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Induction of Apoptosis in neoplastic cells by depletion of vitamin B_{12}

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Abstract

Methionine synthase, a critical enzyme in deoxyribonucleotide biosynthesis for DNA replication, requires vitamin B_{12} as a cofactor. We have tested the hypothesis that depletion of cells of vitamin B_{12} would block growth of neoplastic cells and divert them into apoptosis and could form the basis of a new therapeutic strategy for cancer treatment. Using nitrous oxide to inactivate vitamin B_{12} we show that, in a variety of cell lines *in vitro*, methionine synthase is rapidly inhibited, the cells cease proliferation and undergo apoptosis. The kinetics of cell death, once started, are similar to those observed following methotrexate treatment or serum withdrawal. This is the first observation of apoptosis being induced following depletion of an essential metabolite as opposed to the more conventional strategy of adding a toxic drug to damage cells thereby triggering apoptosis. Moreover, vitamin B_{12} depletion has no effect on the nonproliferating cell population.

Keywords: transcobalamin, growth blocker, apoptosis, methionine synthase, K562

Abbreviations: dTTP, deoxythymidine triphosphate; MeTHF, methyltetrahydrofolic acid; MS, methionine synthase; MTX, methotrexate; N_2O, nitrous oxide gas; TCII, transcobalamin II

Introduction

Vitamin B_{12} is an essential cofactor for two enzymes. Methionine synthase (MS) which catalyses the conversion of homocysteine into methionine using methyl tetrahydrofolic acid (MeTHF) as a carbon donor and methylmalonylCoA mutase which catalyses the conversion of methylmalonylCoA to succinylCoA. Methionine synthase uses methyl-cobalamin as cofactor whereas methylmalonylCoA mutase uses adenosyl-cobalamin. Methionine synthase not only provides methionine for protein synthesis, but also serves as the entry point of dietary folates into the folate cycle. Folates from the diet are converted into MeTHF in the digestive tract during absorption and then circulated as such to peripheral tissues. Since the folate cycle provides reducing equivalents and a methyl group for the conversion of dUMP to dTMP during nucleotide biosynthesis MS activity is also essential for DNA replication. The availability of vitamin B_{12} is, therefore, also crucial for nucleotide synthesis and DNA replication. As with folates, vitamin B_{12} is also obtained from the diet and transported in the blood by its specific carrier, transcobalamin II (TCII). Cellular uptake of vitamin B_{12} requires the expression of a specific vitamin B_{12}-TCII receptor on the surface of cells. Receptor expression is generally coupled to DNA replication (Hall, 1984; Amagaski *et al*, 1990). Peripheral blood lymphocytes, for example, only express a high affinity receptor for cobalamin during the S-phase of the cell cycle. The receptor responsible is rapidly down-regulated in other phases of the cell cycle and when cells undergo differentiation (Lindemans *et al*, 1989).

The folate cycle has long been considered a good target for therapeutic strategies and a number of antifolate analogues, most notably methotrexate (MTX), have proved to be useful. Methotrexate inhibits dihydrofolate reductase, preventing the regeneration of THF, and bringing the folate cycle to a halt. The resulting lack of dTTP causes nucleotide imbalance which leads to misincorporation of nucleotides into DNA and eventually leads to the death of the cell by apoptosis (James *et al*, 1994). Deficiency in dietary folate also leads to apoptosis in primary cultures of proerythroblasts isolated and subsequently incubated in folate-deficient media (Koury and Horne, 1994). Methotrexate, however, is not just specific for proliferating cells and there is a toxic accumulation of the drug in non-proliferating cells such as liver.

Depletion of a cell of vitamin B_{12}, leading to inibition of MS, would be expected to have a similar effect as MTX. Indeed, experiments carried out by Lassen and Kristen (1959), Eastwood *et al* (1963) and Ikeda *et al* (1989), using nitrous oxide gas to cause a whole body B_{12} depletion in patients with leukemias, established that white blood cell counts could be effectively reduced to the point of disease remission. Similar results have been obtained in a mouse ascites tumour model (Parbrook, 1967) and in experimental rat leukemia models (Ermens *et al*, 1989; Abels *et al*, 1990). Nitrous oxide oxidizes CoII to CoIII rendering cobalamin inactive (Banks *et al*, 1968) leading to inhibition of MS activity (Deacon *et al*, 1980; Kondo *et al*, 1981), but not methylmalonylCoa mutase activity (Kondo *et al*, 1981; Chanarin *et al*, 1985) at least during short-term exposures. Therefore, the growth inhibitory effects of nitrous oxide are more likely to act via inhibition of MS than methylmalonylCoA mutase. Although feasibility of this approach has

been demonstrated *in vivo*, consistent results have not been seen *in vitro* using established tumour cell lines and this has hampered progress in this area. Consequently, despite the potential of B_{12} depletion as a therapeutic strategy it has been much less studied than folate depletion.

Since, it is now apparent that apoptosis, as opposed to necrosis, represents the major mechanism by which cells die within the body there is tremendous interest in exploiting the therapeutic potential of apoptosis in cancer therapy (Kerr *et al*, 1994; Hickman *et al*, 1994; Fisher, 1994). Indeed, most current therapeutically useful drugs and metabolic inhibitors have been found to induce apoptosis albeit indirectly (Walker *et al*, 1991; Hickman *et al*, 1994). The true potential of exploiting apoptosis will come from the development of novel strategies that are not directly toxic to cells, but can still activate apoptosis. Cells appear to have sensors that monitor damage or metabolic imbalance and, above a certain threshold, apoptosis is activated and the cells are eliminated. The objective, therefore, is to activate such sensors using protocols that avoid the undesirable side effects encountered in the use of the current generation of nonspecific or broad specificity inhibitors. This may be more readily achievable by specifically removing an essential cellular component rather than trying to inhibit a process or cause intracellular damage. In this study, we have investigated the feasibility of this approach by depleting a variety of neoplastic cells of vitamin B_{12} and show that, under appropriate conditions, it is as effective a trigger of apoptosis as MTX treatment. These results suggest that a new generation of growth-blocker drugs, aimed at reproducibly inducing apoptosis in cancer cells through targeting of vitamin B_{12} metabolism, may be devised.

