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Hypersensitivity to rapamycin of BJAB B lymphoblastoid cells

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SUMMARY

Proliferation of the BJAB B-lymphoblastoid cell line was rapidly and almost completely suppressed by picomolar concentrations of the immunosuppressive macrolide rapamycin (50% inhibitory concentration 10–20 pM for cells reactivated from stationary phase). This cell line was considerably more sensitive to rapamycin than any other B-lymphoblastoid cell line tested, the Jurkat T-cell line or the HL60 promyelocytic cell line. BJAB cell proliferation was not affected by the related immunosuppressive macrolides FK506 or L-685,818, which bind to the immunophilin FKBP12 competitively with rapamycin and also inhibit its peptidylprolyl *cis-trans* isomerase activity. Excess FK506 or L-685,818 added simultaneously competitively antagonized rapamycin's anti-proliferative action. Levels of FKBP12 and uptake of rapamycin from the culture medium were also normal in BJAB cells. The hypersensitivity to rapamycin of BJAB cells thus reflects an unusual dependence on the intracellular signalling system targeted by the rapamycin–FKBP12 complex, and may provide a model system for elucidating the role played by this pathway in lymphocyte activation. The proliferation of BJAB cells reactivated from stationary phase can also be used as the basis for a highly sensitive bioassay for the presence of rapamycin in culture media or other biological fluids.

INTRODUCTION

Rapamycin, a macrolide synthesized by *Streptomyces hygroscopicus*, was initially found to inhibit the growth of some species of yeast *in vitro*, but was then discovered to have strong immunosuppressive activity in mammals and to inhibit the induction of proliferation in cultured T and B lymphocytes.[1,2] Although rapamycin shares its primary target, the immunophilin FKBP12, with other immunosuppressive macrolides such as FK506 and ascomycin,[3,4] it inhibits T- and B-lymphocyte activation at a later stage and by a distinct mechanism.[5–8]

Immunophilins of the FKBP family have peptidylprolyl *cis-trans* isomerase (PPIase) activity that is sensitive to inhibition by rapamycin, FK506, ascomycin and synthetic analogues such as 506BD and L-685,818, but not all effective PPIase inhibitors are immunosuppressive, indicating that immunosuppression is induced by the macrolide–immunophilin complex, rather than simply by inhibition of immunophilin PPIase activity.[9–11] FK506–FKBP12 or ascomycin–FKBP12 complexes bind and sequester a secondary target, the protein serine/threonine phosphatase calcineurin, to prevent the dephosphorylation and activation of a transcription factor (NF-AT) essential for induction of cytokine expression.[12] Rapamycin–FKBP12 sequesters a different secondary target, a mammalian homologue of the protein encoded by the yeast TOR genes.[13,14] By competing for FKBP12, rapamycin and FK506 competitively antagonize each other's effects, while synthetic non-immunosuppressive analogues antagonize the effects of all immunosuppressive ligands.[6–11]

TOR proteins and their mammalian homologues have sequence homology with phosphatidylinositol- and phosphatidylinositide-3-kinases,[13–16] and play an ill-defined role in intracellular signalling pathways required for T-lymphocyte activation and the proliferation of some other mammalian cells and some yeasts. Lymphoid cell proliferation appears to be more sensitive to lower concentrations of rapamycin for reasons that are not currently clear, but which are thought to account for the immunosuppressive action of the macrolide *in vivo*. Addition of rapamycin to lymphoid or non-lymphoid cells results in equally rapid and complete dephosphorylation and inactivation of p70 S6 kinase,[17,18] a serine/threonine protein kinase with substrates that include the S6 ribosomal protein but whose precise physiological role has not been established. The p70 S6 kinase does not interact directly with rapamycin–FKBP12 complexes and, although it is rapidly phosphorylated and activated in response to the addition of many growth factors by a poorly characterized signalling pathway suggested to involve phosphatidylinositide-3-kinases, present evidence suggests that the dephosphorylation of p70 S6 kinase after rapamycin addition may involve different serine and threonine residues from those normally phosphorylated in response to growth factors.[19]

