*Clin. exp. Immunol.* (1989) **76**, 19–23

# Defective DNA synthesis by T cells in acquired 'common-variable' hypogammaglobulinaemia on stimulation with mitogens

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*(Accepted for publication 20 December 1988)*

## SUMMARY

We have studied T cell defects in acquired 'common-variable' hypogammaglobulinaemia (CVH) by measuring the synthesis of DNA, RNA and protein *in vitro* in response to mitogens and to interleukin 2 (IL-2). We have confirmed that some patients have defective DNA synthesis in response to PHA and shown that this extends to responses to cell-derived B cell growth factor (c-BCGF) which is also mitogenic to T cells. DNA synthesis induced by IL-2 was not defective in these patients suggesting IL2-receptor induction is normal. The mitogen-related defect in DNA synthesis was not accompanied by any reduction in synthesis of RNA or of protein. Levels of the rate limiting enzyme (thymidylate synthetase EC. 2.1.1.45) responsible for *de novo* DNA synthesis in the absence of endogenous thymidine were measured following PHA stimulation and found to be in the normal range. In the CVH patients (but not in normal individuals) the relationship between the levels of thymidylate synthetase and DNA synthesis in response to PHA approached significance, suggesting that this pathway becomes more important in CVH patients than in normal individuals perhaps because of defects in the thymidine 'salvage' pathway.

**Keywords** hypogammaglobulinaemia T cell function thymidylate synthetase mitogens interleukin 2

## INTRODUCTION

There are several reports of T cell defects in patients with acquired 'common-variable' hypogammaglobulinaemia (CVH), including a reduced response to mitogens (Webster & Asherson, 1974), and a reduction in proliferation and interleukin 2 (IL-2) production on stimulation with OKT3 antibody (Kruger *et al.*, 1984) or with Concanavalin A (Con A) (Saiki et al. 1984).

We have now assessed the ability of T cells from nine CVH patients and 10 normal individuals to respond to two mitogens, phytohaemagglutinin (PHA) and low-molecular-weight B cell growth factor (c-BCGF, Maizel *et al.*, 1982) as well as to the lymphokine IL-2. The synthesis of DNA, RNA and protein were all measured. Thymidylate synthetase (EC. 2.1.1.45), the rate limiting enzyme in *de novo* DNA synthesis in the absence of thymidine and undetectable in resting cells, was also measured in PHA-stimulated cells to determine whether any failure to respond to mitogenic signals could be attributed to a defect in the *de novo* pathway leading to DNA synthesis.

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## MATERIALS AND METHODS

*Cells and cultures*

Mononuclear cells were obtained from normal human volunteers and from CVH patients. Blood (40 ml) was defibrinated, mixed with medium (3 vols blood: 1 vol medium), layered onto Ficoll-Paque (Pharmacia) and centrifuged at 650 ***g*** for 35 min. The cells harvested from the interface were washed twice and adjusted to approximately $5 \times 10^6$/ml in RPMI 1640 medium to which 10% fetal calf serum, glutamine (2 mM) penicillin (100 iu/ml) and streptomycin (100 iu/ml) had been added. T cells were obtained as follows: to every 3 ml of this cell suspension, 1 ml of 5% neuraminidase-treated sheep red blood cells (n-SRBC) were added. To prepare n-SRBC 1 ml of washed and packed SRBC (Tissue Culture Services) were incubated with 0·3 ml of neuraminidase (1 iu/ml; Sigma) in 10 ml of phosphate-buffered saline (PBS) for 35 min at 37°C and washed three times in PBS. The cell suspension with n-SRBC was centrifuged at 160 ***g*** for 5 min and left at room temperature for between 1 and 16 h. The pellet was gently resuspended and layered onto Ficoll-Paque and centrifuged for 35 min at 650 ***g***. Cells at the interface were discarded. The cell pellet contained T cell rosettes. The T cells were recovered by lysing the n-SRBC with ammonium chloride lysis fluid, washing twice in RPMI 1640 and finally resuspending

