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Altered deoxyribonucleotide pools in T-lymphoblastoid cells expressing the multisubstrate nucleoside kinase of *Drosophila melanogaster*

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The multisubstrate nucleoside kinase of *Drosophila melanogaster* (*Dm*-dNK) can be expressed in human solid tumor cells and its unique enzymatic properties makes this enzyme a suicide gene candidate. In the present study, *Dm*-dNK was stably expressed in the CCRF-CEM and H9 T-lymphoblastoid cell lines. The expressed enzyme was localized to the cell nucleus and the enzyme retained its activity. The *Dm*-dNK overexpressing cells showed ≈200-fold increased sensitivity to the cytostatic activity of several nucleoside analogs, such as the pyrimidine nucleoside analogs (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and 1-β-D-arabinofuranosylthymine (araT), but not to the antiherpetic purine nucleoside analogs ganciclovir, acyclovir and penciclovir, which may allow this technology to be applied in donor T cells and/or rescue graft vs. host disease to permit modulation of alloreactivity after transplantation. The most pronounced effect on the steady-state dNTP levels was a two- to 10-fold increased dTTP pool in *Dm*-dNK expressing cells that were grown in the presence of 1 μM of each natural deoxyribonucleoside. Although the *Dm*-dNK expressing cells demonstrated dNTP pool imbalances, no mitochondrial DNA deletions or altered mitochondrial DNA levels were detected in the H9 *Dm*-dNK expressing cells.

Nucleoside kinases are currently being investigated as suicide genes in gene therapy [1]. Nucleoside kinases phosphorylate nucleoside analog prodrugs into toxic metabolites that will induce cell death in the cells expressing the enzyme. However, the introduction of foreign genes, such as nucleoside kinases, into human cells may affect the metabolism of the target cells in more ways than just the therapeutic purpose of the introduced gene. The normal function of nucleoside kinases is to provide the cells with deoxyribonucleotides for DNA replication and repair. DNA replication is tightly controlled to avoid the introduction of mutations into the growing DNA chain. One level of control is the balanced supply of deoxyribonucleoside triphosphates (dNTPs) available for the DNA synthesis machinery [2]. It is essential that the concentration of each dNTP is maintained in proportion to the abundance of the different nucleotides in the DNA. Unbalanced dNTP pool sizes have been demonstrated to result in increased mutation rates [3]. Although the dNTP pool levels are highly regulated, the sizes of the different dNTP pools in cells differ. Several

Abbreviations
ACV, acyclovir; araT, 1-β-D-arabinofuranosylthymine; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; C-BVDU, carbocyclic (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; *Dm*-dNK, *Drosophila melanogaster* nucleoside kinase; dAdo, deoxyadenosine; dCyd, deoxycytidine; dGuo, deoxyguanosine; dNTP, deoxyribonucleoside triphosphate; dTTP, 2′-deoxythymidine 5′-triphosphate; dNs, deoxyribonucleoside; F-dUrd, 5-fluoro-2′-deoxyuridine; GCV, ganciclovir; GFP, green fluorescent protein; HSV-1 TK, herpes simplex virus thymidine kinase type 1; HU, hydroxyurea; I-dUrd, 5-iodo-2′-deoxyuridine; mtDNA, mitochondrial DNA; PCV, penciclovir.

investigations have demonstrated higher concentrations of 2′-deoxythymidine 5′-triphosphate (dTTP) and 2′-deoxyadenosine 5′-triphosphate (dATP) than of 2′-deoxycytosine 5′-triphosphate (dCTP) and 2′-deoxyguanine 5′-triphosphate (dGTP) in different mammalian cells [2,4]. There is an equilibrium with equal concentrations of dNTPs in the cytosol and in the cell nuclei as a result of the fact that dNTPs diffuse freely through the nuclear pores. However, mitochondria have been shown to have metabolically distinct dNTP pools [5–7], although recent studies indicate an exchange of dNTPs that involves a transporter between the mitochondrial and cytosolic compartments [8,9]. mtDNA (mitochondrial DNA) is replicated continuously throughout the cell cycle and thus needs a constant supply of nucleotides. Unbalanced mitochondrial nucleotide pools have recently been suggested to be involved in the pathogenesis of mitochondrial disorders, causing point mutations and deletions in the mitochondrial genome as well as mtDNA depletion [10,11].

The multisubstrate *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK) is sequence related to the human nucleoside kinases but the enzyme has a broader substrate specificity and higher catalytic activity [12,13]. We have previously shown that *Dm*-dNK can be expressed in human solid tumour cells with retained enzymatic activity and that it increases the sensitivity of the cells to several cytotoxic nucleoside analogs [14]. *Dm*-dNK catalyzes the phosphorylation of all the natural pyrimidine and purine deoxyribonucleosides with equally high turnover and with higher efficiency than the mammalian kinases [12]. Its catalytic rate of deoxyribonucleoside phosphorylation is, depending on the substrate, 10- to 100-fold higher than other studied kinases. This makes *Dm*-dNK a unique nucleoside phosphorylating enzyme and it deserves to be further investigated as a candidate suicide gene. The most studied suicide gene encoding a nucleoside kinase is the herpes simplex virus thymidine kinase type 1 (HSV-1 TK) gene that is used in combination with ganciclovir (GCV) [15]. The use of suicide gene therapy has recently been employed, in clinical trials of allogeneic stem cell transplantation, to permit modulation of alloreactivity after transplantation [16–18]. Donor T cells are genetically modified by insertion of a gene encoding a suicide gene, which makes the cells sensitive to a nucleoside prodrug. The suicide gene activates the prodrug into a highly cytotoxic metabolite that, in the event of graft vs. host disease, allows selective *in vivo* elimination, mediated by immunocompetent donor-derived T lymphocytes that damage the normal tissue in the recipient [19].

We have, in the present study, expressed *Dm*-dNK in T-lymphocytic cell lines and studied the level of enzymatic activity, the effects on nucleoside analog phosphorylation and the effects on the dNTP pools. With the knowledge that altered dNTP pools may damage cell functions, it is important to consider a possible imbalance of the dNTP pools in *Dm*-dNK-transduced lymphoblastoid cells as well as other metabolic effects of suicide genes to be used as therapeutic genes in clinical protocols.