In the course of investigations into the sensitivity of

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cultured lymphoid cells to rapamycin, we have found that many lymphoid cells lines reactivated from stationary phase show sensitivity to rapamycin at concentrations very similar to those necessary to inhibit activation of primary T or B lymphocytes, but that the same cell lines maintained in logarithmic growth are much less sensitive to the macrolide. However, we also identified one B-lymphoblastoid cell line (BJAB) that is hypersensitive to rapamycin. Proliferation of BJAB cells in logarithmic growth is rapidly inhibited by immunosuppressive concentrations of rapamycin, and BJAB cells reactivated from stationary phase have a 50% inhibitory concentration (IC_{50}) an order of magnitude lower than that of primary lymphocytes activated by mitogens. This provides not only a convenient model system for studying rapamycin action but also the basis for a very sensitive bioassay for the presence of rapamycin in culture media or biological fluids.

MATERIALS AND METHODS

Rapamycin was supplied by Dr J. J. Chang (Wyeth Ayerst Research Laboratories, Princeton, NJ), FK506 by Fujisawa Pharmaceuticals (Osaka, Japan) and L-685,818 (18-hydroxy, 21-ethyl FK506) by Dr A. Williamson (Merck Research Laboratories, Rahway, NJ). The BJAB, Raji, Ramos, Loucke and TK6 B-lymphoblastoid cell lines were supplied by Professor M. J. Clemens (St George's Hospital Medical School, London, UK), Bristol 8 B lymphoblastoid cells by Dr B. Adams (University of Sussex, UK), HL60 promyelocytic leukaemia cells by Dr M. Tavassoli (University of Sussex), and the Jurkat J6 T-lymphoblastoid cells by the European Collection of Animal Cell Cultures (Porton Down, UK).

All cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 50 μg/ml gentamicin. Cells were subcultured every 3 days, and log phase cultures were obtained by diluting cultures on the second day after subculture to 2×10^5/ml in fresh culture medium. Cells reached stationary phase after incubation for 5 days, and were then reactivated by dilution to 2×10^5 cells/ml in fresh medium. Inhibitors were added to log phase cells and, unless otherwise indicated, to stationary phase cells at the time of reactivation by dilution. Equivalent amounts of solvent (ethanol) were added to control cultures but the highest concentrations used (0·4%) did not affect cell proliferation. Proliferation was assessed by addition of 0·5 μCi [methyl-^{3}H]-thymidine to each 0·2-ml culture for a terminal 4-hr pulse. Incorporation into DNA was determined using a Skatron Combi 11025 cell harvester (Skatron Instruments Ltd, Newmarket, UK).

Expression of FKBP12 was detected by immunoblotting sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) separated cell extracts with a rabbit antibody raised against recombinant human FKBP12 purified from transformed *Escherichia coli* extracts. The anti-FKBP12 was used at a dilution of 1 : 2000 and antibody binding was detected using the Amersham immunogold detection system (Amersham Int., Little Chalfont, UK). Rapamycin uptake was determined by incubation of cells at a concentration of $1{\cdot}5 \times 10^7$ cells/ml with 600 pM rapamycin at 37° for 1 hr. Cells were collected by centrifugation, and residual rapamycin in the

