Thymidine Kinase of Mouse Spleen Cells in vivo and in vitro

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The thymidine kinase activity of mouse spleen cells was found to parallel their DNA-synthesizing ability, both in vivo and in vitro. In the former case, more than 90% of the activity of these cells was found in the cytoplasmic fraction. This activity was labile, as was its template mRNA, compared with the nuclear component. The nuclear enzyme increased during culture of the lymphocytes in vitro. Mitogenic stimulation with concanavalin A resulted not only in a greater enhancement of the nuclear activity, but also in a marked increase in the amount of cytoplasmic enzyme. This effect appeared to be mediated via stabilization of the mRNA for the cytoplasmic component. These differences have been considered, especially with respect to the cellular changes that occurred during culture and mitogenic stimulation.

Thymidine kinase (ATP-thymidine 5'-phosphotransferase; EC 2.7.1.75) has been investigated in numerous biological systems. Its precise role in the 'salvage' and 'de novo' synthesis pathways of dTTP remains far from clear, although regulatory functions in the synthesis of DNA in certain situations have been proposed (Cooper et al., 1966; Hatanaka & Dulbecco, 1967; Blakley & Vitos, 1968; Gordon et al., 1968; Klemperer & Haynes, 1968; Rothschild & Black, 1970).

The enzyme has been shown to vary in activity under a variety of conditions, mostly relating to the proliferative capacity of the cells studied (Bianchi et al., 1961; Bucher, 1963; Bresnick et al., 1964; Kit et al., 1965b). Thymidine kinase activity is high in mammalian tumours (Bresnick & Thompson, 1965; Bukovsky & Roth, 1965; Gordon et al., 1968; Bresnick et al., 1969; Sneider et al., 1969), after unilateral nephrectomy (Main et al., 1963; Mayfield et al., 1967), in adipose tissue after injection of growth hormone (Epstein et al., 1969), in foetal and regenerating liver (Weissman et al., 1960; Bianchi et al., 1961, 1962; Bresnick et al., 1964, 1970; Maley et al., 1965), and in a variety of DNA virus-infected cells (Nohara & Kaplan, 1963; Dubbs & Kit, 1964; Green et al., 1964; Kit et al., 1965a, 1970; Carp, 1967; Hatanaka & Dulbecco, 1967; Hatanaka et al., 1969). The intracellular distribution of the enzyme has also been shown to be altered as a result of changes in the biological situation (Baugnet-Mahieu et al., 1968; Adelstein et al., 1971).

Although the thymidine kinase activities of spleen (Stirpe & La Placa, 1971) and bone marrow (Feinendegen et al., 1966) have been found to be high, little work has been done on the nature and regulation of the enzyme in lymphoid tissue. The present paper deals with the enzymes found in mouse spleen cells both in vivo and after incubation in vitro, with particular reference to the effects of mitogenic stimulation in the latter case.

Experimental

Chemicals

\[ {\text{[Me}}^{3}\text{H}] \text{Thymidine (17-21 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.} \]

Thymidine, ATP, dTTP, actinomycin D, puromycin and concanavalin A were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; Nonidet P-40 was a gift of Shell Chemical Co., London S.E.1, U.K. Foetal bovine serum and RPMI 1640 culture medium (Moore, 1967) were obtained from Flow Laboratories, Irvine, Ayrshire, U.K. Streptomycin sulphate and benzylpenicillin were obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. All other reagents were of analytical grade.

Animals

Strains CBA and C3H mice were used as the source of spleen cells. Animals 7-12 weeks old were used unless otherwise indicated.

