Eur J Cancer Clin Oncol, Vol. 20, No. 12, pp. 1543–1548, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Production of the Effector Molecule Thymidine by Human Lung Alveolar Macrophages*

ALAIN VERCAMMEN-GRANDJEAN,†‡ ROLAND ARNOULD,† ANITA LIBERT,† PATRICIA EWALENKO§ and FERDY LEJEUNE†

†*Laboratory of Oncology and Experimental Surgery, Department of Surgery and § Department of Anesthesiology, Institut Jules Bordet, Rue Héger-Bordet* 1, 1000 *Brussels, Belgium*

Abstract—*Cultured human alveolar macrophages (HAMØ) were found to produce soluble factors which inhibit tritiated thymidine ([³H]-TdR) incorporation into tumour cells* in vitro. *We present evidence that thymidine (TdR) can be detected in HAMØ culture supernatants by thin-layer chromatography. Moreover, TdR secretion by HAMØ is an active process. Using* [6-¹⁴C]*-orotic acid as an early precursor to TdR and* [³H]*-TMP as the phosphorylation product, we were able to show that cultured HAMØ transformed them into labelled TdR. The very efficient thin-layer chromatography of the labelled metabolites was backed up by high-pressure liquid chromatography of nucleotides. HAMØ produce TdR by* de novo *synthesis and dephosphorylation. This phenomenon is due to the lack of thymidine kinase in normal mature macrophages. Since TdR, in high concentrations, can inhibit DNA synthesis through the 'TdR blockade' phenomenon, it is suggested that TdR secretion by HAMØ is a mechanism of non-specific modulation of tumour cell growth but highly restricted to the immediate macrophage microenvironment* in vivo. *The effectiveness of the thymidine gradient may thus be quite narrow, but is worthy of interest.*

INTRODUCTION

PREVIOUS works have shown that normal mouse peritoneal MØ produce *in vitro* soluble factors inhibiting [³H]-TdR incorporation by melanoma cells [1–3]. Thymidine is a potent cytostatic to which melanoma cells are sensitive in the range of a concentration produced by 10^6 macrophages/ml for 72 hr, which is 4×10^{-5}–1×10^{-5}M of TdR [1]. The range of sensitivity of various cell populations is quite large [4]. The 50% inhibition of DNA synthesis *in vitro* is 3×10^{-3}M for lectin-stimulated lymphocytes, 2.5×10^{-4}M for T1659 and P815 cell lines, 2×10^{-4}M for L1210 cell lines [5], 4×10^{-5}–1×10^{-5}M for melanoma cell lines and 1×10^{-6}M for EL-4 cell lines. Normal macrophages produce cold thymidine because they are thymidine kinase-deficient [4, 6]. Loss of this regulatory enzyme depletes the cell of its retroinhibitory capacity of TdR production by TTP.

Thymidine is thus one of the effector molecules produced by the macrophages which can have a modulatory action on cells present in their microenvironment depending on individual threshold sensitivity to this nucleoside. We use the word modulation for that purpose. The threshold between inhibition and stimulation is difficult to apprehend.

Human alveolar MØ (HAMØ) represent a good source of human tissue MØ. It was interesting to test whether HAMØ produced the nucleoside thymidine, as had been shown for mouse MØ [1]. Thus in order to test the hypothesis of the similitude of metabolism in HAMØ and mouse PMØ, especially those of DNA with nucleosides and nucleotides, we have used a bioassay of incorporation of nucleic acid precursors ([³H]-TdR), the transformation of [6-¹⁴C]-orotic acid into [6-¹⁴C]-TdR by HAMØ and HPLC of different supernatants of cells in culture, and macrophages.

Accepted 21 June 1984.
*This work was supported by grant 3.4526.83 from the Fonds de la Recherche Scientifique Médicale (FRSM) and from the Loterie Nationale through the FRSM.
‡To whom requests for reprints should be addressed.
Abbreviations—MØ: macrophage(s); HAMØ: human alveolar macrophages; TdR: thymidine; FCS: foetal calf serum; PBD: phosphate-buffered saline; BME: basal medium (Eagles); [¹²⁵]I-UdR: iodo deoxyuridine; TCA: trichloroacetic acid; HPLC: high-pressure liquid chromatography; TLC: thin-layer chromatography; H.I.: heat inactivated.

High concentrations of TdR are necessary to inhibit tumour cell growth *in vivo* [7, 8]. Perfusions of g/kg in mice and man for a period of time [9] are necessary to show tumour regression. Furthermore, thymidine alone does not seem to be very promising while co-administration with 5-FU [9–11] or 3'-chloroethylnitrosourea arabinosylcytosine [12] is efficient. Thymidine can also be a rescue drug, as is methotrexate [8].

The modulation of tumour cell growth by TdR produced by macrophages, attractive as it may appear, is probably restricted to a very small gradient of metabolite in the immediate microenvironment of the cell. It is, however, fundamentally interesting to try to measure and describe the effectors produced by these immunological competent cells. Many other substances produced by these cells such as interleukins are being described and analytically defined. Their action toward the high tumour burden may never be as spectacular as those of new drugs, but they may play a role in a more discrete and constant way.

Thymidine is a good example of how a very common and natural metabolite can have the same type of action on various cells but where the sensitivity of the target can vary by 300-fold.