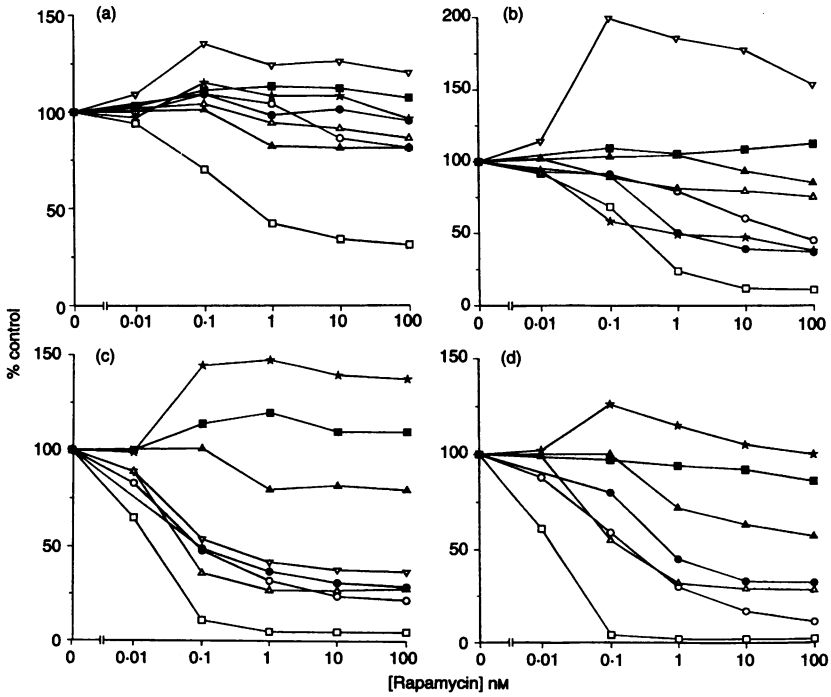

Figure 1. Effect of rapamycin on proliferation of cells maintained in logarithmic growth or reactivated from stationary phase. Cells in logarithmic growth (a,b) or grown to stationary phase (c,d) were diluted to 2×10^5 cells/ml with fresh culture medium and the concentrations of rapamycin indicated added. Cell proliferation was assessed by determining incorporation of [^{3}H]thymidine in a terminal 4-hr pulse 24 hr (a,c) or 48 hr (b,d) after rapamycin addition. Cell lines used were BJAB (□), Jurkat (○), Raji (△), Ramos (▲), Loucke (▽), TK6 (★), Bristol 8 (●) or HL60 cells (■) and data are aggregated from up to 45 similar experiments.

culture medium was detected by bioassay of the effects of a range of dilutions (equivalent to 10 pM–300 pM rapamycin) of the culture medium on the proliferation of reactivated stationary phase BJAB cells, in comparison with a range of standard rapamycin concentrations incubated in culture medium without cells under the same conditions. [^{3}H]thymidine incorporation into DNA was determined as above after 48 hr.

RESULTS

When the effects of rapamycin on the proliferation of a range of lymphoid cell lines were investigated, considerable variation was observed. The extent of inhibition depended on the cell line used, on the stage in the growth cycle at which rapamycin was added and the time at which proliferation was assessed (Fig. 1). The induction of proliferation in lymphoid cells reactivated from stationary phase was usually much more rapidly and severely affected by rapamycin than the proliferation of the same cells in logarithmic growth at the time of rapamycin addition.

All cell lines in exponential growth except BJAB cells were able to maintain normal rates of cell proliferation for at least 24 hr (Fig. 1a). By 48 hr inhibition was apparent in several cell lines, but its magnitude was greater for the BJAB cells (almost 90% inhibition) than any other lymphoid cell line tested (15–65%), while one B-lymphoid cell line, Louckes, showed an anomalous enhancement of proliferation by 100 pM–100 nM rapamycin (Fig. 1b). Proliferation of the myeloid HL60 cells was unaffected under these conditions.

Of the cell lines tested, only TK6 cells and HL60 cells were able to initiate proliferation at rates comparable to normal if rapamycin was added when cells in stationary phase were diluted into fresh culture medium (Fig. 1c, d). Sixty to 80% inhibition of proliferation was seen at 24 hr with most lymphoid cell lines, with IC_{50} in the same range (100 pM–1 nM) as those reported to inhibit activation of primary T and B lymphocytes by mitogens or the interleukin-2 (IL-2) driven proliferation of IL-2-dependent cell lines.[5–8,17] Ramos cells were significantly less sensitive than most other lymphoid cell lines, while TK6 cells proliferated at normal or even enhanced rates in the presence of rapamycin. Of the seven lymphoid cell lines tested, TK6 cells were the only exception to the general rule that proliferation of cells in log phase at the time of rapamycin addition was less sensitive than that of cells activated from stationary phase.

