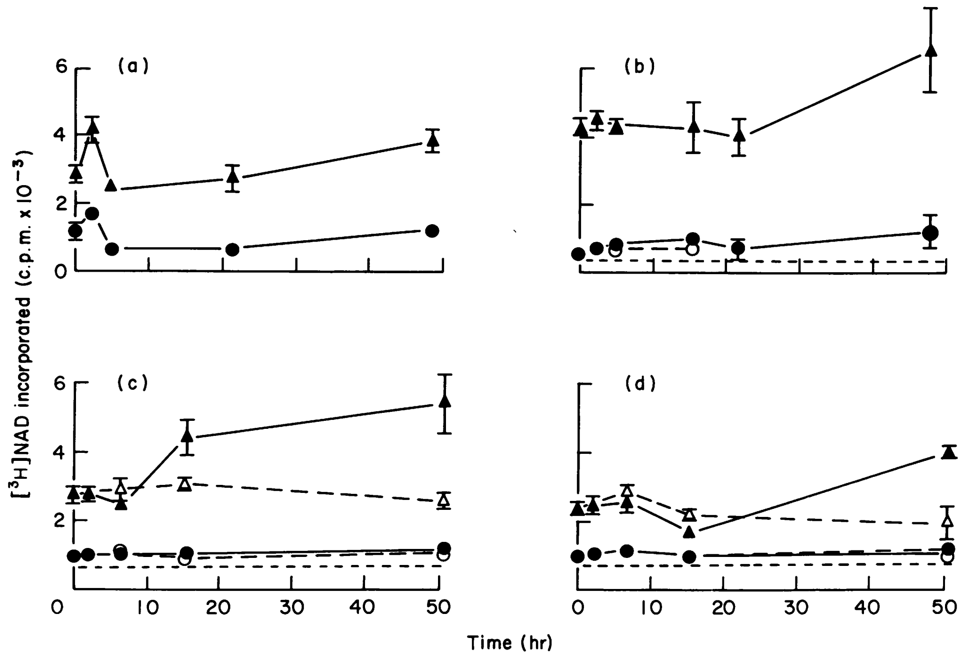

Figure 3. ADPRT activity of human lymphocytes following PHA stimulation. Lymphocytes were incubated either with (solid lines) or without (dashed lines) PHA for the indicated times before washing, permeabilization and assay. The results from four repeated experiments (a)–(d) are presented as incorporation of radioactivity (from [^{3}H]NAD) into acid-insoluble material (mean ± SEM of triplicate determinations)—1000 c.p.m. is equivalent to 17 pmole NAD$^+$ incorporated/10^6 cells (●, ○) intrinsic activity; (▲, △) maximal activity inducible by DNAase I). Background incorporation in the absence of cells is indicated by the horizontal dotted line.

cessation or restarting of growth (Table 1). Parallel cultures were monitored by the dye-binding method to ensure that the cells had indeed stopped and started growth as expected (e.g. the data from Experiment 4 in Table 1 were obtained from the cultures used in Fig. 4).

The K562 cell line originally isolated from a patient with chronic myeloid leukaemia can be induced to synthesize haemoglobin by the addition of haemin (Rutherford *et al.*, 1979). The effect of inhibitors of ADPRT on this process was also investigated for comparison with the lymphocyte and fibroblast data. Addition of haemin caused a four- to five-fold increase in haemoglobin accumulation after 3 days (9·1, 14·8 compared with 2·2, 2·9 μg/10^6 cells; results of two separate experiments). The presence of 2·5 mM 3-methoxybenzamide throughout the cultures had no effect on cell growth, viability or haemoglobin content (8·3, 13·5 μg/10^6 cells).

The possibility that the differing effect of ADPRT inhibitors on different cell types is caused by variations in permeability is unlikely because these chemicals inhibit DNA repair in human fibroblast lines (James & Lehmann, 1982), and prevent differentiation of chick embryo myoblasts (Farzaneh *et al.*, 1980, 1982) and some other cell types (reviewed by Williams & Johnstone, 1983).