Results

Expression of *Dm*-dNK in mammalian lymphoblastoid cells

We used a replication-deficient retroviral vector construct to express the *Dm*-dNK cDNA fused to the green fluorescent protein (GFP) (pLEGFP-*Dm*-dNK) (Fig. 1A). Two human T-lymphoblastoid cell lines – CCRF-CEM and H9 – were transduced with the retroviral vectors. Confocal microscopy of the transduced cells showed that the green fluorescence was localized in the nucleus of cells of both cell lines expressing *Dm*-dNK–GFP (Fig. 1B). After selection of cells that had stably integrated the transgene, flow cytometric analysis showed that $>95\%$ of the cells expressed *Dm*-dNK–GFP (Fig. 1C). The fluorescence level was still constant after several months, indicating an effective stable *Dm*-dNK gene transduction in both cell lines (data not shown).

In order to test the enzymatic activity of the *Dm*-dNK–GFP fusion protein and the level of nucleoside kinase activity in the cells, we determined the phosphorylation of deoxythymidine (dThd) in cell protein extracts. Untransduced cells or cells transduced with the control pLEGFP retroviral vector showed a similar, low level of dThd phosphorylation ($\approx$ 50–100 pmol·mg^{-1} of protein·min^{-1} in CEM and H9 cell lines, respectively). The CEM cells transduced with the pELGFP-*Dm*-dNK vector exhibited $\approx$21-fold higher enzymatic activity (1300 pmol·mg^{-1} of protein·min^{-1}) compared to the untransduced CEM cells, and the *Dm*-dNK expressing H9 cells showed $\approx$76-fold higher enzymatic activity (6000 pmol·mg^{-1} of protein·min^{-1}) compared to the untransduced H9 cells. These data demonstrate that *Dm*-dNK can be expressed with markedly retained enzymatic activity in these human T-cell lines.

Increasing sensitivity to nucleoside analogs in *Dm*-dNK expressing cells

We determined the sensitivity of the untransduced H9 and CEM cells and the cells transduced with either the

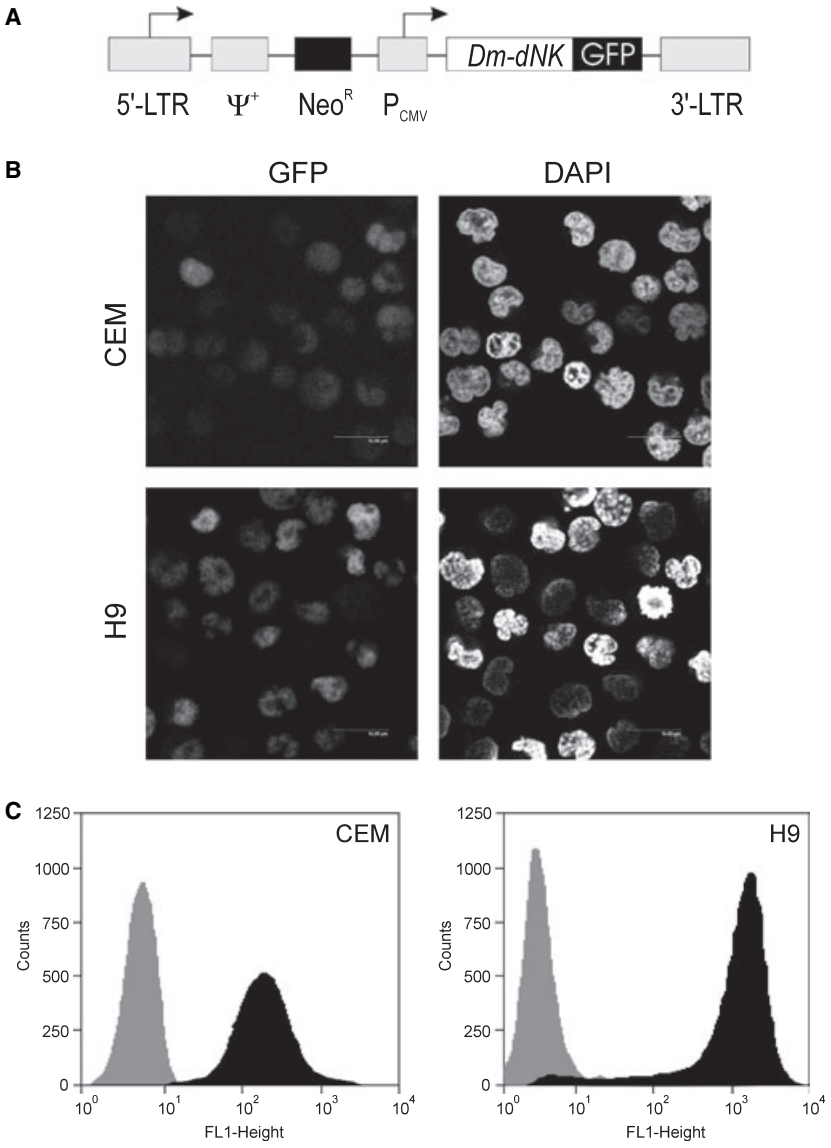

Fig. 1. Expression of *Drosophila melanogaster* nucleoside kinase-conjugated green fluorescent protein (*Dm*-dNK–GFP) in CEM and H9 cell lines. (A) Retroviral vector (pLEGFP-N1) used to insert the *Dm*-dNK cDNA in fusion with GFP. LTR, long-terminal repeat; ψ^+ viral packaging signal; *Neo*R, neomycin resistance gene, $P_{CMV,}$ cytomegalovirus promoter. (B) Confocal microcopy images of cells transduced with the recombinant virus. GFP fluorescence and 4′,6′-diamidino-2-phenylindole (DAPI) nuclear contrastaining showed that the *Dm*-dNK–GFP was located in the nucleus of both cell lines. (C) Flow cytometry analysis of the cells stably expressing *Dm*-dNK–GFP (black) and untransduced control cells (gray).

