Europ. J. Cancer, Vol. 15, pp. 11–16

0014-2964/79/0011-0101 $02.00/0

# A Transplantable Mouse Tumor Deficient in Cyclic-AMP-Dependent Protein Kinase Activity*

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**Abstract**—*A transplantable solid tumor deficient in cyclic-AMP-dependent protein kinase (PK) (ATP: protein phosphotransferase; E.C.2.7.1.37) activity was established* in vivo *following injection into recipient nude mice of mutant (PK⁻)S49 mouse lymphoma cells propagated* in vitro *in suspension culture. Tumor cell growth was not affected by dibutyryl cyclic AMP (DB-cAMP) when transferred back into culture. These results suggest that cyclic AMP (cAMP) is not essential for cellular growth both* in vitro *and* in vivo. *In addition it is suggested that the absence of cAMP dependent protein kinase is related to the tumorigenicity of the cells by rendering them less controlable by environmental stimuli in the host.*

## INTRODUCTION

CYCLIC-AMP is known to affect eukaryotic cells propagated in culture in a multitude of ways among which are cellular differentiation, cell cycle related phenomena and malignant transformation [1]. Cyclic-AMP causes growth arrest followed by cell death 48–72 hr after its administration to S49 mouse lymphoma cells in culture [2]. On the basis of this finding, it has been possible to select for, isolate and describe various mutants [3]. These have been partially characterized to the extent that their biological response to the drug could be directly related to specific lesions in either the membrane bound adenyl cyclase or the cyclic-AMP-dependent protein kinase or to a lesion distal to the protein kinase [4–7]. Cyclic-AMP has also been shown to cause growth arrest at the G-1 phase of the cell cycle. In mutant S49 cells lacking cyclic-AMP-dependent protein kinase activity, cells maintained a normal cell cycle in culture both in the absence and presence of cyclic AMP [8].

It may be argued that the kind of inter-cellular relationship in cells grown in culture does not hold true for the same cells growing *in vivo*. We have therefore undertaken to study the S49 mutant cells with respect to their ability to develop as *in vivo* tumors in recipient mice. Our attempts to grow the S49 cells back in BALB/c mice from which they were originally derived were unsuccessful. It is possible that the cultured S49 cells have changed to the extent that they are immunologically rejected. Therefore, cells were transplanted to congenitally athymic nude mice. We report here, the first successful induction of a transplantable tumor that lacks cyclic-AMP-dependent protein kinase activity from cultured lymphoma cells with the same phenotype. We suggest that cyclic-AMP is non-essential for the growth of this mutant both *in vivo* and *in vitro*.

Accepted 28 April 1978.

*This work was supported by grants from the Israel Commission for Basic Research, and the United States–Israel Binational Science Foundation (no.—1470) to J.H.

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## MATERIALS AND METHODS

*Cell culture*

Wild type S49 mouse lymphoma cells [9] and the variant clone-kin C [5] lacking detectable cyclic-AMP-dependent protein kin ase activity ($PK^-$) were maintained in stationary suspension culture in Dulbecco's modified Eagle's medium with 10% heat inactivated horse serum in a humidified atmosphere at 37°C containing 5% $CO_2$.

*Animals*

Congenitally athymic (nude) mice 8–10 weeks old (obtained from the animal breeding facilities of the Weizmann Institute, Rehovot) were inoculated intraperitoneally with $1 \times 10^7$ mutant ($PK^-$) S49 cells (80–90% viable cells as measured by trypan blue exclusion). Tumors appeared within 4–6 weeks following injection. When tumors exceeded 2 cm in diameter (5–10 g tumor weight), animals were killed and the tumor tissue removed. One part of the tumor was fixed by 10% formalin and stained with hematoxylyn and Eosin; another part was stored at $-70°C$ and used for protein kinase determination. The rest of the tumor was resuspended and grown in culture medium for further investigation.

*Protein kinase and cyclic-AMP binding assays*

Protein kinase was determined by measuring the transfer of $^{32}P$ from ($\gamma^{32}P$)-ATP (New England Nuclear) to histone in the absence and in the presence of $10^{-5}$ M cyclic-AMP as previously described [5]. Binding of [$^3$H] cyclic-AMP (New England Nuclear) to cell extracts and regulatory subunit of the protein kinase was measured by a modification of the Millipore® filter technique [10]. Binding activity was determined in the presence of $2 \times 10^{-7}$ M [$^3$H] cyclic-AMP. Protein was determined by the method of Lowry *et al.*, with bovine albumin as a standard [11].