DISCUSSION

The present study of mitogen-stimulated mouse lymphocytes confirms our earlier work on human T cells (Johnstone & Williams, 1982; Johnstone, 1984) and, in essence, agrees with the mouse data of Greer & Kaplan (1983, 1985). The blocking by 3-methoxybenzamide of the response to LPS as well as to Con A shows that B- as well as T-cell activation is prevented by inhibitors of ADPRT. This agrees with a recent report of 3-methoxybenzamide blocking an *in vivo* antibody (plaque-forming cell) response to a simple hapten in mice, but only if administered around the time of immunization (Broomhead & Hudson, 1985). Several groups have

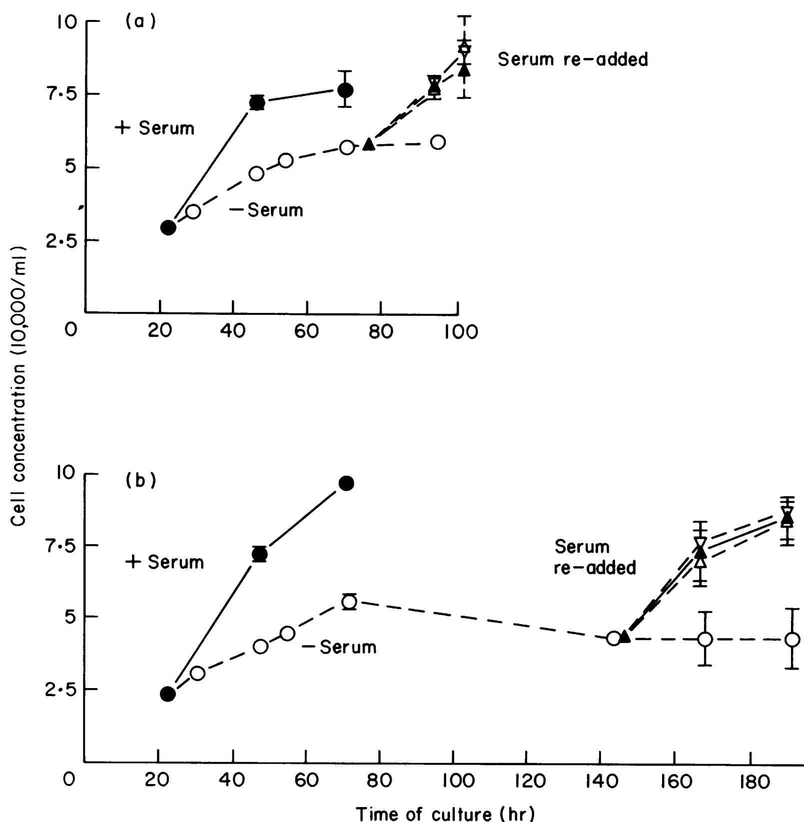

Figure 4. Effect of ADPRT inhibitors on fibroblast growth. At zero time, replicate cultures of (a) 3T3 or (b) 210C cells were plated in 10% serum, and after 24 hr the medium was changed to no serum (○- - -○) or left as 10% serum (●——●). The cell concentration was monitored at the indicated times and, when the serum-starved cultures had stopped growing, some were re-stimulated by changing to medium containing 10% serum in the absence of ADPRT inhibitors (▲——▲), in 2·5 mM 3-methoxybenzamide (▽- - - -▽), or in 5 mM 3-aminobenzamide (△- - - -△). The mean and range of duplicate assays is presented.

reported that lymphocyte ADPRT activity, both the maximal present and intrinsic value being expressed, increases three- to 10-fold over the period 24 hr to 72 hr following stimulation with PHA (Lehmann *et al.*, 1974; Berger *et al.*, 1978; Rochette-Egly *et al.*, 1980). We have confirmed this increase, but could detect no change before this (Fig. 3) during the period up to 16 hr when ADPRT inhibitors are effective (Johnstone & Williams, 1982; Johnstone, 1984). Resting lymphocytes have a very low intrinsic level of ADPRT, only 1·5–2 times background with no cells added (Fig. 3), and so fluctuations of up to 50% are not readily detectable by the assay in these cells. Thus, the enzyme can fulfil its function early in the activation process without any large change in the level of its activity. Greer & Kaplan (1985) report an increase of about 50% in ADPRT activity following Con A stimulation of mouse lymphocytes, peaking at 40 min and returning to resting levels at 1 hr. Whether these authors are referring to intrinsic or maximal activity is not stated, and their value for resting cells (440 pmoles NAD incorporated/10^6 cells) is quite different from those of the other workers (e.g. 10–30 pmoles/10^6 cells/30 min calculated from the data of Berger *et al.*, 1978; 5–20 pmoles/10^6 cells/10 min, Fig. 3; total NAD content is only about 70 pmole/10^6 cells, Berger *et al.*, 1982).