Spleen cell cultures

Spleens were obtained from mice immediately after cervical dislocation. All subsequent procedures were carried out at 4°C under sterile conditions, unless otherwise indicated. The spleen cells, in 5 ml of RPMI 1640 culture medium containing penicillin (600 µg/ml), streptomycin (100 µg/ml) and 5% (v/v) foetal bovine serum, per 10⁸ cells, were sieved through a 60-gauge stainless-steel mesh. After centrifugation at
700g for 10min, the suspension was sieved as before and centrifuged again. After resuspension in culture medium, the lymphocyte concentration was determined by haemocytometer counting of a suitable dilution of cells fixed in 1.5% (v/v) acetic acid. The concentration was adjusted to 3 x 10^6 cells/ml by the addition of complete medium. A portion of the suspension was removed for use as the 'in vivo' (non-incubated, day 0) sample. Portions (1 ml) were dispensed for incubation into flat-bottomed plastic vials with loose-fitting aluminium foil caps. The cultures were maintained in an atmosphere of 5% CO_2 in air at 37°C for 48h, unless otherwise indicated. Concanavalin A was included as indicated, at a final concentration of 4 μg/ml, to induce transformation.

**Rate of [3H]DNA synthesis**

[3H]Thymidine (2 μCi; 17 Ci/mmol) was added to duplicate cultures containing 3 x 10^6 cells/ml. These were incubated for 2h, then harvested by centrifugation as described. The cultures were resuspended in 1 ml of medium and centrifuged again. After resuspension in the same volume of medium, 0.1 ml of each was placed on Whatman 3MM discs. These were dried, washed twice with 10 ml of cold 10% (w/v) trichloroacetic acid per disc, and then with methanol. They were air-dried and the radioactivity was determined.

The rate of [3H]DNA synthesis was calculated on the basis of radioactivity (c.p.m.) incorporated into trichloroacetic acid-insoluble material/h per 10^6 cells. DNA synthesis was linear with time during this period at the cell concentration used.

**Preparation of cell extracts**

Suspensions of mouse spleen cells were centrifuged and resuspended at a concentration of 1 x 10^7-5 x 10^7 cells/ml in 0.25M-sucrose-3.3 mM-CaCl_2-0.25% (v/v) Nonidet P-40 (lysis buffer). After 15 min to allow for disruption of the cell membrane, the lysate was centrifuged at 700g for 10min. The crude cytoplasm was removed and used for assay of enzyme activity. The pellet contained the nuclei as well as a small proportion of intact cells (less than 1%). This crude nuclear fraction was resuspended in the lysis buffer and used for assay of enzyme activity. Recovery of the nuclei was never less than 90-95% as determined by direct haemocytometer counts after fixation.

Fractionation of the cytoplasm was accomplished by centrifugation at 10000g for 15 min to sediment the mitochondria, and at 105000g for 60 min to remove the microsomal fraction. These pellets were gently resuspended in the lysis buffer for the determination of thymidine kinase activity.

The crude nuclear pellet was purified by gently layering the suspension over 2 vol. each of 30 and 60% (w/v) sucrose containing CaCl_2 and detergent. After centrifugation at 700g for 10min, the nuclei-free supernatant was removed, and the purified nuclei were obtained from the 30% sucrose layer and 30/60% sucrose interface. The recovery of nuclei in this procedure, in terms of recovery of radioactive DNA by using cells pre-labelled with [3H]thymidine as described, was never less than 80%. Unbroken cells migrated to the bottom of the tube. The purified nuclei were diluted in the lysis buffer, centrifuged at 700g for 10 min and suspended in this buffer at a concentration of 2 x 10^7-1 x 10^8 nuclei/ml, for enzyme assay.