In both growth conditions the BJAB B-lymphoblastoid cell line stood out as hypersensitive to the effects of low concentrations of the immunosuppressive agent. Figure 1a shows that proliferation of log-phase BJAB cells was uniquely sensitive at 24 hr, while the induction of proliferation in reactivated BJAB cells was exquisitely sensitive to rapamycin, with complete abolition of proliferation at rapamycin concentrations above 1 nM and an IC_{50} of 15 ± 5 pM (Fig. 1c, d). The onset of inhibition of [^{3}H]thymidine incorporation in this cell line could be observed within 6 hr of rapamycin addition (Fig. 2). Determinations of cell numbers confirmed these results and direct observation of the cells revealed that rapamycin was cytostatic for most cell lines studied but cytotoxic for BJAB cells. The BJAB cell line was thus considerably more sensitive to rapamycin than any other B-lymphoblastoid cell line tested, or than the Jurkat T-cell line and the HL60 promyelocytic cell line.

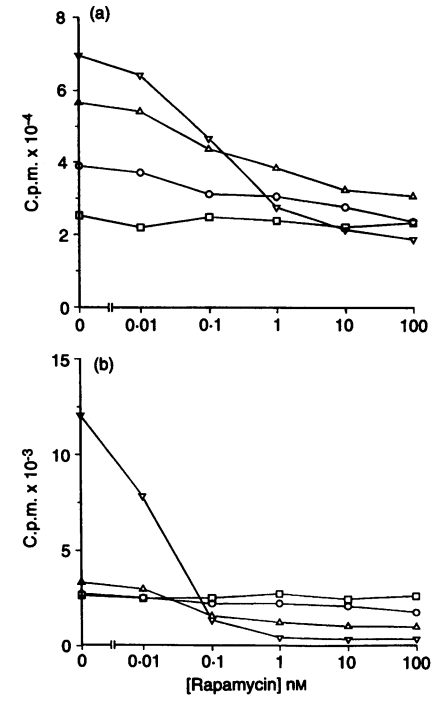

Figure 2. Onset of inhibition of BJAB cell proliferation after rapamycin addition. BJAB cells in logarithmic growth (a) or grown to stationary phase (b) were diluted to 2×10^5 cells/ml with fresh culture medium and the concentrations of rapamycin indicated added. Cell proliferation was assessed by determining incorporation of [^{3}H]thymidine in a terminal 2-hr pulse ending 2 hr (□), 6 hr (○), 10 hr (△) or 24 hr (▽) after rapamycin addition.

BJAB cell proliferation was not affected significantly by the related immunosuppressive macrolides FK506 or L-685,818 at concentrations more than 10 000-fold higher than the effective concentrations of rapamycin. Similar results were obtained with other lymphoid cell lines (data not shown). However, high concentrations of FK506 or L-685,818 competitively antagonized the anti-proliferative action of rapamycin (Fig. 3a, b). Attempts to rescue rapamycin-inhibited BJAB cultures with FK506 showed that even short periods of incubation with rapamycin were sufficient to cause irreversible effects, with addition of FK506 at 3 hr after rapamycin less than half as effective as simultaneous addition, and addition after 12 hr almost completely ineffective (Fig. 3c).

There were no obvious differences between BJAB cells and the other cell lines studied to account for their hypersensitivity to rapamycin. When the cell concentrations in the assays were varied there was a proportionate change in IC_{50} for rapamycin, but BJAB cells were of similar size to the other cells studied. Assessment by forward light scatter in a Coulter Epics FACS gave a mean size of 640 arbitrary units for BJAB cells in logarithmic growth compared to 630 and 660 arbitrary units for Jurkat and Raji cells, respectively, and BJAB cells in exponential growth contained 9·3 pg RNA/cell compared to 8·4 pg/cell in Jurkat cells. BJAB cells proliferated in culture with a normal growth pattern, indistinguishable from that of the other B-cell lines studied.