Results

Effects of nitrous oxide on cell growth

Mouse L1210 cells were plated at 0.2×10^6 cells/ml in complete RPMI or in media depleted in either B_{12} (-B_{12}) or both folate and B_{12} (-folate/B_{12}) and incubated in either air-CO_2 (cells in complete medium) or the N_2O gas mixture (cells in B_{12} or folate/B_{12}-depleted media). Some cells were also plated in serum-free complete medium. Growth was followed over a 15 day period with the cells being split back to their starting cell density every 3–4 days (Figure 1). Under these conditions, the cells plated in complete medium and incubated in air-CO_2 completed approximately three cell divisions every 3–4 days while producing negligible amount of dead cells (Figure 1A). In contrast, cells incubated in serum-free RPMI underwent one cell cycle during the first 3 days and then rapidly lost viability by day 6. Attempts to adapt L1210 cells to the serum-free medium, supplemented solely by bovine serum albumin as described by Kondo *et al* (1989) were unsuccessful. The growth of cells incubated in RPMI -B_{12} (in the presence of N_2O) was not significantly different from those of the controls in normal RPMI until after day 8 when growth became slightly, but consistently reduced (Figure 1A). However, cells plated in media depleted in both folate and B_{12} grew poorly after day 3 (Figure 1B) and the culture rapidly lost viability after 10 days in either of these media (Figure 1C).

Similar results were obtained for two other cell lines, human K562 (Figure 2A and B) and mouse BW5147.3 cells (Figure 2C and D). In both cases, the cells quickly lost viability in serum-free media which we have previously shown to be via apoptosis (Walker *et al*, 1995). After completing two cell cycles, the cells were unable to proliferate further in medium depleted of both folate and vitamin B_{12}. After 5 or 6 days in depleted media (in the presence of the N_2O gas mixture) both cultures lost viability. Over the 8 days of the experiment, growth of

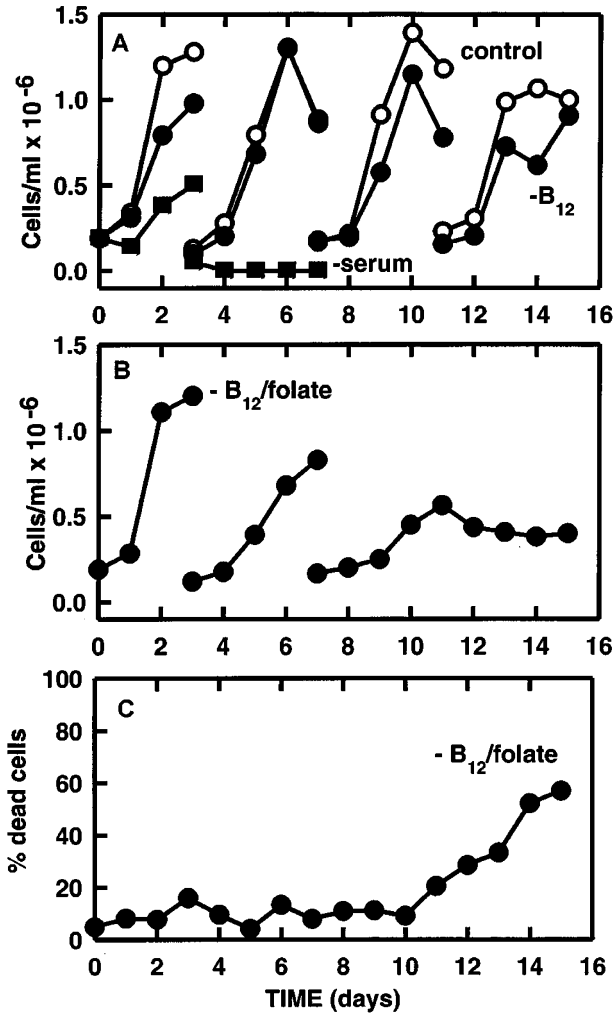

Figure 1 Growth of L1210 cells in normal or serum-free media (-serum), or media depleted of either only vitamin B_{12} (-B_{12}), or depleted of both vitamin B_{12} and folate (-B_{12}/folate). In A, cells were plated at 0.2×10^6 cells/ml in normal RPMI +10% FBS (open circles, control), in the same medium without serum (closed squares, -serum) or in B_{12}-depleted medium +10% FBS in the presence of N_2O (closed circles, -B_{12}). Cells were split back to the starting density as indicated. In B and C, cells were plated as above in medium without folic acid and B_{12} (-B_{12}/folate) and incubated in the presence of N_2O. A and B show cell growth, C shows the percentage of dead cells for the experiment in B.