retroviral GFP vector alone or the *Dm*-dNK–GFP encoding vector to several cytotoxic nucleoside analogs (Table 1). The two T-cell lines that expressed *Dm*-dNK showed an increase in sensitivity towards several nucleoside analogs. The highest increase in sensitivity for the *Dm*-dNK expressing CEM cells was detected for (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and 1-β-d-arabinofuranosylthymine (araT), with an ≈200-fold decrease in the inhibitory concentration required to inhibit cell proliferation by 50% (IC_{50}) as compared to the GFP transduced control cells. 1-(2-Deoxy-2-fluoro-β-d-arabinofuranosyl)-5-iodouracil (Fialuridine, FIAU) showed a reduction of ≈28-fold in the IC_{50}, whereas 5-fluoro-2′-deoxyuridine (F-dUrd), 5-iodo-2′-deoxyuridine (I-dUrd) and carbocyclic BVDU (C-BVDU) showed a seven- to ninefold decrease in the IC_{50} compared with the control CEM cells, but not with H9 cells where there were no marked differences in cytostatic activity against the transfected vs. non-transfected cells. The molecular basis of the latter phenomenon, which was consistent for 5-F-dUrd and 5-I-dUrd, is still unclear. GCV, acyclovir (ACV) and penciclovir (PCV) were not markedly toxic to the CEM cells at the investigated concentrations. The highest increase in sensitivity for the H9 cells was observed for pyrimidine nucleoside analogs, in particular the dUrd analogs BVDU (with a > 300-fold increase in sensitivity) and FIAU (fialuridine) (with a 100-fold increase in sensitivity), whereas the sensitivity of dCyd analogs or any of the purine nucleoside analogs such as GCV, ACV, PCV and other drugs tested was not enhanced by *Dm*-dNK expression in this T-cell line.

Table 1. Sensitivity (IC_{50}) of green fluorescent protein (GFP) and *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK) transduced H9 and CEM cells to several nucleoside analogs. Values represent the IC_{50} (μM) ± SD of at least two to four independent experiments. IC_{50}, inhibitory concentration required to inhibit cell proliferation by 50%. 2-Chloro-dA, 2-chloro-deoxyadenosine; 5-F-dUrd, 5-fluoro-2′-deoxyuridine; 5-I-dUrd, 5-iodo-2′-deoxyuridine; ACV, acyclovir; araC, 1-β-D-arabinofuranosylcytosine; araG, 9-β-D-arabinofuranosylguanine; araT, 1-β-D-arabinofuranosylthymine; BVaraU, (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; C-BVDU, carbocyclic (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; ddC, 2′,3′-dideoxycytidine; dFdC, 2′,2′-difluorodeoxycytidine; dFdG, 2′,2′-difluorodeoxyguanosine; FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (fialuridine); GCV, ganciclovir; PCV, penciclovir.

	H9-GFP	H9-*Dm*-dNK-GFP	CEM-GFP	CEM-*Dm*-dNK-GFP
5-F-dUrd	0.013 ± 0.007	0.030 ± 0.003	0.017 ± 0.005	0.0024 ± 0.0006
5-I-dUrd	57 ± 30	30 ± 6	12 ± 2	1.80 ± 0.42
BVDU	> 500	1.47 ± 0.66	260 ± 66	1.25 ± 0.18
C-BVDU	> 500	428 ± 26	> 500	56 ± 12
BVaraU	> 500	> 500	279 ± 30	387 ± 93
FIAU	61 ± 10	0.61 ± 0.40	4.3 ± 0.1	0.14 ± 0.01
araT	> 500	24 ± 4	21 ± 3	0.086 ± 0.005
araC	0.030 ± 0.002	0.036 ± 0.008	0.038 ± 0.012	0.045 ± 0.020
ddC	30 ± 10	28 ± 16	1.9 ± 0.8	1.09 ± 0.061
dFdC	0.0090 ± 0.0032	0.0063 ± 0.0009	0.071 ± 0.006	0.057 ± 0.009
2-Chloro-dA	0.068 ± 0.012	0.11 ± 0.01	0.18 ± 0.01	0.12 ± 0.07
araG	149 ± 98	58 ± 20	0.39 ± 0.10	0.31 ± 0.01
dFdG	0.062 ± 0023	0.079 ± 0.089	0.035 ± 0.030	0.023 ± 0.014
GCV	147 ± 23	490 ± 13	240 ± 32	270 ± 146
ACV	> 500	> 500	282 ± 5.0	154 ± 23
PCV	> 500	> 500	244 ± 74	128 ± 23

Effects of *Dm*-dNK expression on dNTP pools

Dm-dNK has a higher catalytic activity compared to the endogenous deoxyribonucleoside kinases present in human cells [12,13]. The higher *Dm*-dNK activity may accordingly affect the dNTP pools and we decided to determine the steady-state intracellular dNTP concentrations in the cell lines. The dNTP concentrations were determined in cells cultured under three different conditions: medium supplemented with dialyzed serum that was devoid of exogenous nucleosides (Fig. 2A); medium with dialyzed serum supplemented with 1 μm dThd, deoxyadenosine (dAdo), deoxyguanosine (dGuo), and deoxycytidine (dCyd) (Fig. 2B); and medium containing dialyzed serum and 100 μm of the ribonucleotide reductase inhibitor, hydroxyurea (HU) (Fig. 2C). For the CEM cells grown in dialyzed medium and in medium containing 100 μm HU, the levels of dNTP pools were not significantly altered by the presence of the *Dm*-dNK activity, as compared to the untransduced control cells. However, the transduced *Dm*-dNK cells, grown in culture medium supplemented with 1 μm deoxyribonucleoside (dNs), showed a significant increase in the dTTP pool size ($P = 0.01$), twofold higher than the control (Fig. 2B).

The dNTP pools in each H9 cell line grown under normal culture conditions (medium supplemented with dialyzed serum) were highly asymmetric in the manner expected (dTTP > dATP > dCTP > dGTP) [20], whereas the *Dm*-dNK transduced cells showed a threefold increase ($P < 0.05$) in the dTTP pool compared to the control cell lines (Fig. 2A). The dCTP/dTTP ratios were 1 : 2 to 1 : 4 in these cells. In the presence of exogenous dNs, the *Dm*-dNK expressing H9 cells showed a significant increase of 10- and sixfold of the dTTP pool ($P < 0.01$) and of the dGTP pool ($P = 0.01$), respectively, compared with the control H9 cells (Fig. 2B). This changed the previous dNTP asymmetric order to dTTP > dGTP > dCTP > dATP. The dCTP/dTTP ratio in the *Dm*-dNK expressing H9 cells was 1 : 22. The dTTP pools in cells grown in dialyzed medium are probably derived predominantly from dTMP that has been synthesized through the de novo thymidylate synthesis. The increased dGTP levels can be attributed to a stimulatory effect of ribonucleotide reductase-catalyzed GDP reduction to dGDP by the higher dTTP levels.