When levels of FKBP12 were investigated by separation of cell extracts on SDS–PAGE and Western blotting with an

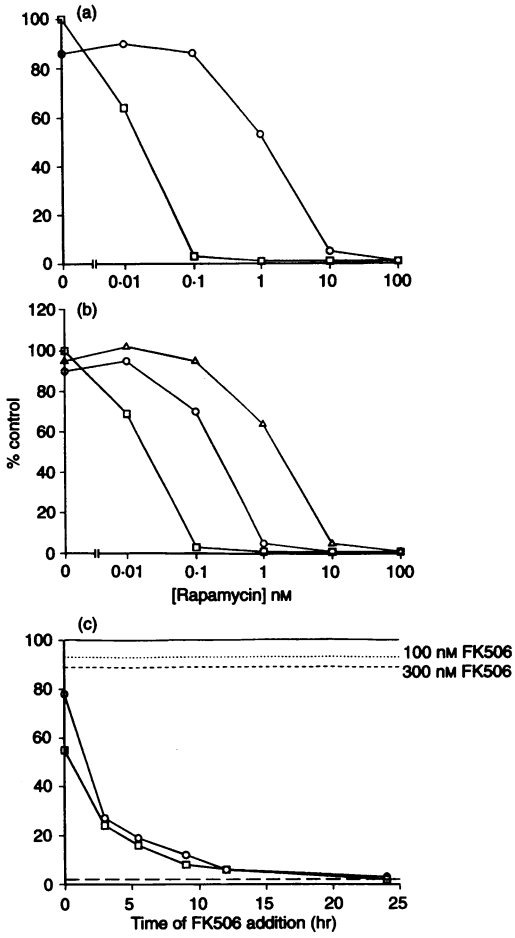

Figure 3. Antagonism of the inhibitory action of rapamycin by FK506 and L-685,818. BJAB cells grown to stationary phase were diluted to 2×10^5 cells/ml with fresh culture medium and the concentrations of rapamycin indicated added, alone (□) or with the simultaneous addition in (a) of 100 nM FK506 (○), or in (b) of 100 nM (○) or 1 μM (△) L-685,818. In (c) similar BJAB cultures were incubated with 30 pM rapamycin (added at the time of reactivation) and 100 nM (□) or 300 nM (○) FK506 at the indicated time later. Cell proliferation was assessed by determining incorporation of [^{3}H]thymidine in a terminal 4-hr pulse ending 48 hr after rapamycin addition. Lines indicate incorporation by control cultures (——), or cultures incubated with 30 pM rapamycin (– – –), 100 nM FK506 (- - - -) or 300 nM FK506 (— — —) only.