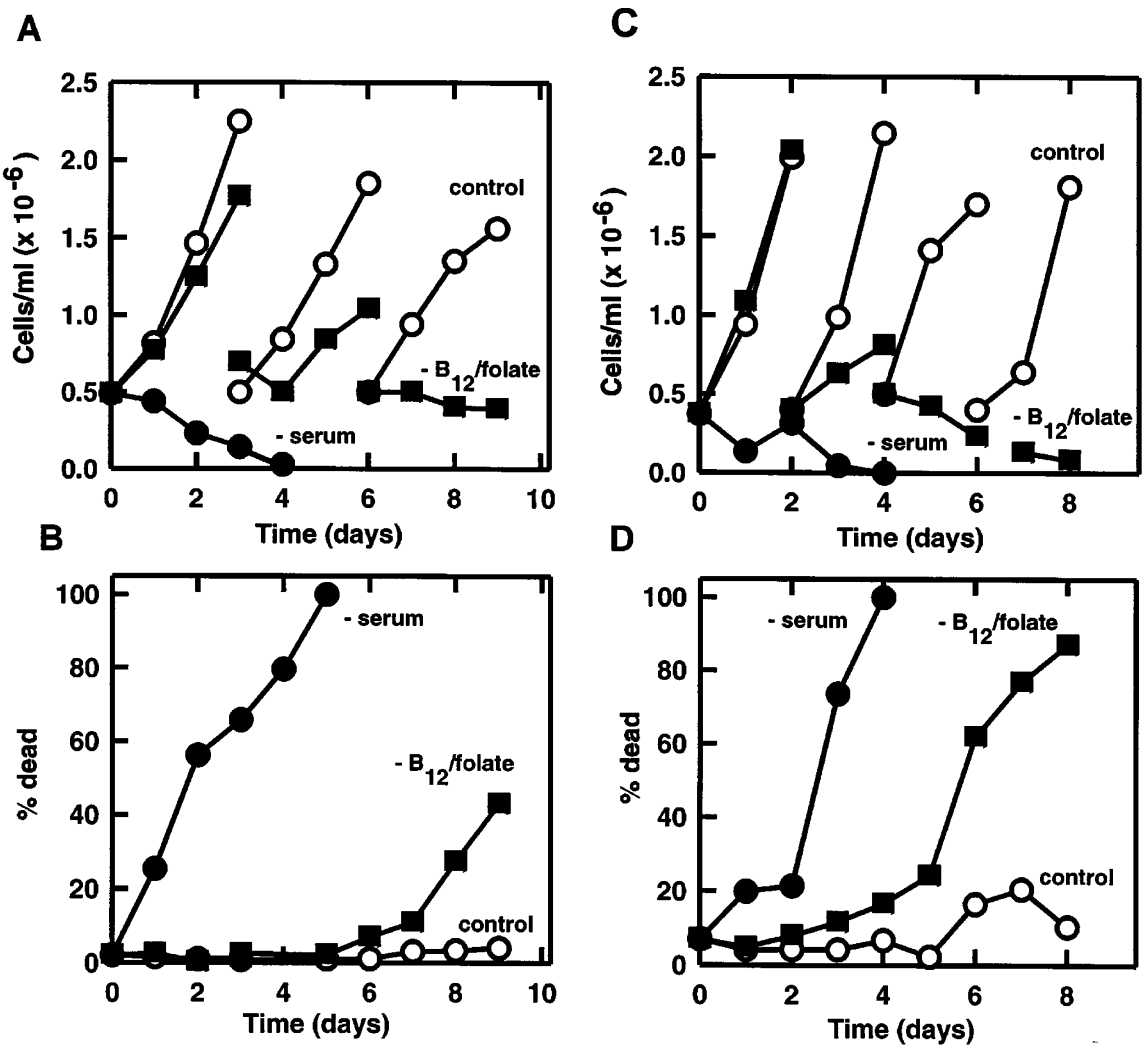

Figure 2 Growth of K562 (A,B) and BW5147.3 (C,D) cells in normal medium (with air-CO_2) or medium depleted in both vitamin B_{12} and folate (-B_{12}/folate+N_2O gas mixture). K562 cells were plated at an initial density of 0.5×10^6 cells/ml and split back to this density every 3 days (with the exception of serum depleted cells which were fed, but not split). Growth (A) and the percentage of dead cells (B) were determined every day. BW5147.3 cells were plated at an initial cell density of 0.4×10^6 cells/ml and fed and/or split every 2 days (C,D). Open circles, control cells; closed circles, cells in serum-free medium; closed squares, cells in B_{12}\folate-depleted medium.

each cell line was only marginally affected by depletion of B_{12} alone (data not shown).

DNA fragmentation in cells incubated in depleted media

Cells undergoing apoptosis show characteristic high molecular weight and internucleosomal DNA fragmentation patterns (Walker *et al*, 1995) and the extent of DNA fragmentation seems to be a characteristic of each specific cell type. In this experiment K562 and BW5147.3 cells, grown under conditions described in Figures 1 and 2, were examined for evidence of apoptotic cell death by analysis of DNA isolated at various times after treatment (Figure 3). K562 cells undergo apoptosis following serum withdrawal, but degrade their DNA primarily to high molecular weight fragments only (Walker *et al*, 1995) as shown in Figure 3A (lanes 2–5). Cells deprived solely of vitamin B_{12} showed no DNA degradation during the first 4 days of culture (lanes 6–9). However, cells depleted of either folate or both vitamin B_{12} and folate showed considerable DNA degradation, typical of apoptosis, by 11 days of culture (Figure 3B). BW5147.3 cells, which degrade their DNA much more extensively to produce internucleosomal DNA fragments, also underwent apoptosis when depleted of either serum or folate and vitamin B_{12} (Figure 3C).

Dissection of folate and vitamin B_{12} growth requirements

The data described above suggested that when folic acid is present in the medium the cells have little requirement for vitamin B_{12}. To evaluate this BW5147.3 cells were grown in medium deficient in either folate, vitamin B_{12} or both (Figure 4). As in the experiment described in Figure 2, the cells

depleted of both folate and B_{12} lost viability after 6 days, whereas cells depleted in folate alone did not begin to lose viability until 2 days later. In this longer-term experiment, cells incubated in medium depleted of vitamin B_{12} (and exposed to the N_2O gas mixture) eventually ceased proliferating and began to lose viability, but not until after 10–12 days in culture. Similar results were obtained following prolonged incubations of K562, L1210 and U937 cells (data not shown).

In a further experiment to more directly demonstrate B_{12}-dependent growth, K562 cells were adapted to grow in a serum-free medium so that the levels of folate and vitamin B_{12} which the cells were exposed to could be controlled completely (fetal bovine serum can contribute sufficient B_{12} to support growth as well as substantial amounts of folate). This experiment also allowed us to determine the rate of depletion of intracellular B_{12} by incubating the cells in the presence or absence of N_2O (Figure 5). Cells incubated in serum-free medium, without folic acid and supplemented with 5 μM MeTHF, and also containing vitamin B_{12} (control cells) completed two cell cycles in the 5 days of the experiment and this was not significantly affected by leaving vitamin B_{12} out of the medium (Figure 5A). Clearly, there is sufficient endogenous B_{12} to support at least two cell cycles. However, when the cells were also exposed to N_2O at the time of removal of B_{12} the population only doubled and there was no further net accumulation of cells. Flow cytometric analysis of the cell population showed an accumulation of some cells in early S-phase (data not shown). The culture also began to lose viability almost immediately suggesting that some cells were still in cycle whereas others were undergoing apoptosis. Indeed, when the morphology of these cells was examined by fluorescence microscopy (Figure 5C) apoptotic cells with highly condensed nuclei as well as apoptotic bodies were readily seen.