Unlike Con A on mouse or human cells and PHA on human cells (Fig. 2 and Johnstone, 1984), no reproducible change in the number of single-strand DNA breaks could be detected by nucleoid sedimentation analysis following LPS stimulation of mouse splenic lymphocytes (data not shown). Using a different

Table 1. Nucleoid sedimentation of quiescent and growing 3T3 fibroblasts*

Cultures analysed†		Experiment number 1	2	3	4
Day 1	Without serum	0·26–0·27	0·27–0·28	0·33–0·34	ND‡
	With serum	0·26	0·29–0·31	0·31–0·33	
Day 2	Without serum	0·30–0·32	0·30–0·32	0·27–0·28	0·29
	With serum	0·32	0·32–0·37	0·30–0·32	0·27
	Serum re-added	0·28–0·30	0·26–0·30	0·33–0·35	ND
Day 3	Without serum				0·26–0·27
	With serum	ND	ND	ND	0·27–0·38
	Serum re-added				0·32–0·40

* The distance sedimented is expressed as a fraction of total length of the gradient (range of duplicate determinations for each independent experiment).

† The medium in replicate cultures was changed at Day 0 to zero or 10% serum (1 day after initial plating in 10% serum at $2{\cdot}5\times10^4$/ml). Cultures were continued for the indicated times before analysis, in the absence or presence of serum or with 10% serum re-added to starved cultures 1 day before analysis.

‡ ND, not determined.

assay, Greer & Kaplan (1985) reported a change in the number of breaks after LPS stimulation of mouse spleen cells comparable with that observed after Con A; they reported a much smaller, barely significant, change after LPS stimulation of nude mouse spleen cells. The lack of detection of a change in the number of breaks does not mean that changes are not occurring—if there is breaking and rejoining at different sites, the nett change in numbers might be zero or very small as discussed by Williams & Johnstone, 1983. A similar situation exists for the differentiation of the human HL60 cell line (F. Farzaneh, personal communication).

There are several discrepancies between the present report and the mouse studies of Greer & Kaplan (1983, 1985). These workers describe a very rapid change in the number of breaks (plateau reached 2 hr after Con A addition) compared with our data [still decreasing after 24 hr (Fig. 2)—approximately 16 hr slower than human lymphocytes stimulated with PHA]. The reason for these discrepancies is not clear. Young BALB/c mice were used in both studies, although our preparation of lymphocytes using density fractionation should be cleaner, more viable and less disturbed than the other workers who removed red blood cells by ammonium chloride lysis. Different assays were used by the two laboratories to assess strand breaks, although in a different study we have employed the fluorometric analysis of DNA unwinding technique used by Greer & Kaplan and obtained essentially the same results as nucleoid analysis, albeit with lower sensitivity and reproducibility (Johnstone, 1984).

Thus, the addition of inhibitors of ADPRT to lymphocytes at the time of stimulation with mitogen prevents the associated increase in DNA and protein synthesis (Fig. 1, and Johnstone, 1984), rejoining of strand breaks (Johnstone, 1984) and secretion of γ-interferon (Bhayani, Williams & Johnstone, 1985). At the same concentration, these chemicals do not block some early plasma membrane events (Johnstone, 1984) and they have only a small effect on the continued stimulation of activated blasts by medium containing IL-2 (Fig. 1). Furthermore, response to IL-2 does not involve a detectable change in DNA breaks (Fig. 2). Hence, the rejoining of strand breaks associated with a requirement for ADPRT activity appears to be involved only in the initial stimulation by mitogen and not in the response to the subsequent signals from interleukins. This approach might allow the further dissection of the complex processes of lymphocyte activation.

In this regard, the present report clearly distinguishes between activation of quiescent lymphocytes, which requires ADPRT activity and involves

rejoining of strand breaks (Figs 1 and 2), and some other cellular induction systems, fibroblasts and an erythro-myeloid cell line, which do not (Fig. 4, Table 1). This difference between cell types rules out the possibility that these phenomena are merely consequences of changes from the resting to the growing state, and supports the concept that they are involved in eukaryotic differentiation, possibly related to changes in gene expression (Farzaneh *et al.*, 1982; Williams & Johnstone, 1983). The induction of increased haemoglobin synthesis in K562 cells is reversible and appears only to involve an increase in mRNA species already present, not new expression of genes (Dean *et al.*, 1983). Inhibitors of ADPRT do not, therefore, block all increases in gene expression in all circumstances.

ACKNOWLEDGMENTS

We thank Gwyn Williams, Peter Knox, Colin McInnes and David Winterbourne for valuable advice and discussion, and Robin Thorpe and Veronica Britten for gifts of characterized IL-2 preparations. This work was supported by grants from the Wellcome Trust and the Medical Research Council.

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