**Determination of thymidine kinase activity**

The incubation mixture contained the following components in a final volume of 100 μl: enzyme extract, 50 μl; ATP, 0.5 μmol; Tris-HCl, pH 8.0, 1.0 μmol; MgCl_2 1.0 μmol; NaF, 1.5 μmol; [Me-3H]-thymidine, 2.0 nmol (specific radioactivity 250 mCi/ml). The reaction was initiated by transfer from 4°C to a 37°C water bath. The incubation was carried out for 15 min, after which the tubes were placed in a boiling-water bath for 2 min. A 2.5 cm-square piece of DEAE-cellulose paper (Whatman DE-81) was folded and inserted into each tube. Absorption of the phosphorylated products was allowed to proceed for 5 min. The tubes were filled with water and placed in a boiling-water bath to a depth of approx. 2.5 cm above the bottom of the tube, for 5 min. The elute, containing non-absorbed substrate, was decanted from each tube, and the washing process repeated two more times. The squares were then washed with methanol, dried at 60°C and placed in counting vials with 10 ml of scintillation fluid [0.0125, 1,4-bis-(5-phenyloxazol-2-y1)benzene and 0.6% 2,5-diphenyloxazole in toluene]. All assays were done in duplicate, which did not differ by more than 10%.

The radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer, model 3385. The amount of label retained on the DEAE-cellulose in the absence of enzyme was less than 0.6% of the input into the assay. This blank value was subtracted from all samples.

Under the conditions of assay, the rate of formation of dTMP was constant for at least 30 min and was proportional to the enzyme concentration. dTMP accounted for at least 95% of the phosphorylated products in control experiments, as determined by t.l.c. on polyethyleneimine-cellulose plates (Schleicher and Schull, Dassel, W. Germany) in a solvent of 0.02 M-NaHCO_3—0.005 M-Na_2B_4O_7.

One unit of thymidine kinase activity is defined as that quantity of enzyme which converts 1 pmol of thymidine into dTMP/min under the conditions of
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assay. The specific activity is defined as units per mg of protein, determined by the method of Lowry et al. (1951) with bovine γ-globulin as a standard.

Results

Thymidine kinase activity during development

The activity of thymidine kinase in extracts of mouse spleen lymphocytes varied as a function of age, as shown in Fig. 1. The specific activities of both the cytoplasmic and nuclear fractions were greatest soon after birth, and steadily declined thereafter. More than 90% of the total activity was found in the cytoplasmic fraction at all ages. Similar changes in the 'soluble' thymidine kinase activity have been found during the development of mammalian liver (Klem perer & Haynes, 1968; Adelstein et al., 1971). Fig. 2 shows the incorporation of [3H]thymidine into mouse spleen DNA in vitro, also as a function of age. This pattern was identical with the rate of [3H]DNA synthesis measured by injection of [3H]thymidine into intact mice (I. Olsen & G. Harris, unpublished work), and both parallel the activity of thymidine kinase.

Subcellular distribution of thymidine kinase activity

Although the soluble supernatant has been used as the source of enzyme in most studies, the presence of a particulate form of enzyme has been reported in mouse (Adelstein et al., 1971), rat (Baugnet-Mahieu et al., 1968; Stirpe & La Placa, 1971) and human (Taylor et al., 1972) liver, and rat adrenal tissue (Masui & Garren, 1971). No activity was found by these investigators in the nuclear fraction. To determine whether the low enzyme activity associated with mouse lymphocyte nuclei was due to cytoplasmic contamination and/or intact cells, the nuclei were purified by centrifugation through a sucrose gradient. Table 1 demonstrates that the major proportion of the activity in this fraction remained nuclear-bound, and also that only negligible amounts were associated with the small number of intact cells which migrated to the bottom of the gradient.

The phosphorylated products of the activity of this nuclear fraction have also been analysed, as described in the Experimental section, and have been found to consist of more than 95% dTMP. The absence of nuclear activity observed by other workers may have been due either to possible disruptive effects resulting from the homogenization techniques used by these workers, which we have avoided by the use of the Nonidet detergent, or to a real difference between the tissues.

Fractionation of the cytoplasm by high-speed centrifugation to remove mitochondria and microsomal fraction showed that the activity was not particulate (Table 1), in contrast with previous studies with mammalian liver mentioned above.

![Fig. 1. Thymidine kinase activity of mouse spleen cell extracts as a function of age](image1)

Details are given in the Experimental section. The left-hand axis shows the specific activity of the cytoplasmic enzyme (●) and the right-hand axis the nuclear enzyme (○).