Effects of nitrous oxide on intracellular methionine synthase activity

Although N_2O has been shown to alter MS activity *in vitro* (Deacon *et al*, 1980; Kondo *et al*, 1981; Christensen and Ueland, 1993) the kinetics of its effect on cultured cells has not been examined. To establish the kinetics by which nitrous oxide was able to inactivate vitamin B_{12} resulting in a loss of MS activity the enzyme was assayed in a number of cell lines at various times after the onset of exposure to the gas mixture (Figure 6). Both apo- and holoenzyme activities were determined. Apoenzyme activity indicates the immediate effects of N_2O on vitamin B_{12}-bound enzyme, i.e. the effects of inactivation of functional activity, whereas holoenzyme

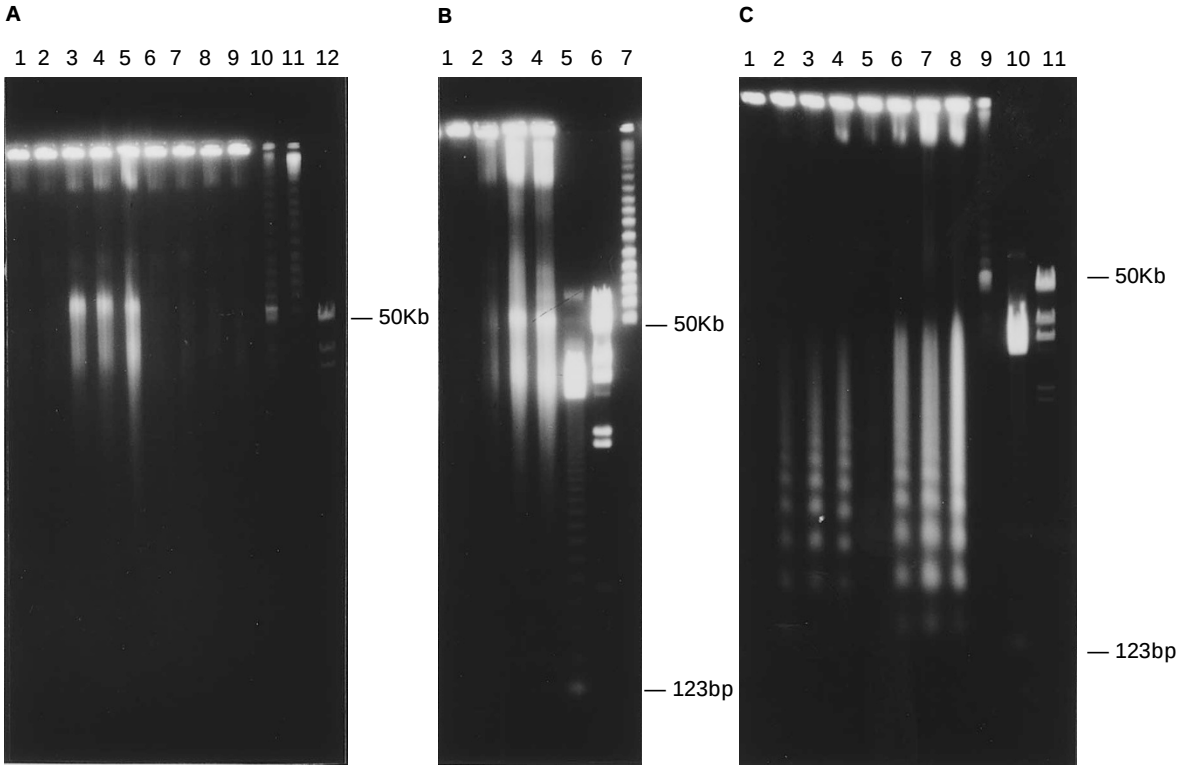

Figure 3 DNA fragmentation in K562 (A,B) and BW5147.3 (C) cells determined by pulsed field gel electrophoresis. In A, lane (1)=DNA from starting cell culture; lanes 2–5, DNA from cells incubated for 24, 48, 72 and 96 h in serum-free medium; lanes 6–10, DNA from cells incubated in medium - B_{12} (+N_2O). Lanes 10–12, molecular size markers; Yeast chromosomes, Lambda ladder (multimers of 48.5 Kb) and HindIII/lambda DNA digest, respectively. The sizes of representative markers are shown to the right. In B, lane 1=DNA from starting culture; lanes 2–4, DNA from cells grown for 11 days in complete medium, folate-depleted medium or B_{12}/folate depleted medium, respectively. Lanes 5–7, molecular size markers; 123 bp ladder, HindIII/lambda DNA digest and Lambda ladder, respectively. In C, DNA from BW5147.3 cells grown for 1, 2, 3 or 4 days in serum-free medium (lanes 1–4) or medium depleted in B_{12} and folate (lanes 5–8). Lanes 9–11, molecular size markers; Yeast chromosomes, 123 bp ladder and, HindIII/lambda DNA digest, respectively.

activity reflects the effects of N_2O on total enzyme protein. In all cases, apoenzyme activity was rapidly lost and became undetectable within 6–12 h. Interestingly, holoenzyme activity also declined throughout the 72 h exposure to gas, particularly in the case of K562 cells. A similar loss of holoenzyme activity was seen in the liver of rats exposed to N_2O for prolonged periods (Kondo *et al*, 1981). The different cell types had substantially different levels of MS activity and the K562 cells had a much higher MS activity and a much higher fraction (35%) of the enzyme was occupied by B_{12} compared with the other cells (5–10%, Table 1).