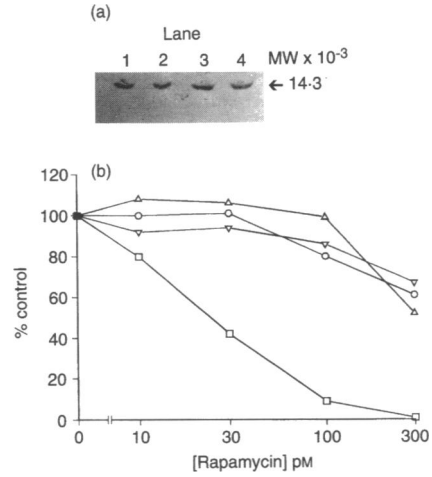

Figure 4. Presence of FKBP12 in BJAB, Jurkat and Louckes cells. (a) Extracts from 5×10^5 stationary phase (lane 1) or log phase (lane 2) BJAB cells or stationary phase (lane 3) or log phase (lane 4) Jurkat cells were separated on 15% SDS–PAGE gels, immunoblotted and probed with a 1 : 2000 dilution of rabbit anti-human FKBP12. FKBP12 bands were visualized by the immunogold detection system. (b) BJAB cell bioassay to detect absorption of rapamycin from culture media by BJAB, Jurkat or Louckes cells. Culture medium containing 600 pM rapamycin was incubated for 1 hr at 37° without cells (□) or with log phase BJAB cells (○), Jurkat cells (△) or Louckes cells (▽) at $1{\cdot}5 \times 10^7$ cells/ml. The cells were then collected by centrifugation and discarded, and to assess the residual rapamycin remaining in the culture medium it was added at final concentrations of 50%, 15%, 5% and 1·5% to fresh BJAB cells grown to stationary phase and then diluted to a final concentration of 2×10^5 cells/ml. Control cultures contained equivalent amounts of medium preincubated with or without cells but without rapamycin. Proliferation of the indicator BJAB cells was determined after incubation for 48 hr, and the percentage inhibition by residual rapamycin determined.

antibody raised against recombinant human FKBP12, similar levels of FKBP12 expression were detected in BJAB cells as in Jurkat cells (Fig. 4a). To establish that the BJAB cell FKBP12 functioned normally, uptake of rapamycin from the culture medium by BJAB cells, Jurkat cells and Louckes cells was compared. Cells in logarithmic growth at $1{\cdot}5 \times 10^7$ cells/ml were incubated with 600 pM rapamycin for 1 hr at 37°. The cells were then collected by centrifugation and discarded, and residual rapamycin in the culture medium was assessed by comparing the abilities of different dilutions of the culture media to inhibit the proliferation of fresh BJAB cells reactivated from stationary phase. Figure 4b shows that all three cell lines were able to deplete the rapamycin concentration in the culture medium with similar efficiency. Incubation of rapamycin under these conditions in culture medium alone only slightly reduced its inhibitory activity (apparent IC_{50} 25 pM). However, incubation with cells increased the apparent IC_{50} to over 300 pM, indicating that over 90% had been removed. Control experiments have confirmed that under these conditions the inhibitory activity of the macrolides can be recovered from lysates of the cells used for absorption,[20] indicating that the disappearance of rapamycin from the culture medium is due to uptake rather than degradation.

DISCUSSION

These results establish clearly that the sensitivity of lymphoid cell proliferation to rapamycin depends both on the stage of the cell growth cycle at which rapamycin is added and the particular cell line studied. Typical T- and B-lymphoid cell lines, such as Jurkat, Raji, Louckes and Bristol 8, are able to continue proliferating normally for 1–2 days if rapamycin is added to cells in logarithmic growth. Ramos cells in logarithmic phase at the time of rapamycin addition have previously been reported to proliferate normally for up to 3 days,[21] and were here significantly less sensitive than the lines above. However, addition of rapamycin to such cells at the time of reactivation from stationary phase causes severe inhibition of proliferation. The concentrations of rapamycin necessary to inhibit the induction of proliferation of these cell lines (within the range 0·1–1·0 nM) are comparable to those necessary to prevent the

induction of proliferation in mitogen activated peripheral blood lymphocytes[5–8] or IL-2-induced proliferation of IL-2-dependent T-cell lines,[5,17] suggesting that the sensitivity to rapamycin of such lymphoid cells is a general feature of their progression from stationary phase into the cell cycle, rather than a function of the specific growth factor(s) initiating the response. The response of IL-2-dependent T-cell lines to IL-2 also becomes insensitive to rapamycin once the cells have entered logarithmic growth.[22]