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![Fig. 2. Synthesis of [3H]DNA by mouse spleen cells in vitro](image2)

Freshly prepared suspensions of mouse spleen cells were cultured in duplicate and the rate of [3H]DNA synthesis was measured as described in the Experimental section.
Table 1. Intracellular distribution of thymidine kinase activities in mouse spleen cells

The distribution of activity was determined in cells obtained directly ('in vivo') and after incubation for 2 days in the presence of concanavalin A. In view of the low nuclear activity, approx. 10 times the number of cells were used for the nuclear fractionation as for the cytoplasmic, in the case of the non-incubated cells. Details of the preparations are given in the Experimental section.

<table>
<thead>
<tr>
<th>Enzyme fractions</th>
<th>In vivo (+concanavalin A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic preparation</td>
<td></td>
</tr>
<tr>
<td>Crude cytoplasm</td>
<td>1.55</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.14</td>
</tr>
<tr>
<td>Mitochondria-free supernatant</td>
<td>1.29</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>0.07</td>
</tr>
<tr>
<td>105,000 g supernatant</td>
<td>1.07</td>
</tr>
<tr>
<td>Nuclear preparation</td>
<td></td>
</tr>
<tr>
<td>Crude nuclei</td>
<td>0.98</td>
</tr>
<tr>
<td>Non-particulate fraction</td>
<td>0.14</td>
</tr>
<tr>
<td>(0.25 M sucrose)</td>
<td></td>
</tr>
<tr>
<td>Purified nuclei (30% sucrose plus 30/60% sucrose interface)</td>
<td>0.68</td>
</tr>
<tr>
<td>Pellet</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Thymidine kinase activity during culture of mouse spleen lymphocytes

Mouse spleen cells cultured in vitro can be stimulated by various mitogenic substances to develop from inactive mononuclear cells into large transformed blast cells very active in DNA synthesis [for review, see Müller (1972)]. The rate of DNA synthesis in the cultures transformed with concanavalin A was at least 10-fold greater than that in the non-stimulated cultures, after 2 days of incubation (Fig. 3). A progressive loss of DNA-synthesizing ability was observed in these latter cultures. Transformed cells have in addition been found to have vastly increased thymidine kinase activity. Fig. 4 shows the changes in the cytoplasmic and nuclear activities during culture. The specific activities of the nuclear fractions increased markedly and were at a maximum after 2 days of incubation, in both the absence and the presence of concanavalin A (although the increase was greater in the latter case). In contrast, although the cytoplasmic activity also increased in transformed cells, and reached a maximum after 2 days, the non-stimulated culture showed a progressive decay of the specific activity initially found in this fraction. This observation is consistent with the decline in the DNA-synthesizing ability of these cultures, and demonstrates that this synthesis is probably related to the cytoplasmic, but not the nuclear, thymidine kinase activity. A similar decay of the activity of the 'soluble' enzyme has also been reported during long-term culture of mouse fibroblasts (Weissman et al., 1960; Littlefield, 1965).

The changes in intracellular distribution were also reflected by the observation that whereas 90–95% of the total units of enzyme activity were found in the cytoplasm of non-incubated ('in vivo') cells, this fraction accounted for only 45 and 75% of the total activity of lymphocytes incubated for 2 days in the absence and presence of concanavalin A respectively.

Fractionation of the cytoplasmic and nuclear activities from mouse spleen cells cultured for 2 days in the presence of concanavalin A demonstrated that, as with the enzyme found in vivo, the cytoplasmic activity was not particulate and that the high nuclear activity was not due to cytoplasmic contamination and/or intact cells (Table 1).