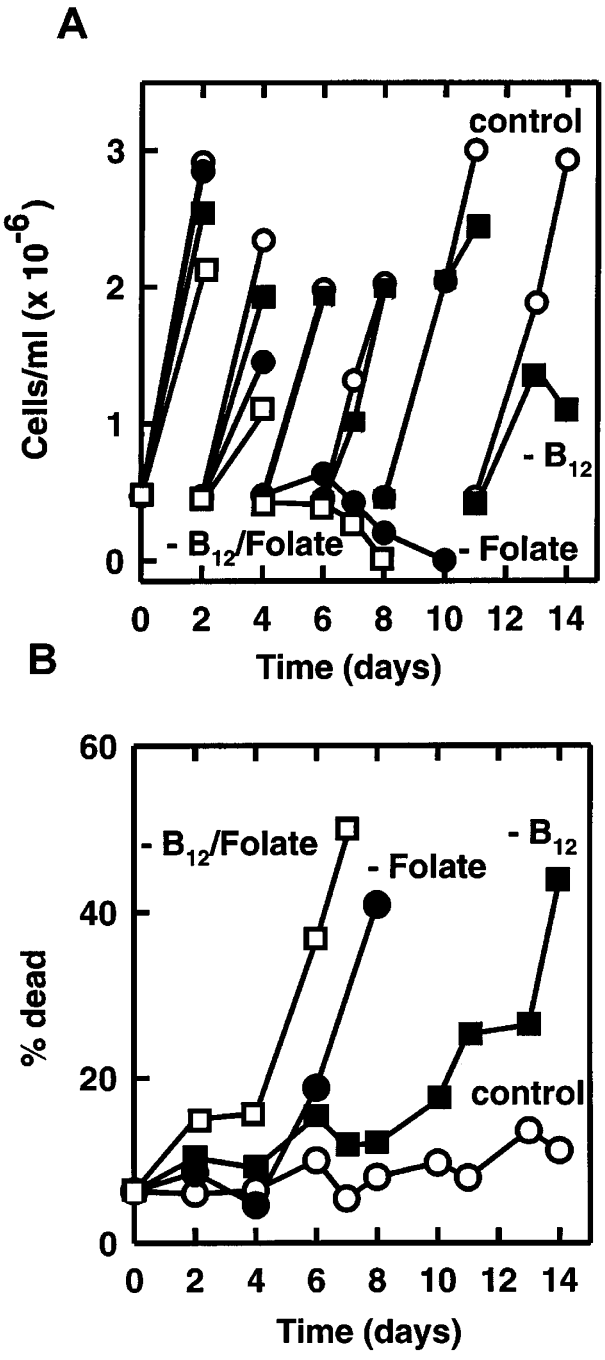

Figure 4 Growth of K562 cells in normal medium or medium depleted in either B_{12} (-B_{12}) or folate (-Folate) or both (-B_{12}/Folate) as indicated. Cells were plated at 0.5×10^6 cells/ml and split and fed every 2 days until growth ceased. A, live cell counts/ml of medium. B, Percentage of trypan blue positive cells. Open circles, control cells; closed circles, folate-depleted; closed squares, B_{12}-depleted medium; open squares, B_{12}\folate-depleted medium.

Comparison of kinetics of cell death induced by B_{12}-depletion and MTX

The rate of cell death seen in K562 cells following B_{12}/folate-depletion seen in Figure 2B was compared with the rate at which MTX killed the cells (Figure 7A). In this experiment cells were exposed to 100 nM MTX and the percentage of trypan blue positive cells counted each day. A significant increase in cell death was observed 3–4 days after treatment and by day 8 the entire cell population was dead. In contrast, cell death began almost immediately after serum withdrawal, but did not start until 6–7 days after B_{12}/folate-depletion. However, once started the rate of cell death following depletion was similar to that seen in MTX-treated cells (Figure 7B). In this figure the rate of cell death of K562 cells is compared for serum withdrawal, MTX treatment and B_{12}/folate depletion. Regression analysis showed that upon serum withdrawal the culture began to undergo apoptosis almost immediately with 19% of the cells being lost/day. In the presence of MTX approximately 16% of the cells died/day after a 2 day delay in onset. Similarly, 16% of the cells were lost/day in cells depleted of B_{12}/folate, but there was a delay of 6 days before cell death commenced. Similar results were obtained with the other cell lines (data not shown).

Discussion

The only documented effect of incubating cells or tissues with N_2O, in the presence of normal oxygen levels (20%) is the oxidation of CoII to CoIII resulting in an effective depletion of vitamin B_{12}. In this manuscript we have shown that depletion of functional vitamin B_{12} with N_2O in a variety of transformed cell lines triggers apoptosis. Given that vitamin B_{12} depletion is specific, at least in the short-term, for proliferating cells it can form the basis of a therapeutic strategy aimed at elimination of cancerous cells by specifically inducing apoptosis. Until now, only a cumbersome, irreproducible method of B_{12} depletion was available, i.e. N_2O inhalation. This was the only reason for the abandonment of this therapeutic approach, despite documented efficacy in leukemia patients with minimal toxicity (Lassen and Kristen, 1959; Eastwood *et al*, 1963; Ikeda *et al*, 1989). We are developing 'growth blockers' aimed at antagonising the uptake of vitamin B_{12} via the proliferation-associated B_{12}/TCII receptor. We are using two approaches: one called receptor modulation (Pathare *et al*, 1996) aimed at interfering with the expression and intracellular routing of B_{12} receptors (and thereby delivery of B_{12} to intracellular methionine synthase). The second approach uses monoclonal antibodies to TCII (Quadros *et al*, 1996; McLean *et al*, 1996), the B_{12} serum transport protein, to inhibit binding to the cell receptor and thereby producing a depletion of B_{12}.