A few lymphoid cell lines, such as Ramos and TK6, and the non-lymphoid HL60 cells are notably less sensitive or insensitive to rapamycin. Rapamycin-insensitive sublines of Jurkat and Raji cells can also be readily selected by extended culture in the presence of high concentrations of rapamycin,[23] as previously reported for the murine YAC-1 T-cell line.[24] The induced proliferation of some non-lymphoid cell lines has also been reported to be slowed by rapamycin,[18,25–28] although the effects are usually less dramatic than in normally sensitive lymphoid cells. In other cell lines, such as the murine MEL erythroleukaemia cell line, induced proliferation may even be enhanced by rapamycin,[29] as found for Loucks cells in logarithmic growth here. In most cases, including MEL cells, rapamycin still maintains effective inhibition of p70 S6 kinase activation, indicating that cell proliferation is not invariably dependent upon this signalling pathway. However, in YAC-1 cells[24] and Jurkat cells selected for rapamycin resistance[23] rapamycin–FKBP12 complexes are unable to sequester the mammalian TOR homolgue and thus to inactivate p70 S6 kinase, and the possibility that other resistant cell lines may have undergone a similar change cannot be disregarded.

However, BJAB cells are much more sensitive to rapamycin than any other lymphoid cell line tested. Proliferation of BJAB cells in logarithmic growth is blocked within a few hours by immunosuppressive concentrations of rapamycin, while the reactivation of stationary phase BJAB cells is completely inhibited by such concentrations. This hypersensitivity is associated with marked cytotoxicity and attempts to select rapamycin-insensitive BJAB sublines were unsuccessful. Like most lymphoid cell lines, BJAB cell proliferation is unaffected by high concentrations of other FKBP PPIase inhibitors, but hypersensitivity to rapamycin appears to be mediated by FKBP12 (or some similar immunophilin that binds rapamycin and FK506 with comparable affinities), as proliferation could be rescued by addition of an excess of competing FKBP ligands, such as FK506 or L-685,818. High concentrations of the rescuing ligand are required, but the excess (100–1000-fold) is no greater than that necessary to achieve similar reversals of rapamycin action in normal lymphocytes.[6–8]

It is not clear at present whether the hypersensitivity of BJAB cells (or the relative insensitivity of some other lines such as Ramos) is a property of the specific human B-lymphocyte subpopulation from which they arose, and thus may be relevant to *in vivo* immunosuppression induced by rapamycin, or whether it is due to some abnormality that has arisen during their transformation or *in vitro* culture. The BJAB cell line has normal levels of apparently normally functional FKBP12, and it has been reported to also contain levels of the mammalian TOR homologue capable of interacting with rapamycin–FKBP12 complexes comparable to those found in normal human T lymphocytes and the T-lymphoid cell line Molt 4.[30] High level expression of the 160 000 MW P glycoprotein associated with multi-drug resistance was not detected by immunoblotting with the mouse anti-human P glycoprotein antibody C219 in BJAB, Jurkat, Ramos, TK6 or HL60 cells, although expression was found in Raji cells (M.C. Smith & A. Wellhausen, unpublished data). The hypersensitivity cannot be explained by any unusual sensitivity of the BJAB p70 S6 kinase to rapamycin as, in agreement with previous reports with a range of lymphoid and non-lymphoid cells,[17–19,22,26,27] this enzyme is inhibited down to undetectable levels by the concentrations of rapamycin used here even in the less sensitive Jurkat and Raji cells (data not shown). It seems likely that BJAB cells are more dependent than other cells on the intracellular signalling pathway mediated by the mammalian TOR homologue and p70 S6 kinase and blocked by FKBP12–rapamycin complexes. They may be a valuable tool for elucidating the nature and significance of this pathway.

In addition, the inhibition of proliferation of reactivated stationary phase BJAB cells is sufficiently sensitive to rapamycin to provide the basis of a bioassay for the detection of rapamycin in culture media or other biological fluids (Fig. 4b). The IC_{50} (in the range 10–20 pM) is an order of magnitude lower than the rapamycin concentrations required for immunosuppression and two orders of magnitude below the lower end of the linear range of published high-performance liquid chromatography (HPLC) assays.[31,32] Such assays are potentially capable of high sample throughput, and would detect any immunosuppressive metabolites formed during incubation in aqueous media in addition to rapamycin itself.

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