Half-life of thymidine kinase and its template

In view of these changes observed in the thymidine kinase activities of mouse spleen lymphocytes cultured in vitro, the stability of the enzymes and their
Mouse spleen cells were incubated in vitro as described in the Experimental section. The changes in the cytoplasmic (■, △) and nuclear (□, △) specific activities were determined after 1, 2, and 3 days of culture in the absence (□, ■) and presence (△, △) of concanavalin A (Con A). The results are presented on a logarithmic scale as the percentage of the original non-incubated enzyme activity. These were 39 and 3.6 units/mg of protein for the cytoplasmic and nuclear enzymes respectively.

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For this purpose actinomycin D and puromycin were used at concentrations that inhibited [3H]uridine incorporation into RNA and [3H]leucine incorporation into protein in these cells by 94 and 96%, respectively, within 1.5 h. As shown in Fig. 5, the cytoplasmic activity decayed with a half-life (t4) of approx. 2.9 h when the cells were initially placed under tissue culture conditions. This decrease was unaffected by the presence of actinomycin D or puromycin.

The nuclear activity, on the other hand, increased after an initial lag period lasting about 1.5 h, and was unaffected by the presence of actinomycin D during 6 h of culture (Fig. 5). Puromycin was observed to prevent the continued synthesis of this enzyme after 4.5 h. Splenic lymphocytes would therefore appear to have relatively stable templates for the nuclear thymidine kinase component. Concanavalin A had little if any effect on the changes in either cytoplasmic or nuclear activities during the first 6 h of culture, in contrast with the reported very rapid stimulation by this mitogen of 'early' lymphocyte protein synthesis (Wettenhall & London, 1974).

Fig. 6 shows that, after 2 days of incubation in vitro, the cytoplasmic enzyme decayed with a t4 of 3.6 h and its mRNA with a t4 of 5.9 h. In concanavalin A-stimulated lymphocyte cultures, however, although the t4 of the enzyme was similar (2.75 h), there was no detectable effect on enzyme activity resulting from the presence of actinomycin D during the 6 h period of incubation. These results are consistent with those presented above, showing a net loss of cytoplasmic activity in non-stimulated cultures and its enhancement in mitogen-transformed lymphocytes after 2 days of incubation in vitro. Further, these data suggest that the concanavalin A effect is mediated via stabilization of mRNA, and provides support for the postulate that the stability of the mRNA template is related to the DNA-synthesizing ability of the cell (Bresnick et al., 1967).

The values obtained in this work for the t4 of the cytoplasmic enzyme are in good agreement with previous estimates of 2.6–3.8 h found in vivo in neonatal, regenerating, and adult rat livers (Bresnick et al., 1967; Bresnick & Burleson, 1970). The mRNA for this enzyme has been reported to have a t4 of 6.8 (Adelstein et al., 1971) and 7.5 h (Bresnick et al.,...
Mouse spleen cells were cultured for 44 h as described previously. Actinomycin D (10 μg/ml) (■, □) or puromycin (50 μg/ml) (▲, △) was added, and a suitable number of cultures was removed for enzyme assay at the time-periods indicated. The results are shown, on a logarithmic scale, as a percentage of the activity present at the time of addition of the inhibitors. In the absence of the inhibitors, the enzyme activities changed less than 10% during the course of the experiment, in both the non-stimulated (■, ▲) and concanavalin A (Con A)-transformed (□, △) cultures.

1967) in mouse liver and regenerating rat liver respectively, although it is important to note that in the less proliferative adult rat liver a value of 3.0h has been found (Pitot et al., 1965; Bresnick et al., 1967).

The high nuclear activities of both non-stimulated and mitogen-transformed lymphocyte cultures were completely unaffected by the presence of actinomycin D and puromycin over a 6 h period during the second day of culture. Similar stability of activity in the absence of new protein synthesis has also been noted for the particulate (mitochondrial) enzyme of mouse liver (Adelstein et al., 1971).

**Enzyme stabilization in tissue culture**

Thymidine kinase has previously been shown to decay during incubation of mouse fibroblasts in vitro. The loss of activity was prevented by the inclusion of the substrate, thymidine, in the culture medium, resulting in enzyme stabilization (Littlefield, 1965) rather than induction (Weissman et al., 1960).