Nitrous oxide completely inhibits functional MS (apoenzyme) activity in these cultured cells with kinetics similar to

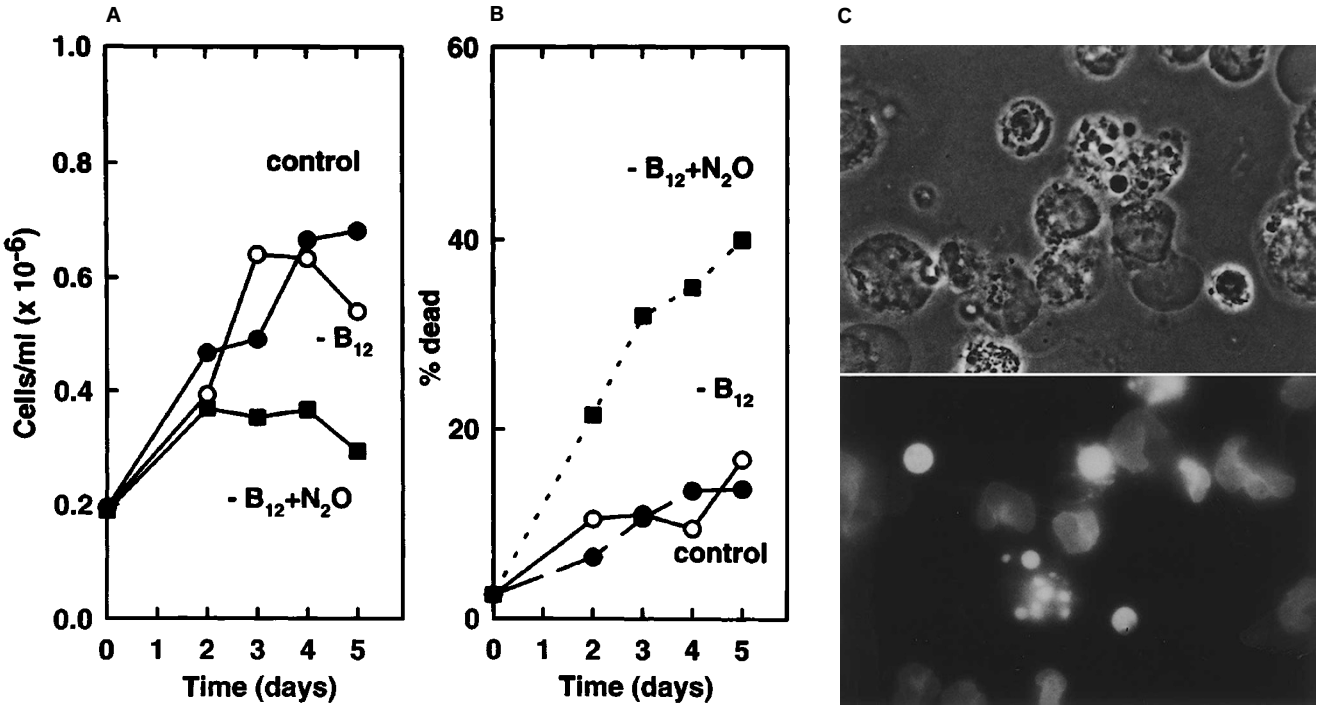

Figure 5 Growth and apoptosis of K562 cells in serum-free medium. Cells were plated at 0.2×10^6 cells/ml in either the serum-free medium (-folate+MeTHF+B_{12}, closed circles) described in the Materials and Methods or the same medium without vitamin B_{12} and incubated in air-CO_2 for 5 days (open circles). Parallel cultures were set up in medium without vitamin B_{12} and incubated in the N_2O mixture for 5 days (closed squares). Live cell counts (A) and the percentage of trypan blue positive cells (B) were determined each day. C, Phase contrast microscopy (upper panel) and Hoechst staining (lower panel) of cells 5 days after incubation in medium without vitamin B_{12} and incubated in the N_2O mixture. The data are representative of several such experiments.

those seen *in vivo* (Kondo *et al*, 1981; Chanarin *et al*, 1985). It has been shown that *in vivo* there is a subsequent loss of folates (Chanarin, 1982) and derangements in deoxyribonucleotide pools (Chanarin *et al*, 1985; Wickramsinghe and Fida, 1993). Deoxyribonucleotide pool imbalance results in a lowered dTTP pool and misincorporation of nucleotides into DNA during replication (Wickramsinghe and Fida, 1993) eventually triggering cell death (Yoshioka *et al*, 1987). Since methylmalonylCoA mutase activity is not affected by short-term exposure to N_2O (Kondo *et al*, 1981; Chanarin *et al*, 1985) it is unlikely to contribute to the death of the cell.

Reproducible *in vitro* effects of vitamin B_{12} depletion on cell growth and cell death have been difficult to obtain because the composition of most cell culture media do not reflect the cellular environment *in vivo*. For example, most cells *in vivo* are not normally exposed to folic acid (Chanarin, 1982). Folates from the diet are converted to MeTHF and transported to peripheral tissues in this form. Thus, cells *in vivo* are completely dependent upon the MS reaction to convert MeTHF to THF and replenish the folic acid cycle. Cells in culture, on the other hand, are exposed directly to folic acid as the normal supplement of most media. Because cells cannot directly convert folic acid to MeTHF the folate is believed to enter the folate cycle by being successively reduced to dihydro- and then tetrahydrofolic acid. Since in complete RPMI medium the level of folic acid (2.3 μM) is orders of magnitude higher than serum folates cells *in vitro* have little or no requirement for MS enzyme (or vitamin B_{12}) to maintain adequate folate cycle activity to support DNA replication. The slightly reduced growth rate seen when cells are depleted only of B_{12} probably reflects the accumulation of some folate as MeTHF (the methyl folate trap, Chanarin, 1985) which may eventually block proliferation (Figure 4). When folic acid is removed from the medium the cells undergo apoptosis and this is accelerated by also inactivating cobalamin (Figure 4). However, a full dependency of proliferation on B_{12} availability is only seen under serum-free conditions (in media supplemented with MeTHF) since even 10% fetal bovine serum can contribute substantial amounts of folates (Figure 5). Thus, our *in vitro* model was

Table 1 Methionine Synthase activity in cultured cells

Cell Type	$-B_{12}$ (dpm $\times 10^{-2}/10^6$ cells)	$+B_{12}$	% Holoenzyme
L1210	109	1094	10
BW5147	162	1523	11
CEM	55	1018	5
HL60	70	507	14
K562	2099	5930	35

Cells were assayed for MS activity +/−50 μM exogenous cynaocobalamin prior to exposure to the N_2O mixture. % holoenzyme is the activity observed in the absence of added cyanocobalamin as a fraction of total activity.