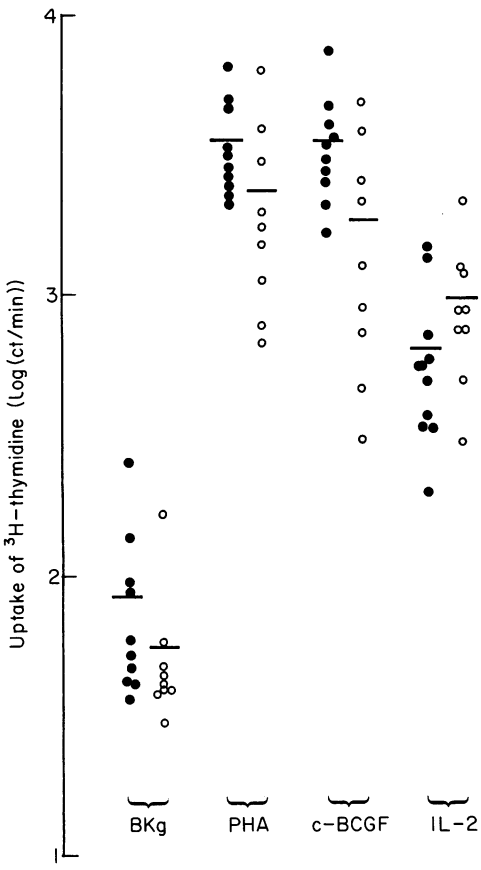


**Fig. 1.** DNA synthesis. Incorporation of $^3$H-thymidine into DNA (mean log ct/min) by T cells from 10 normal individuals (●) and nine common variable hypogammaglobulinaemia (CVH) patients (○) in response to stimulation by PHA (1 μg/ml), c-BCGF (10%) and IL-2 (500 u/ml). The mean incorporation for each group is indicated by a horizontal bar. Numbers of patients giving responses below the control range were six for PHA; five for c-BCGF and none for IL-2.

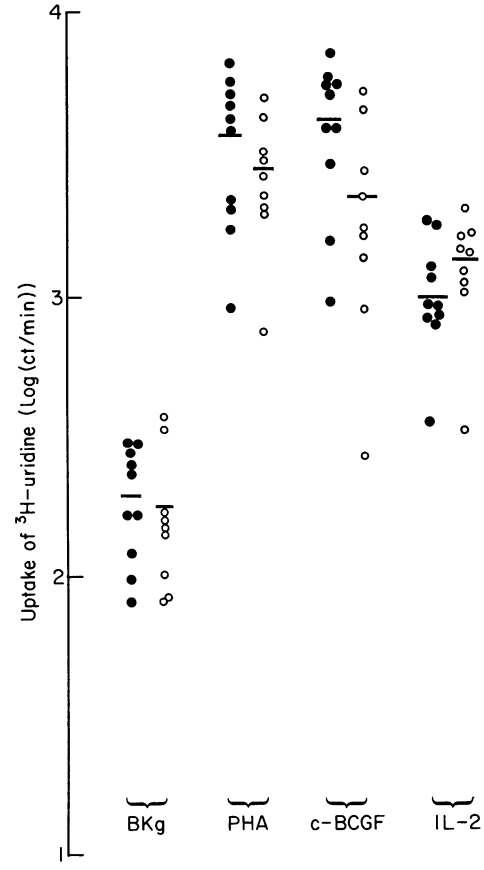


**Fig. 2.** RNA synthesis. Uptake of $^3$H-uridine (mean log ct/min) by T cells from 10 normal individuals (●) and nine CVH patients (○). For other details and symbols see Fig. 1. Responses were in, or close to, the control range except for one patient whose cells were not stimulated with c-BCGF.

in complete Iscove's medium adapted for human cell culture (Farrant *et al.*, 1984). FACS analysis showed that, for both normal and patient, contamination with other cell types was low i.e. NK cells (CD16) < 5%, monocytes (CD14) < 1%, B cells (CD19) < 0·5%. The CD3 levels were > 83%.

Aliquots (20 μl) of T cells at $4 \times 10^6$/ml were dispensed into Terasaki plates and stimulants were added in a 1 or 2 μl volume to give the required concentration with six replicates for each sample. The cells were cultured by inverting the Terasaki plates and placing them in humidified boxes in a 5% v/v $CO_2$ in air incubator as previously described (Farrant *et al.*, 1980).

*Stimulants*

Phytohaemagglutinin (PHA) (Wellcome) was obtained in freeze-dried form reconstituted in sterile water, stored at −70°C and used at 1 μg/ml. Human-B-cell-derived growth factor c-BCGF (Sera-lab) was used at a final concentration of 10% (2 μl/well). Recombinant IL-2 (Biogen) was reconstituted according to the manufacturer's instruction and stored at −70°C. Dilutions in medium were made immediately before use, and a final concentration of 500 U/ml was used.