The presence of HU resulted in similar dNTP pool levels of the *Dm*-dNK expressing H9 cells, as found in the same cells grown in dialyzed medium without HU.

Effects on mtDNA

In the light of the changed dNTP pools in *Dm*-dNK expressing H9 cells, we wanted to investigate whether the dTTP pool imbalance may have effects on the

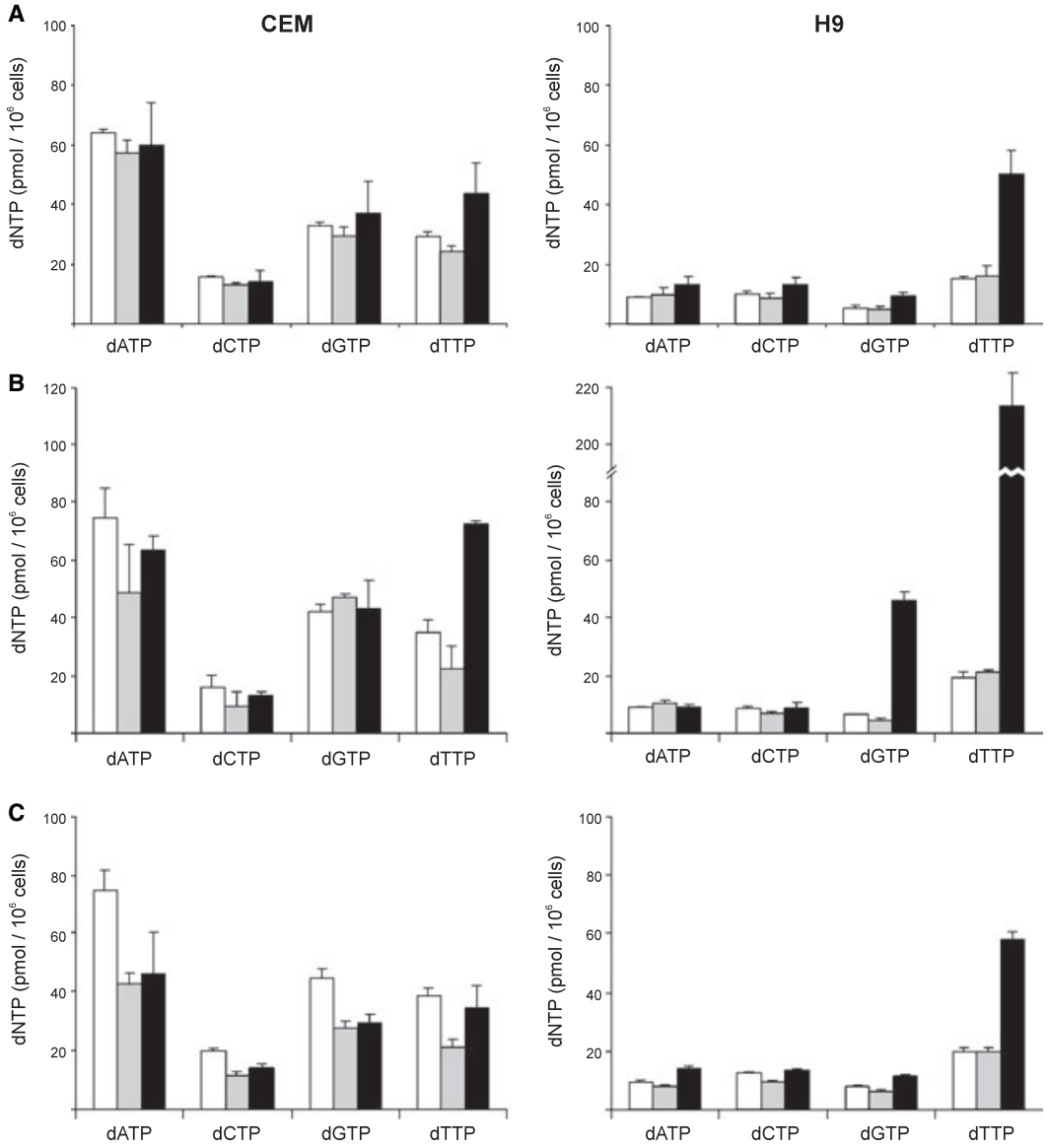

Fig. 2. Deoxyribonucleoside triphosphate (dNTP) pools in cells overexpressing *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK). Cells were cultured in normal culture medium, as described in the Experimental procedures, and supplemented with (A) only dialyzed serum, (B) dialyzed serum and 1 μM each of dThd, dAdo, dGuo and dCyd; or (C) dialyzed serum and 100 μM hydroxyurea (HU). The dNTP concentrations were determined in wild-type cells (open bars), cells transduced with a green fluorescent protein (GFP)-expressing vector alone (gray bars), and cells expressing *Dm*-dNK–GFP (black bars). Each data point represents the mean value ± SD of two separate experiments carried out in duplicate.

mtDNA. It has been suggested that a dTTP pool imbalance could account for replication errors in the mitochondrial genome, leading to both deletions and point mutations [11]. mtDNA of the three different H9 cell lines was analyzed by Southern blot (Fig. 3A) and quantitative real-time PCR (Fig. 3B). The cells had been grown for ≈10 months and analyzed regularly for the expression of *Dm*-dNK–GFP or the control GFP. The phenotype of the cells was found to be very stable during this time (data not shown). We were unable to detect any alteration in the mtDNA concentration, either by Southern blotting or by real-time PCR, and did not find an increase of mtDNA deletion in *Dm*-dNK-transduced H9 cells compared to the control cells. However, a faint band that hybridized with the mtDNA probe was visible in