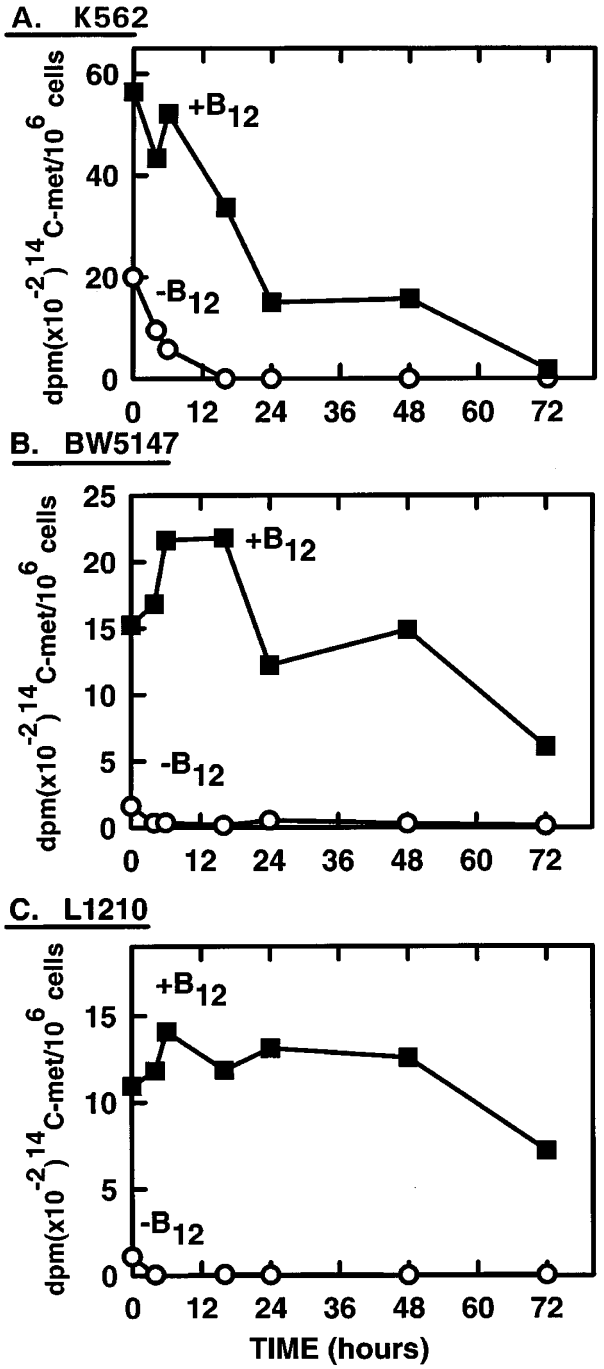

Figure 6 Methionine synthase (MS) activity of (A) K562, (B) BW5147.3 and (C) L1210 cells during 72 h of exposure to the N_2O mixture. Cells were grown in vitamin B_{12}-containing media. Cell samples were taken at the times indicated and assayed for activity +/− 50 μM exogenous cobalamin (solid squares, open circles, respectively). Activity is expressed as the dpm of [^{14}C]-methionine produced/10^6 cells.

designed to approach the *in vivo* conditions in which full dependency on B_{12} as a co-factor is the rule.

Cells depleted of all growth and survival factors by serum withdrawal, treated with MTX or depleted of B_{12}/folate begin to undergo apoptosis after different lag times. This indicates that a critical threshold or point of commitment to cell death must be achieved and that this point is reached with different kinetics depending on the nature of the inducer. However, once reached the cell population undergoes apoptosis at comparable rates (Figure 7B). All cells must continuously repress apoptosis and serum withdrawal depletes the cells of all the growth and survival factors involved in repression. The cells stop cycling and begin to undergo apoptosis almost immediately. In the case of MTX treatment, the cells also stop cycling almost immediately, but there is a delay of 2 days before the cells respond to the cell cycle block by activating apoptosis. The exact nature of the sensor is unknown. The cells may be sensitive to a prolonged nucleotide pool imbalance, or to the DNA damage that results from misincorporation of nucleotides. Two days are necessary for the threshold to be reached. In the case of N_2O treatment the cells do not reach this point for 6 days. This, in turn, reflects the additional time required for the cells to be completely depleted of folates which appears to take 3–4 cell cycles. The rate of depletion, and hence the time required to reach the commitment point, is dramatically affected by exogenous folates, even the small amounts present in 10% serum. In addition, cells grown in the presence of MeTHF can still undergo several cycles unless intracellular levels of B_{12} are inactivated by N_2O. In the complete absence of medium and serum folates and B_{12} the cells begin to undergo apoptosis within 2 days, similar to the delay seen with MTX. The effects of MTX are not dramatically altered by the presence or absence of exogenous folates since it prevents the folate cycle from operating.

Since the rate of apoptosis, once it commences, is independent of the inducer the most successful strategy, from a therapeutic point of view, is the one that drives cells to the threshold of apoptosis activation with the minimum of cellular damage and the highest degree of selectivity. Since vitamin B_{12} participates in only two cellular reactions, one of which is essential for proliferation, its depletion may represent one of the least disruptive means of blocking growth and inducing apoptosis in cancer cells. Moreover, whereas cells may be able to develop resistance to drugs designed to inhibit or damage specific intracellular targets, it is impossible to overcome the lack of an essential cofactor.

Materials and Methods

Cell cultures

The following human and murine cell lines were tested in this study; K562 human chronic myelogenous leukemia, CCRF-CEM human acute lymphoblastic leukemia, U937 human histiocytic lymphoma, BW5147.3 mouse lymphoma and L1210 mouse lymphocytic leukemia. All the cells were maintained in normal or modified RPMI media (Gibco BRL, Burlington, Ontario) containing 10% (v/v) Fetal Bovine Serum (Unipath, Nepean, ON). Since complete RPMI medium contains 3.7 nM B_{12} and 2.3 μM Folic acid, a modified medium without folic acid and vitamin B_{12} was purchased and supplemented with either folic acid or vitamin B_{12} as required for specific experiments. In other experiments, K562 cells were propagated in serum-free RPMI (modified or complete) medium supplemented with 0.1% bovine