*Proliferation*

On day 3 some wells were pulsed with either methyl-$^3$H-thymidine (specific activity 2 Ci/mmol, 0·4 μCi/well) or dual-labelled with 5-$^3$H-uridine (specific activity 33 Ci/mmol, 0·2 μCi/well) and L-$^{35}$S methionine (specific activity > 800 Ci/nmol, 0·2 μCi/well). After 2 h cultures were harvested onto glass fibre discs and washed sequentially with PBS, 5% w/v trichloroacetic acid and methanol (Farrant *et al.*, 1980). RNA and protein (as well as DNA) are insoluble under these conditions. The discs were dried and counted in 1 ml Liquiscint (National Diagnostics) in a liquid scintillation spectrometer. Time course experiments (data not shown) indicated that day 3 was optimal for all labelled substrates both for normal individuals and patients.

*Thymidylate synthetase*

T lymphocytes (5 ml) at $2 \times 10^6$/ml in Iscove's medium were stimulated with PHA (1 μg/ml) and cultured in Falcon tissue culture flasks at 37°C in 5% v/v $CO_2$ in air. After 3 days the cells were harvested, washed twice with ice-cold saline and resuspended in 200 μl of 50 mM Tris HCl buffer pH 7·5. Cells were disrupted by sonication at 0°C in an MSE Soniprep 150 sonicator at amplitude 8 nm for two bursts of 20 seconds with a break of 20 seconds.

Thymidylate synthetase activity was measured by a modification of the method of Smith-Lomax & Greenberg (1967).

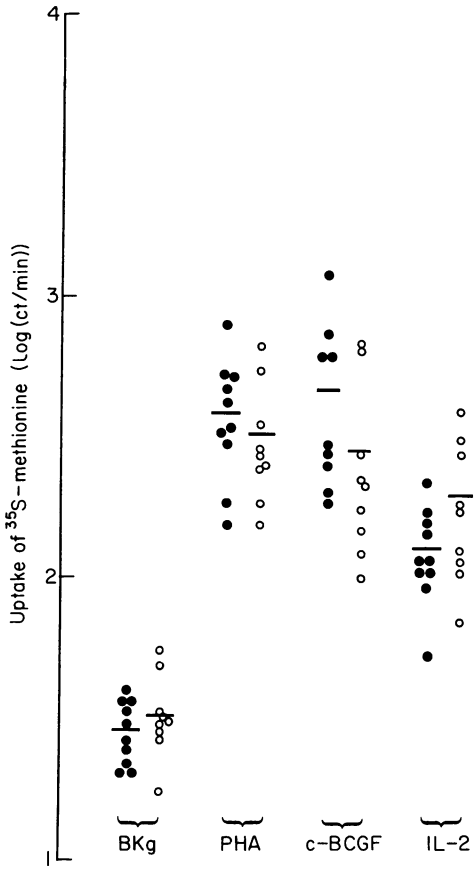


**Fig. 3.** Protein synthesis. Uptake of $^{35}$S-methionine (mean log ct/min) by T cells from 10 normal individuals (●) and nine CVH patients (○). For other details and symbols see Fig. 1. Responses were not below the control range except for three or four patients whose cells were stimulated with c-BCGF.

Briefly, the release of tritium as tritiated water from 5-$^{3}$H-deoxyuridine 5′-monophosphate (d-UMP) occurs when the substrate is converted to deoxythymidine monophosphate (dTMP) by thymidylate synthetase. The assay mixture (total volume 200 $\mu$l) contained: Tris HCl buffer (25 mM, pH 7·5), formaldehyde (BDH, 5 mM), tetrahydrofolic acid (Sigma, 0·5 mM), 2-mercaptoethanol (Sigma, 50 mM), d-UMP (Koch-Light, 0·025 mM (5-$^{3}$H)-d-UMP (Amersham) 0·25 $\mu$Ci specific activity, 10·9 Ci/mmol) and 200–500 $\mu$g T cell protein from the cell sonicate.