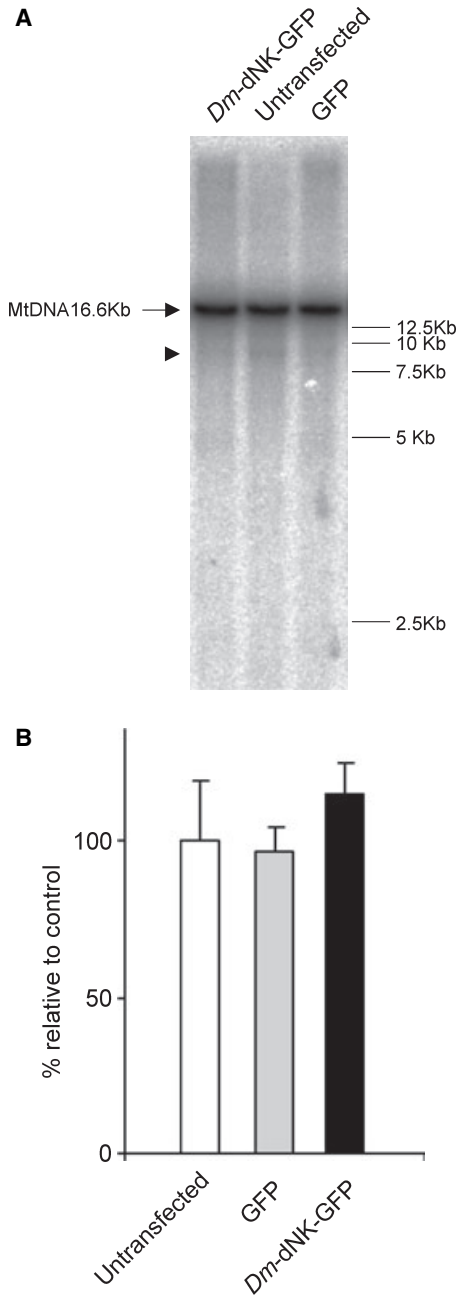

Fig. 3. Mitochondrial DNA (mtDNA) in H9 cells overexpressing *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK) (A) Southern blot analysis of the *Bam*HI mtDNA digest. (B) Quantification of mtDNA levels relative to controls. Results represent the mean value ± SD of two separate experiments carried out in quadruplicate (see the Experimental procedures).

mtDNA preparations from the wild-type control H9 cells as well as from the GFP- and *Dm*-dNK-expressing H9 cells. We estimated its molecular mass to be between 7.5 and 10 kb.

DNA, RNA and protein synthesis in the *Dm*-dNK gene transduced cells

The extent of DNA, RNA and protein synthesis in *Dm*-dNK expressing cells was compared to that in control cells by measuring the amount of incorporation of radiolabeled dThd and dCyd (DNA synthesis), Urd (RNA synthesis) or leucine (protein synthesis) in trichloroacetic acid-insoluble material after 20 h of cell incubation. Whereas there were no measurable differences in RNA or protein synthesis between the *Dm*-dNK expressing cells and their corresponding parental cell lines, the incorporation of dCyd was increased in the *Dm*-dNK expressing cells (by 1.6-fold for CEM *Dm*-dNK and by 3.3-fold for H9 *Dm*-dNK) and the incorporation of dThd was increased by 1.9-fold in H9 *Dm*-dNK cells, but not in CEM *Dm*-dNK cells (0.95-fold) (Fig. 4A,B). BVDU was also incorporated to a much greater extent into DNA of *Dm*-dNK-GFP gene expressing cells than into DNA of the parental

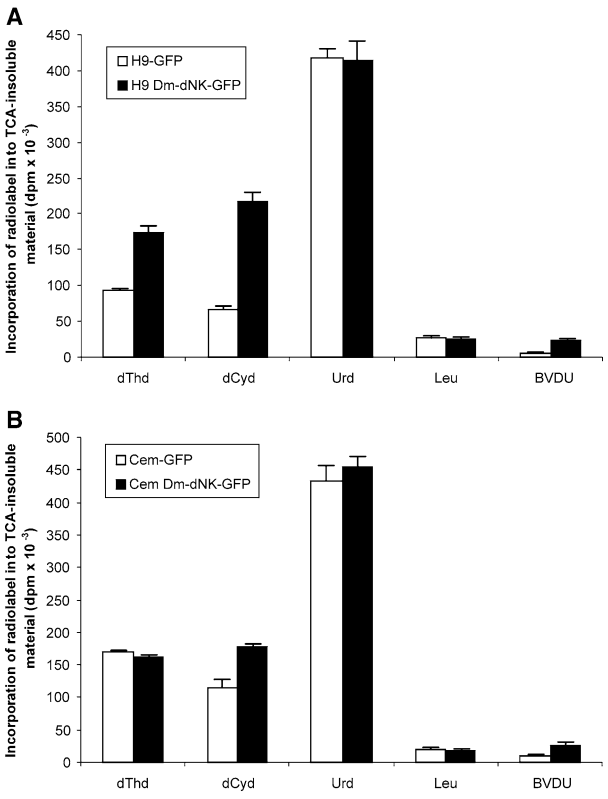

Fig. 4. Incorporation of macromolecular precursors in trichloroacetic acid-insoluble cell material. H9 (A) and CCRF-CEM (B) T-lymphoblastoid cell lines. Open bars represent the cells transduced with a green fluorescent protein (GFP)-expressing vector alone; closed bars represent the *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK)–GFP gene-transduced cell lines. Data represent the mean value ± SD of three experiments.

CEM and H9 cell lines (2-fold and 4-fold, respectively). However, when compared with dThd, BVDU was incorporated 4.6–5.1-fold less in the trichloroacetic acid-insoluble material of CEM and H9 cells.