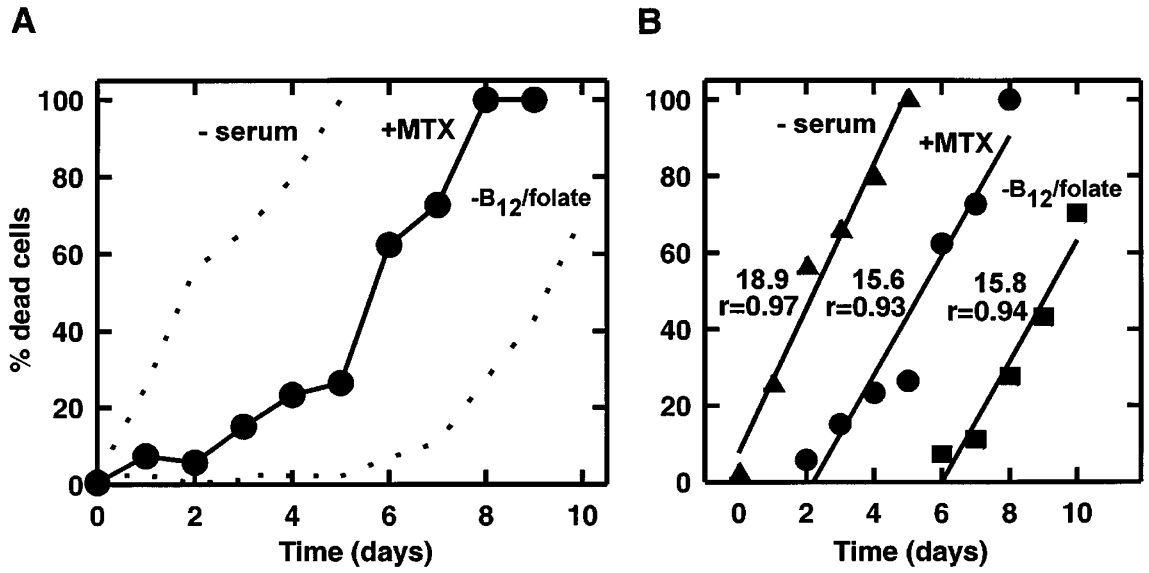

Figure 7 Kinetics of MTX-induced cell death of K562 cells. Cells were treated with 100 nM MTX and the percentage of trypan blue positive cells determined every day (A). Curves for the kinetics of cell death following serum or B_{12}/folate depletion are indicated by dotted lines. B, regression analysis of the rates of cell death following the three treatments. The slope as well as the correlation coefficient for each curve are indicated.

serum albumin, 5 μg/ml insulin, 5 μg/ml apotransferrin, 5 ng/ml selenium and 10 mM hepes buffer, pH 7.2 (Sigma Chemical Co. St Louis, MO).

Nitrous oxide and drug treatments

Culture media and serum were saturated with a mixture of 50% N_2O, 20% O_2, 25% N_2 and 5% CO_2 before being added to cells. Cultures were set up at $0.2-0.5\times10^6$ cells/ml in this media and the flasks of cells were flushed with the same gas mixture, sealed and placed in the incubator. Cells were subsequently fed or split using media saturated as above. Parallel cultures, not exposed to N_2O were set up in the usual way and placed in the same incubator and exposed to 95% air, 5% CO_2.

Some cultures were treated with MTX at a dose of 100 nM from a 5 mM stock in dimethylsulphoxide as indicated in the Figure legends.

Analysis of apoptosis

Cell death by apoptosis was established using morphological and biochemical criteria. For fluorescence microscopy, cells were attached to poly-L-lysine coated coverslips (Sigma, St Louis, MO), fixed for 5 min in 3% paraformaldehyde in PBS and rinsed twice briefly with PBS. The coverslips were immersed for 1 min in 1 μg/ml of Hoechst 33258 in PBS, mounted onto slides and examined using an Olympus Bmax fluorescence microscope as previously described (Weaver *et al*, 1996). DNA fragmentation, a characteristic of apoptotic cell death, was examined by pulsed field gel electrophoresis (PFGE) as described previously (Walker *et al*, 1993). Under these electrophoresis conditions both high molecular weight and oligonucleosomal fragments can be resolved on the same gel. Briefly, 2×10^6 cells were immobilized in a low melting point agarose plug and deproteinised for 3 h at 37°C in 300 μl of buffer containing 4 μg of proteinase K, 1% SDS, 10 mM Tris-HCl, pH 9.5, 25 mM EDTA, 1 mM EGTA and 10 mM NaCl. The plugs were rinsed in 10 mM Tris-HCl, pH 8.0+1.0 mM EDTA and loaded into the wells of an 0.8% agarose gel and electrophoresis was carried out using a Q-Life (Kingston, Ontario) Autobase PFGE system. After electrophoresis the gels were stained with ethidium bromide and photographed on a transilluminator using a Polaroid DS-34 camera and positive/negative film no. 55.

Cell counts were performed using a haemocytometer and cell viability was determined by trypan blue dye exclusion.

Methionine synthase activity

Methionine synthase activity was assayed essentially as described by Christensen *et al* (1992). Briefly, $2-5\times10^7$ cells were harvested, pelleted and lysed in 100 μl of 50 mM Potassium phosphate buffer, pH 7.4 containing 100 mM NaCl, 10 mM dithiothreitol and 0.05% Triton X100. Following centrifugation (30 s in a microfuge), 50 μl of extract was assayed in a final reaction volume of 100 μl that contained 50 mM potassium phosphate buffer, pH 7.4, 125 mM β-mercaptoethanol, 0.4 mM DL-homocysteine, 0.3 mM s-adenosyl methionine, 1 μM (0.2 μci) N^5-[methyl-^{14}C] methyltetrahydrofolic acid (Amersham, Oakville, ON) +/− 50 μM cyanocobalamin. The reaction mixture was overlayed with 50 μl of bisphthalate (Fluka, Caledon laboratories, Georgetown, ON) and incubations carried out at 37°C for 2 h in the dark. The reaction was stopped by adding 400 μl of ice cold water and the radioactive methionine product separated by AG1-X8 (200–400 mesh) column chromatography (Biorad, Mississauga, ON) and counted in a Beckman LS3801 liquid scintillation counter. The data is expressed as dpm ^{14}C-methionine produced/10^6 cells.

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