*Recombination of catalytic and regulatory subunits*

Separation of the regulatory and catalytic subunits of the enzyme was carried out essentially as previously described [7, 12]. A constant amount of catalytic subunit activity (4 nmole/10 min/tube) from S49 cells with wild type protein kinase was incubated with varying amounts of separated binding protein from the tumor. Recombination was carried out for 20 hr at 4°C in a total volume of 350 μl. Percentage of recombination = $100 \times$ [A–B]/C, where A and B represent the kinase activity (after recombination) measured in the presence and absence of 10 μM cyclic-AMP respectively, and C is the amount of catalytic subunit added. Prior to recombination, the regulatory subunit was tested and found to be devoid of both cyclic-AMP-independent and cyclic-AMP-dependent kinase activities. The catalytic subunit was found to be devoid of both cyclic-AMP binding and cyclic-AMP-dependent kinase activities.

## RESULTS

Within 4–6 weeks 17 out of 20 mice inoculated developed swellings in their lower abdomen later manifested as solid tumors. Tumor tissue consisted mostly of lymphocytes, and cellular infiltration of skeletal muscle was quite prominent in most tumors (Fig. 1). Further analysis demonstrated that the tumors have retained the phenotype of the original mutant cells with respect to both biochemical and biological responses to cyclic-AMP. Thus, Table 1 demonstrates that the mutant lymphoma cells prior to transplantation into nude mice ($PK^-$), the tumor tissue itself, and the tumor cells after their transfer back into culture, all lack cyclic-AMP-dependent protein kinase activity. In addition, dibutyryl-cyclic-AMP (Db-cAMP) had no effect on the growth rate in culture of mutant cells whether or not they had previously been transplanted into mice (doubling time 16–18 hr). In the wild type cells, however, DB-cAMP is cytolytic and cells die within 48–72 hr following their exposure to the drug [2, 8]. That the

*Table* 1.

| | Protein kinase activity (nmole $^{32}P$/10 min/mg protein) | | | |
|---|---|---|---|---|
| | Cyclic-AMP-independent | Cyclic-AMP-dependent | Activity ratio +cAMP/−cAMP | Cyclic-AMP binding Pmole/mg protein |
| Wild type | 1.65 | 11.3 | 6.9 | 7.77 |
| Protein kinase deficient ($PK^-$) | 1.35 | <0.2 | — | 0.66 |
| Tumor | 1.54 | <0.2 | — | 0.70 |
| Post tumor cultured cells | 1.57 | <0.2 | — | 0.72 |

Protein kinase activity and cyclic-AMP binding in S49 lymphoma cells. Data are shown for wild type cells, $PK^-$ cells, transplanted tumor cells and tumor cells transferred back to culture, respectively.

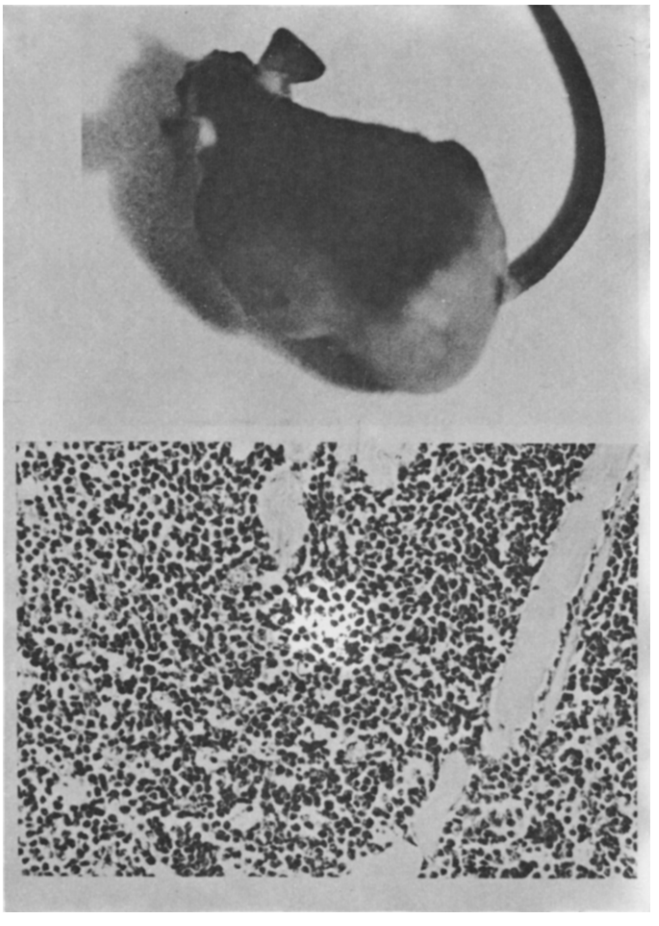

*Fig. 1. Nude mouse with a tumor induced by the inoculation of S49 cells lacking cyclic-AMP-dependent protein kinase activity (top) and a low power (×200) photomicrograph of the tumor (bottom).*

$PK^-$ phenotype is a stable somatic trait is manifested by the finding that as much as 11 consecutive passages of the tumor cells within nude mice did not alter their protein kinase deficient phenotype.