Discussion

It has been previously shown that *Dm*-dNK can be expressed in solid tumor cells, such as human osteosarcoma cells [14]. In the present study we showed that *Dm*-dNK can also be stably expressed in T-lymphoblastoid cells with retained enzymatic activity. Our observations, and previous data on solid tumor cells, suggest that in addition to its use as a suicide gene in combined gene/chemotherapy of cancer, *Dm*-dNK can also be applied in donor T cells as a rescue in graft vs. host disease to permit modulation of the alloreactivity after transplantation. Indeed, the most efficient compound to be used in combination with *Dm*-dNK is BVDU, which is a selective anti-HSV compound that is nontoxic in cells not expressing *Dm*-dNK or HSV-TK. However, a major difference between *Dm*-dNK and HSV-TK is that *Dm*-dNK does not recognize the antiherpes purine nucleoside analogs GCV, ACV and PCV. The pronounced increased toxicity demonstrated for BVDU, FIAU and araT, but not for the acyclic purine nucleoside analogs GCV and ACV, correlate well with the pronounced substrate activity of purified *Dm*-dNK against the pyrimidine nucleoside analogs vs. the virtual inactivity of the purine derivatives as alternative substrates. Therefore, it could be an advantage to use *Dm*-dNK in donor T cells for bone marrow transplantation applications. Immunosuppressed patients often suffer from herpesvirus infections, such as HSV, varicella zoster virus and cytomegalovirus. If GCV or ACV is used to treat these infections, the compounds will also become activated in the suicide gene carrying T cells if HSV-TK is used as the suicide gene. If, instead, *Dm*-dNK is used as the donor T-cell suicide gene, only BVDU (not GCV, ACV or PCV) will affect these cells. This could be a very favorable characteristic for *Dm*-dNK as a suicide gene in well-defined applications such as allogenic stem cell transplantations. For suicide gene therapy of cancer, however, the aim is to kill as many cancer cells as possible. In such cases other properties, like the efficient bystander effect of GCV, may be more important and the HSV TK/GCV approach may be more relevant.

We also investigated the effects of a stable expression of *Dm*-dNK on nucleotide metabolism. If suicide genes are to be used as a potential rescue mechanism in cell transplantation and other cell therapy systems, it is important to establish whether such genes will affect cell metabolism. The *Dm*-dNK is indeed a highly active multisubstrate enzyme and our study demonstrates, for the first time, the pronounced effect that this enzyme activity has on the dNTP pools. As it has been shown that there is an equilibrium and exchange of nucleotide pools between the cytosolic and nuclear compartments, we believe that the data obtained for the nuclear expression of *Dm*-dNK in this study would not be significantly different if the enzyme had been expressed in the cytosol. Recent studies suggest that long-term alterations of nucleotide pools may cause damage, especially to mitochondria [21]. The most pronounced effect was found for the dTTP pool, whereas the dATP and dCTP pools seemed to be highly regulated to maintain their levels. The dGTP pool was increased in the H9 cells, but not in the CEM cells.

Defects in nucleotide metabolism are known to cause certain immunological disorders, such as adenosine deaminase deficiency where increased dAdo is believed to cause immune cell toxicity. The most recent disorder suggested to be caused by nucleotide imbalance is mitochondrial neurogastrointestinal encephalomyopathy, an autosomal recessive disorder associated with multiple deletions and depletion of mtDNA in skeletal muscle [22] as well as mtDNA point mutations [23]. The disease is believed to be caused by mutations in the nuclear gene for thymidine phosphorylase, which results in increased levels of thymidine. This enzyme catalyzes the phosphorolysis of thymidine to thymine and deoxyribose 1-phosphate, and a deficiency of thymidine phosphorylase results in increased circulating levels of thymidine and deoxyuridine [24]. The toxic effects caused by thymidine phosphorylase deficiency are suggested to be through misincorporations in mtDNA as a result of the increased dTTP pool. As we found high dTTP pool levels that could mimic the situation in the mitochondrial neurogastrointestinal encephalomyopathy syndrome, we investigated whether we could detect any deletions in the mtDNA of *Dm*-dNK expressing H9 cells. Despite a dCTP/dTTP pool imbalance in the *Dm*-dNK expression in H9 cells, no alteration in mtDNA was observed compared to its parental cell line. There may be several reasons for the discrepancy between our results and those of previous reports. One of the most important differences may be the cell type used in the different studies. The toxicity of nucleosides, as well as the sensitivity towards cytotoxic nucleoside analogs, shows large variations between different cell types that may reflect the cell-specific pathology in patients with disorders in nucleotide metabolism.

The increased incorporation of dThd, dCyd and BVDU in DNA of the *Dm*-dNK gene-transfected cells

can be attributed to an increased preferential phosphorylation by *Dm*-dNK through the salvage pathway, as the doubling time of *Dm*-dNK is not essentially different from that of normal cells.

In conclusion, we have shown that human T-lymphoblastoid cells can be stably transduced with the *Dm*-dNK gene, resulting in pronounced expression of the enzyme. The *Dm*-dNK gene transduced cells are sensitive to the cytostatic activity of BVDU, but not to that of the antiherpetic drug, GCV. This property argues for *Dm*-dNK as an attractive alternative gene to control adverse reactions after cell transplantation, where patients may need treatment with GCV or ACV as a result of herpesvirus infections, without activation of the suicide gene induced toxicity. Our data also demonstrate effects on nucleotide metabolism in *Dm*-dNK expressing cells. It should be further investigated whether this imbalance of the nucleotide pools can cause damage in cells after long-term expression of *Dm*-dNK.

Experimental procedures

Construction of a retrovirus vector expressing *Dm*-dNK

We used a retrovirus vector, based on the Moloney murine leukemia virus, to generate a replication-deficient recombinant retrovirus containing the deoxyribonucleoside kinase cDNA of *Drosophila melanogaster*. Oligonucleotide primers containing engineered *Xho*I and *Bam*HI restriction enzyme sites were used to clone the open reading frame of *Dm*-dNK cDNA into the *Xho*I–*Bam*HI site of the pLEGFP-N1 vector (Clontech, Mountain View, CA, USA). The plasmids were purified by using the NucleoBond plasmid purification kit (Clontech). The DNA sequences of the constructed plasmids were verified by sequence determination using an ABI310 automated DNA sequencer (PerkinElmer Life Sciences, Boston, MA, USA).