In cultures of mouse spleen lymphocytes, thymidine at concentrations between 50 and 500 μM resulted in enhancement of the nuclear activities of both non-stimulated and transformed cells, to more than 250% of the value found in the respective (no thymidine) control cultures (Fig. 7). In contrast, incubation in the presence of low concentrations of the substrate led to an initial large decrease in the activity found in the cytoplasmic fraction. This may have resulted from an increase in the intracellular pools of thymidine or thymidylate, which had not been washed out of the cells and thereby interfered in the assay itself. At higher thymidine concentrations, the cytoplasmic activities increased in a manner similar to that observed in the nuclear activities at these substrate concentrations. These changes may be associated with the inhibition of growth caused by high thymidine concentrations, as reported in cultured Chang liver cells (Eker, 1968a). Alterations in the intracellular concentrations of metabolites that repress the synthesis of thymidine kinase, as a result of drug-induced inhibition of DNA synthesis, have been proposed to explain these findings (Eker, 1968b).

**Discussion**

Lymphocytes isolated from mouse spleen have been shown in these investigations to contain both soluble and nuclear-bound thymidine kinase activities. These were distinguishable on the basis of enzyme and template stabilities, and of the marked changes that occurred in subcellular distribution during incubation of these cells in vitro.
The activity of cytoplasmic enzymes reflected more precisely the rates of DNA synthesis, which declined, from the values estimated at isolation of these spleen cells from the mouse, in the absence of mitogenic stimulation, and which was enhanced during their transformation in vitro by concanavalin A. In this latter situation, the mRNA for the cytoplasmic thymidine kinase showed a far greater stability, on the basis of lack of inhibition in the presence of actinomycin D.

The increase in the activities of the nuclear enzymes has hitherto not been described. Further studies are required to determine whether these increases resulted from mitogen-induced quantitative alterations in the amounts of pre-existing enzymes, or from the appearance of entirely new protein species. Preliminary experiments suggest that these enzymes are biochemically distinguishable, as has also been shown for foetal and regenerating liver compared with normal adult liver (Bresnick et al., 1967; Klemperer & Haynes, 1968; Taylor et al., 1972), of DNA virus-infected compared with uninfected cells (McAuslan, 1963; Frearson et al., 1965; Kit & Dubbs, 1965; Kit et al., 1966, 1970; Sheinin, 1966; Carp, 1967; Klemperer et al., 1967; Hatanaka & Dubbecco, 1969; Munyon et al., 1971), and of neoplastic compared with normal tissues (Gordon et al., 1968). Further, it is possible that the nuclear and cytoplasmic thymidine kinase activities may reflect varying degrees of subunit aggregation and may be interconvertible, as has been shown for DNA polymerase (Lazarus & Kitron, 1973).

In view of the enzymic changes described here, it is important to consider the cellular changes that occurred during the incubation of mouse spleen lymphocytes in vitro. The composition of the mouse spleen is highly heterogeneous with respect to cell types. As well as initially inactive cells of the lymphoid series, precursors for myelopoiesis, erythropoiesis, monocytopoiesis and platelet production are also present. Thus the thymidine kinase analysed initially represented mainly enzyme derived from a variety of these proliferating precursor cells. In contrast with this, the responding population in cultured spleen cells, with respect to DNA synthesis, are mainly composed of the small lymphocytes which were not active in this respect at the time of initiation of the culture.

The major cell response to concanavalin A stimulation is considered to be that of thymus-derived small lymphocytes ('T'-cells). Their role in the immune response has been widely documented [for review, see Möller (1972)]. After stimulation with concanavalin A, these cells transform into large blast cells very active with respect to DNA synthesis. It is primarily the enzymes found in these cells which have therefore been studied here. Even in the absence of the mitogen, incubation in the presence of foetal bovine serum has been shown to evoke low 'spontaneous' transforma-

References


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