A stock solution of tetrahydrofolate, formaldehyde and 2-mercaptoethanol was prepared immediately before use and kept on ice under a gentle stream of argon. All the assay tubes were set up on ice and gassed for 10 seconds each with argon. The assay mixture was incubated for 15 min at 37°C and the reaction was terminated by the addition of 100 $\mu$l 1 M HCl. Unreacted (5-$^{3}$H)-d-UMP was removed by the addition of 200 $\mu$l of activated charcoal 100 mg/ml (Norit PM5, BDH). Samples were mixed, left to stand on ice for 30 min, mixed again and spun for 3 min at 11050 ***g*** in a micro-centrifuge and 100 $\mu$l aliquots of the supernatant were added to 2 ml Liquiscint (National Diagnostics). To one pair of blank tubes 200 $\mu$l of water were added instead of the charcoal to give the total counts per assay. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

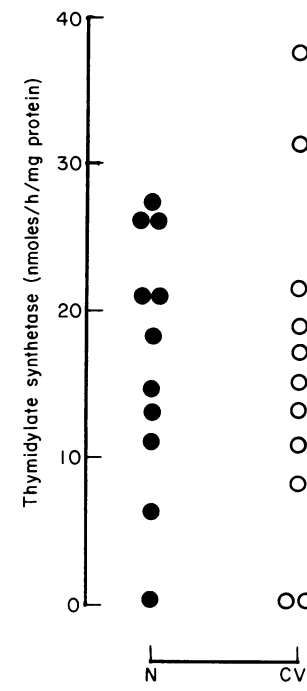


**Fig. 4.** Thymidylate synthetase. Levels of thymidylate synthetase (nmoles/h/mg protein) by PHA-stimulated T cells from 11 normal individuals (●) and 11 CVH patients (○). The CVH data were within the control range except for two patients with higher levels of the enzyme. One normal individual and two patients had undetectable levels of the enzyme on stimulation with PHA. Unstimulated cells showed no enzyme activity (data not shown).

## RESULTS

Nine CVH patients and 10 normal individuals were studied for T cell responses to PHA, low-molecular-weight c-BCGF and IL-2 as assessed by the incorporation of $^{3}$H-thymidine ($^{3}$H-Tdr, Fig. 1), $^{3}$H-uridine (Fig. 2) and $^{35}$S-methionine (Fig. 3). Results are plotted as the log mean of six replicates for each variable.

In relation to the control range, six of the nine CVH patients had reduced uptake of $^{3}$H-Tdr on stimulation with PHA, and five of these also showed reduced uptake with c-BCGF. There was no difference in the uptake of $^{3}$H-Tdr following stimulation with IL-2 when the patients were compared with normal subjects. With c-BCGF, only one CVH patient showed reduced $^{3}$H-uridine incorporation (Fig. 2) and three or four patients had reduced $^{35}$S-methionine uptake (Fig. 3). In all other respects no differences were seen between the CVH patients and the normal individuals.

Figure 4 shows that on stimulation with PHA there was no difference in the activity of thymidylate synthetase between the patients and normal individuals. One normal subject and two CVH patients had undetectable activity; on one of these patients the measurement was repeated on another occasion with the same result. Regression of PHA-stimulated incorporation of $^{3}$H-Tdr and levels of thymidylate synthetase for normal individuals (Fig. 5a) showed no relationship (correlation coefficient $= -0{\cdot}14$, $P > 0{\cdot}9$). With the CVH patients (Fig. 5b), the regression approached significance (correlation coefficient $= 0{\cdot}73$, $P < 0{\cdot}1$). However, the slope of the linear regressions of the normals and patients were significantly different ($P < 0{\cdot}02$), suggesting that in patient cells for increased DNA synthesis, a rise in thymidylate synthetase activity was needed.