Cell culture, production of viral particles and viral transduction of cell lines

RetroPack PT67 packaging cells (Clontech) were cultured in Dulbecco's modified Eagle's medium. The human T-cell lines, CCRF-CEM and H9 (American Type Culture Collection, Manassas, VA, USA), were grown in RPMI 1640 medium. The medium was supplemented with 10% (v/v) heat-inactivated dialyzed fetal bovine serum (Life Technologies Inc., Gaithburg, MD, USA), 100 $U \cdot mL^{-1}$ penicillin, and 0.1 $mg \cdot mL^{-1}$ streptomycin. All cells were grown at 37 °C in a humidified incubator with a gas phase of 5% CO_2. The cell cultures were tested for the absence of mycoplasma by using the Mycoplasma Plus PCR primer set (Stratagene, La Jolla, CA, USA).

The constructed pLEGFP and pLEGFP-*Dm*-dNK plasmid vectors were transfected into the packaging cells by using FuGENE 6 transfection reagent (Roche, Brussels, Belgium), according to the protocol provided by the supplier. Virus vector particle-containing supernatant was produced at 32 °C in tissue culture bottles (75 cm^2) and harvested 48 h after plasmid transfections. Virus supernatant was clarified by filtration through a 0.45 μm filter and immediately used to transduce the lymphoid target cells in 24-well tissue culture plates coated with RetroNectin 20 $\mu g \cdot cm^{-2}$ (Takara, Kyoto, Japan). Two days after transduction, the selection of T lymphocytes was started with 1 $mg \cdot mL^{-1}$ geneticin (Gibco, Paisley, UK) and was continued for 2–3 weeks. GFP positive cells were sorted by using a fluorescence activated cell sorter (FACaliber; Becton-Dickinson, Franklin Lakes, NJ, USA). The nuclei of the cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI). GFP and DAPI fluorescence was observed by using a Leica TCS SP2 confocal microscope.

Enzyme assays

Cell protein extracts were prepared as described previously [25]. Briefly, the assays were performed in a total volume of 50 μL containing 50 mm Tris/HCl, pH 7.6, 5 mm $MgCl_2$, 2.5 mm ATP, 5 mm dithiothreitol, 15 mm NaF, 100 mm KCl, 0.5 $mg \cdot mL^{-1}$ BSA, 0.5 μg of protein extract and 3 μm [methyl-^{3}H]dThd (Moravek Biochemicals, Brea, CA, USA). Aliquots of the reaction mixture were spotted onto Whatman DE-81 filters after 10 or 20 min of incubation at 37 °C. The filters were washed three times in 5 mm ammonium formate. The nucleoside monophosphates were eluted from the filter with 0.5 m KCl, and the radioactivity was determined by scintillation counting.

Compounds

The following compounds were used in the study: Fialuridine (FIAU), C-BVDU (P. Herdewijn, Rega Institute, Leuven, Belgium), araT (Sigma, St Louis, MO, USA), GCV (Roche), ACV (the former Wellcome Research Laboratories, Research Triangle Park, NC, USA), PCV (I. Winkler at that time at Hoechst, Frankfurt, Germany), BVDU (P. Herdewijn, Rega Institute, Leuven, Belgium), and F-dUrd (Aldrich Chemical Co., Milwaukee, WI, USA), I-dUrd (Sigma), 2′,3′-dideoxycytidine (D.G. Johns, at that time at the NIH, Bethesda, MD, USA), 1-β-d-arabinofuranosylcytosine (araC) (Upjohn, Puurs, Belgium), 9-β-d-arabinofuranosylguanine (araG) (R.I. Chemical, Inc., Orange, CA, USA), 1-beta-d-arabinofuranosyl-E-5-[2-bromovinyl] uracil (BV-araU) (provided by H. Machida, Yamasa Shoyu Co, Choshi, Japan), 2′,2′-difluorodeoxyguanosine (dFdG) (J. Colacino, at that time at Eli Lilly, Indianapolis, IN, USA), 2′,2′-difluorodeoxycytidine (dFdC) (J. Colacino, at that time at Eli Lilly), and 2-chloro-2′-deoxyadenosine (CdA) (Sigma).

Cell proliferation assays

Approximately 2.5×10^5–3×10^5 cells·mL^{-1} were seeded in 200 μL wells of 96-well microtiter plates in the presence of serial fivefold dilutions of the test compounds. The cells were then allowed to proliferate at 37 °C for 72 h. After this time period, control cells (in the absence of test compounds) were almost at the end of the exponential growth phase. The cell number was determined by use of a Coulter counter type ZM (Coulter Electronics, Luton, UK).

Analyses of dNTP pools

Extracts of dNTPs were prepared from CEM and H9 cells grown under the following different conditions: in normal culture medium [RPMI containing 10% (v/v) dialyzed serum, penicillin and streptomycin], in culture medium containing 1 μm dNs (dAdo, dCyd, dGuo, dThd), and in culture medium containing 100 μm HU. Twenty-four hours later, 1 μm dNs was added again to the cells that grew in the presence of dNs. For the preparation of extracts, after incubation for 48 h, 2×10^6 logarithmically growing viable cells from each cell line were harvested and washed several times with ice-cold NaCl/P_i. The cell pellets were dissolved in 100 μL of 0.3 M perchloric acid and incubated on ice for 20 min. After 3 min of centrifugation at 16 000 ***g***, 100 μL of TOF-neutralization buffer [1.5 mL of tri-*n*-octylamine (Sigma) and 3.5 mL of 1,1,2-trichlorotrifluoroethane (Fluka, St Louis, MO, USA)] were added to the supernatants, which were then shaken on ice for a further 20 min. The samples were then centrifuged for 3 min at 16 000 ***g***, and the upper aqueous phase of each sample was collected and snap-frozen in dry ice before storage in a −80 °C freezer until required for analysis.

A primer template mix was prepared through the ligation of a tailor-made oligo template (T; 5′-TTTGTT TGTTTGTTTGTTTGGGCGGTGGAGGCGG-3′) with a 14-mer primer (P; 5′-CCGCCTCCACCGCC-3′) in a ratio of 2 : 1 [26]. The ligation was performed in a buffer containing 50 mm Tris/HCl and 50 mm NaCl, pH 7.0, at 95 °C for 5 min·s^{-1} and thereafter slowly cooled to room temperature. The generated T/P mix was diluted to concentrations of 12–6 μM and stored at −20 °C until use.