A small amount of cyclic-AMP binding activity (10%) relative to wild type cells was consistently found in all $PK^-$ cells (Table 1) grown both *in vitro* (suspension culture) and *in vivo* (tumor). The large amount of tumor tissue now available permits the investigation of the molecular nature of the cyclic-AMP binding capacity in the tumor cells in a detailed manner. Does that binding represent an authentic regulatory subunit of cyclic-AMP-dependent protein kinase? To clarify this question, we separated the cyclic-AMP binding protein from the tumour by affinity chromatography on a cyclic-AMP-Sepharose column and subjected it to recombination with the catalytic subunit of cyclic-AMP-dependent protein kinase prepared from wild type enzyme. Figure 2 demonstrates that the

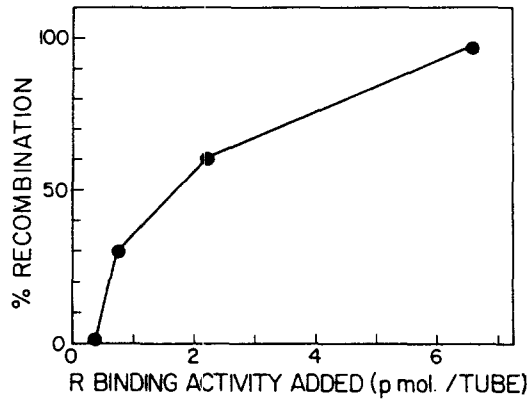


*Fig. 2. Recombination of catalytic subunit from S49 cells with cyclic-AMP binding protein from a tumor lacking cyclic-AMP-dependent protein kinase activity. A constant amount of catalytic subunit activity* (4 nmole/10 min/tube) *from* 549 *cells with wild type protein kinase was incubated with varying amounts of separated binding protein from the tumor for* 20 hr. *% Recombination was calculated as explained in the Method section.*

binding protein from the tumor cells recombines with the catalytic subunit, thus rendering it completely cyclic-AMP-dependent. A similar recombination curve was obtained upon recombination of wild type catalytic subunit with wild type regulatory subunit [7]. We therefore conclude that the binding activity in the tumor indeed represents the regulatory subunit of cyclic-AMP-dependent protein kinase.

## DISCUSSION

*In vivo* analysis of the S49 lymphoma cells was hampered due to the fact that both wild type and mutant cells did not develop as tumors in BALB/c mice from which they were originally derived. It is possible that during the years that these cells were propagated in culture [9] their histocompatibility antigen(s) have changed. Therefore, this may account for their inability to develop in BALB/c mice. We overcame the problem by growing the S49 cells in congenitally athymic nude mice which are immune deficient.

The finding that cells which lack any detectable cyclic-AMP-dependent protein kinase activity can develop as a solid (transplantable) tumor in nude mice, suggests that cyclic-AMP is a non-essential growth regulator in S49 mutant tumor cells both *in vivo* and *in vitro*. Recently we were able to induce transplantable tumor with wild type PK phenotype in nude mice (unpublished).

Various observations have suggested an inverse correlation between cyclic-AMP-dependent protein kinase and malignancy [13–16]. Our findings permit, for the first time, to directly investigate the relationship between the regulation of cyclic-AMP dependent PK and the *in vivo* control of tumor growth (manuscript in preparation).

An important observation in this paper is the finding that the $PK^-$ tumor has cyclic-AMP binding activity capable of recombining with the catalytic subunit of cyclic-AMP-dependent protein kinase. This suggests that $PK^-$ cells do contain a small amount of authentic regulatory subunit of protein kinase. It appears that the cell lacks PK activity, then, because it lacks C subunit activity.

However, it is not clear at the present moment whether the basis of the observed lesion is of a structural nature as previous PK mutants were shown to be [6, 7] or whether the lesion is of a regulatory nature.

**Acknowledgements**—We thank Rivka Mukan for skilful technical assistance. We wish to thank Drs. Bourne, Coffino and Melmon from the University of California, San Francisco, for kindly supplying us with the original S49 mutant cells.

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