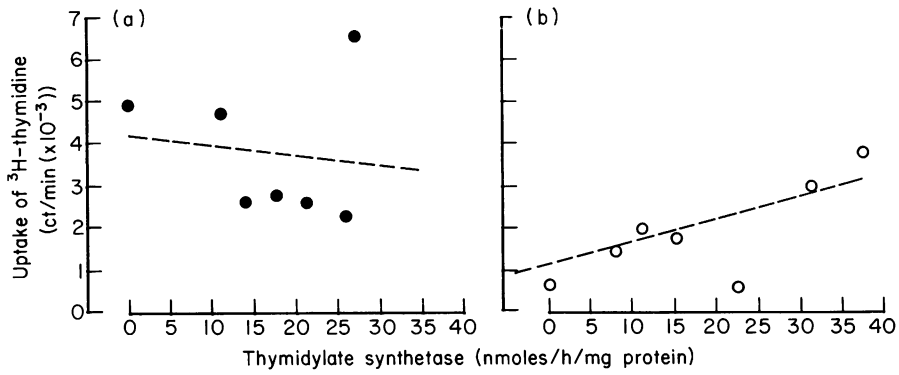


**Fig. 5.** *Thymidylate synthetase and DNA synthesis.* Linear regression (dashed line) of PHA-stimulated uptake of $^3$H-thymidine (log ct/min) (ordinate) and PHA-stimulated levels of thymidylate synthetase (nmoles/h/mg protein) for seven individual normals (a) and seven individual CVH patients (b). For the normals there was no relationship between DNA synthesis and enzyme levels (correlation coefficient $= -0{\cdot}14$, $P > 0{\cdot}9$). In the T cells of CVH patients there was again no significant correlation (correlation coefficient $= 0{\cdot}73$, $P < 0{\cdot}1$) but significance was approached. However, the slope of the linear regressions of the normals and patients were significantly different ($P < 0{\cdot}02$), suggesting that in patient cells a rise in thymidylate synthetase levels was needed for increased DNA synthesis.

## DISCUSSION

We have confirmed the observation of Webster & Asherson (1974) and Kruger *et al.* (1984) that T cells from some CVH patients have a depressed response to the mitogen PHA as measured by incorporation of $^3$H-Tdr into DNA. We have now shown that most of the patients who had low responses to PHA also exhibited reduced DNA synthesis to another T cell mitogen c-BCGF (Maizels *et al.*, 1982). c-BCGF has been shown to act as a potent mitogen on purified T cells in addition to its effects on B cells (Maizels *et al.*, 1982). Kruger *et al.* (1984) have also shown defective DNA synthesis in T cells of CVH patients stimulated with the mitogenic monoclonal antibody OKT3.

The mechanism of this defect to mitogen-induced DNA synthesis is not clear, although it could be reversed by phorbol myristate acetate (Fiedler *et al.*, 1987) which is known to activate protein kinase C (Nishizuka, 1984). ConA stimulation of CVH cells induces subnormal levels of expression of the Tac antigen (IL-2 receptor) (Malkovsky *et al.*, 1986) and Kruger *et al.* (1984) showed that the suppressed DNA synthesis on stimulation with mitogens was accompanied by a reduction in both IL-2 production and the expression of the IL-2 receptor. They showed that this could be restored to normal levels in a sub-group of patients by adding IL-2, suggesting that in these patients the upregulation of its own receptor by IL-2 (Depper *et al.*, 1985) remains normal. This is supported by our own experiments in which IL-2 was used as a T cell stimulant in the absence of mitogens and induced DNA synthesis by CVH cells within the normal range.

It is not yet clear whether the differences between DNA synthesis in response to mitogens and IL-2 are because the overall responses to IL-2 are low, other signals being required to induce maximal T cell proliferation, or whether they are due to different pathways of activation. The difference between the mitogens and IL-2 is not due to an alteration in the time course of DNA synthesis either for CVH patients or normal subjects (data not shown).

Despite the defect in DNA synthesis, our data show that in almost all patients the mitogen-induced synthesis of RNA and protein were in the control range. This agrees with Fiedler *et al.* (1987) who showed that mRNA for both IL-2 and IL-2R is synthesized normally in CVH cells stimulated with OKT3 and suggested that the defect could be post-transcriptional. Two pathways contribute to the production of deoxythymidine triphosphate (d-TTP) for incorporation into DNA. The 'salvage' pathway using thymidine kinase converts thymidine into deoxythymidine monophosphate (d-TMP) and the *de novo* pathway involving thymidylate synthetase converts deoxyuridine monophosphate (d-UMP) to d-TMP. A close correlation has been reported between thymidine kinase and DNA synthesis on stimulation with PHA (Munch-Petersen & Tyrsted, 1977).

Our data relate to the *de novo* pathway. We show that PHA-induced levels of thymidylate synthetase in CVH T cells fall within the control range suggesting that the *de novo* pathway is intact. However, the suggestion of a correlation between thymidylate synthetase levels and DNA synthesis in response to PHA seen in cells from CVH patients but not in normal cells suggests that the activity of the 'salvage' pathway is impaired in CVH and that the cells in patients are relying on the *de novo* pathway. This merits further study.

## ACKNOWLEDGMENTS

We thank Mrs A. Leversha and Mrs M. Moriarty for secretarial assistance.

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