The assays were performed in a final volume of 50 μL, and the assay mix contained 50 mm Tris/HCl, pH 8.3, 1 mm dithiothreitrol, 5 mm $MgCl_2$, 0.25 mg·mL^{-1} BSA, and 2.5 U·mL^{-1} complementary template [poly(dA-dT)-poly(dA-dT) for dATP and dTTP, poly(dI-dC)-poly(dI-dC) for dGTP and 0.5–0.25 μm T/P template for dCTP] [27]. In addition, the assays contained 1.1 μm of 9.1 Ci·mmol^{-1} [^{3}H]dTTP for dATP, [^{3}H]dCTP for dGTP and [^{3}H]dATP for the dTTP and dCTP assays, respectively. The reaction components were mixed together with 5 μL of a dNTP standard (0, 0.25, 0.5, 1, 2 or 4 pmol), or with 5–10 μL of cell extract, at 4 °C. The reactions were then started by the addition of 0.2 U *Escherichia coli* DNA Polymerase Klenow fragment and subsequent transfer to 37 °C. After 30 min (dATP and dTTP) or 60 min (dGTP and dCTP), 20 μL of the reaction mixtures were spotted onto Whatman DE81 filters. When the filters were dry they were washed three times (for 5 min each wash) in $NaHPO_4$, then rinsed quickly in milliQ-water and then in 70% (v/v) ethanol. The radioactivity that remained on the filters after washing was measured in 3 mL of Ready Safe liquid scintillation cocktail per filter by using a liquid scintillation counter. The data are shown as pmol per 1×10^6 cells normalized to the respective standard curve [28,29].

Data represent the mean of one representative experiment out of two. Each independent experiment was run in duplicate. Significant differences were compared with the control (wild type) and analyzed by the Student's *t*-test ($P < 0.05$).

Quantification of mtDNA

Extraction of genomic DNA was performed by using the Easy-DNA Kit (Invitrogen, Carlsbad, CA, USA). For each genomic DNA extract, the nuclear gene for the β-actin and the mitochondrial gene cytochrome *c* oxidase subunit I were quantified separately by real-time quantitative PCR. Primers were designed by using the software Primer Express (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). The primer sequences were: β-actin (Fwd: 5′-TCCTCCTGAGCGCAAGTACTC-3′; Rev: 5′-GCATTT GCGGTGGACGAT-3′; Probe: 5′-TGTGGATCAGCAAG CAGGAGTATGACGAGT-3′) and Cyto B (Fwd: 5′-CCG CTACCTTCACGCCAAT-3′; Rev: 5′-TGCAAGCAGGAG GATAATGC-3′; Probe: 5′-TCTTCCTACACATCGGGC GAGGCC-3′). 4,7,2′,7′-Tetrachloro-6-carboxy-fluorescein (TET) was chosen as the reporter dye for β-actin and 6-carboxy-fluorescein (FAM) as the reporter dye for cytochrome *c*. Reactions were carried out by using the TaqMan Universal PCR master kit (Perkin-Elmer Applied Biosystems) and the data were collected by using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The reaction volume was 50 μL, containing 25 μL of 2× TaqMan buffer, 0.2 μm forward primer, 0.4 μm reverse primer, 0.1 μm probe and 50 ng of DNA. Initial steps of the PCR were 2 min at 50 °C for AmpErase UNG enzyme activation, followed by a 10 min hold at 95 °C for its deactivation. Cycles ($n = 40$) consisted of a 15 s melt at 95 °C, followed by 1 min of annealing/extension at 60 °C. The final step was a 60 °C incubation for 1 min. A standard curve of 800, 400, 200, 100, 50 and 12.5 ng of genomic DNA of control H9 cells was included in each run, and the same genomic DNA values were used for both the nuclear and the mitochondrial gene quantifications. Each assay included genomic DNA standards (each concentration measured three times), nontemplate controls and the genomic DNA tested: H9

WT, GFP and *Dm*-dNK transduced cells (each sample measured four times).

β-Actin was used as an internal reference control to normalize relative levels of gene copy number. The data were analyzed by using the second-derivative maximum of each amplification reaction and relating it to its respective standard curve. The results from the quantitative PCR were expressed as the ratio of the mean mtDNA value to the mean nuclear DNA (nDNA) value for a given extract (mtDNA/nDNA). Furthermore, these values were expressed as a percentage related to the value resulting from the designated calibrator sample (wild type).

Southern blot analyses of mtDNA

Six micrograms of genomic DNA from each H9 cell line, purified by using the Easy-DNA Kit (Invitrogen), was digested with *Bam*HI and separated by electrophoresis on a 0.8% (w/v) agarose gel. The gel was blotted onto a nylon membrane (Hybond-XL; Amersham, Piscataway, NJ, USA). The filter was hybridized with a [^{32}P]dCTP[γP]-labelled near-full-length mtDNA probe (16.3 kb) [26]. Prehybridization, hybridization and washing were performed according to the instructions of the manufacturer of the membrane (Amersham). The washed membrane was analyzed by PhosphorImager.

Incorporation of precursors of macromolecular cell material in trichloroacetic acid-insoluble cell materials

To each well of a microtiter plate were added 10^5 H9-GFP, H9-*Dm*-dNK–GFP, CEM-GFP or CEM-*Dm*-dNK–GFP cells and 0.25 μCi [methyl-^{3}H]dThd (89 Ci·mmol^{-1}), 1 μCi [5-^{3}H]dCyd (18.4 Ci·mmol^{-1}), 1 μCi [8-^{3}H]BVDU (14.6 Ci·mmol^{-1}), 1 μCi [5-^{3}H]Urd (27 Ci·mmol^{-1}) or 1 μCi [4,5-^{3}H]leucine (152 Ci·mmol^{-1}). The cells were allowed to proliferate for 20 h at 37 °C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the content of the wells (200 μL) were transferred onto 25-mm glass fiber filters, mounted on a Millipore (Billerica, MA, USA) 3025 sampling Manifold apparatus. The filters were washed twice with cold NaCl/P_i, twice with cold 10% (v/v) trichloroacetic acid, twice with cold 5% (v/v) trichloroacetic acid and once with cold ethanol (70%; v/v). The radioactivity precipitated on the filters was then counted in a High-Safe II cocktail (Perkin-Elmer).

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