MATERIALS AND METHODS

Peritoneal macrophages

Macrophages were harvested from C57BL, BALB/c, BDF1 (Charles River Inc.) and GIF (CNRS Gif-sur-Yvette) mice by peritoneal washing with 3 ml of Dulbecco's modified medium containing 25 mM HEPES, L-glutamine and 10% H.I. FCS (Gibco-Biocult.). Following adherence of the cells to the plastic of the culture vessel, non-adherent cells were washed out by rinsing three times with PBS [13–15].

Transformed macrophages

IC21 [16] was a gift from Dr Mauel, Epalinges-Lez-Lausanne, Switzerland; J774A and P388D1 were gifts from Dr Van Loveren, Utrecht University. These cells were grown as monolayers in 75-cm^2 Nunc culture bottles with Dulbecco's modified Eagle's medium containing 25 mM HEPES and 10% H.I. FCS, and passaged as the other tumour cells.

Tumour cells

The HM6 human melanoma cell line established in our laboratory from subcutaneous metastasis and the B16 mouse melanoma cell line from C57BL tumour-bearing mice at passage 5–10 *in vitro* were used. The monolayers were cultured in HAMF 10 (Gibco-Biocult.) with L-glutamine and 10% FCS. Passage of the cells was done with a solution of trypsin (0.05%) and EDTA (0.025%) in PBS.

L cells: L 925/25 cells were cultured in Dulbecco's modified medium with antibiotics and 10% FCS. Cells were passaged by trypsinization with trypsin 1/300 in PBS without EDTA.

Human alveolar macrophages

HAMØ were isolated by bronchoalveolar lavage after informed consent from patients undergoing surgery for stage I curable melanoma or benign tumours. Briefly, after intravenous hypnotic drug injection the trachea was intubated and the lungs mechanically ventilated with a mixture of oxygen and nitrous oxyde.

A fibreoptic bronchoscope (Olympus ITR) was inserted through the intratracheal tube into the lungs and wedged in a subsegmental bronchus of the lingula or the right middle lobe.

Fifty millilitres of sterile saline were instilled with a syringe through the suction part of the fibrescope, immediately re-aspirated with a suction pump and collected in a sterile trap kept on ice. This process of lavage and suction was repeated six times to a total amount of 300 ml. The first aliquot was discarded because of contamination with upper airway secretions or ciliary bronchial cells. The washing liquid was filtered through sterile gauze in order to eliminate most of the mucus. After three washings in PBS the HAMØ suspension was adjusted to 10^6 cells/ml in BME (Gibco Europe) with 10% H.I. FCS (Gibco Europe) [17, 18].

Production of macrophage supernatants

Mouse peritoneal macrophages, transformed MØ and human alveolar macrophages, after washing with PBS, were cultivated for 72 hr at 37°C at a density of 10^6 cells/ml in BME without FCS in order to avoid background thymidine that is present in small amounts in FCS.

Bioassay of the inhibition of incorporation of nucleic acid precursors

A bioassay of the inhibition of incorporation of nucleic acid precursors [^{3}H]-TdR (Amersham, England) was added to obtain a final specific activity of the conditioned fluids of 2 μCi/ml. [^{125}I]-UdR (Amersham, U.K.) was added in the same manner. The final specific activity was 0.5 μCi/ml. HM6 cells were plated in the microplaque wells (Nunclon Delta-multidish 96, Nunc, Denmark) at a cell density of 8×10^4 cells per well. Each well contained 200 μl of suspension fluid. The cells were left to adhere overnight and the plating medium was replaced by the conditioned media containing the tritium (^{3}H)-labelled precursor. Normal medium was used as

control. After 4 hr of incubation the supernatant was discarded and the cells were washed three times with PBS. The cells were then lysed with SDS 3% and placed in vials for counting. The results are expressed as the percentage inhibition of incorporation as compared to the control. Experiments were done in triplicate. The standard deviation was usually below 10%.

Transformation of [6-^{14}C]-*orotic acid into* [6-^{14}C]-*TdR by mouse and human macrophages*

This technique enables us to measure qualitatively the transformation by cellular synthesis of orotic acid into thymidine released into the supernatant.

Normal macrophages (BALB/c), transformed macrophages (IC21) and tumour cells (HM6) were plated in Nunc wells at a density of 2.5×10^4 cells per well in Dulbecco's medium with [6-^{14}C]-orotic acid (Amersham, U.K.) for 72 hr. Ten microlitres of supernatant were tested on a thin-layer chromatography plaque. The cellulose plaque was divided into a 1-cm^2 area which was scraped off and counted for ^{14}C activity in scintillation vials packed with Pico Fluor 15 scintillation fluid [19].

HPLC of nucleosides

Flasks with cellular supernatants were cooled for 10 min in iced water. A one-quarter volume of 40% TCA was added and nucleosides extracted with occasional mixing for 30 min at 4°C. Precipitated material was removed by centrifugation at 5000 *g* for 15 min. The supernatant was neutralized by vigorously mixing it with 2 vol of trioctylamine-Freon (1:4 v/v). The aqueous phase was clarified and recovered by centrifugation. The final neutralized extracts were assayed for ribonucleosides. The HPLC was carried out using instrumentation from Perkin-Elmer, Norwalk City. HPLC of TCA extracts of supernatants was performed with a series of 2/2 double-pump chromatographs. Samples were injected into the volume using a Rheodyne model 7105 high-pressure valve. Separated components were detected using two LC-15 u.v. detectors at 254 and 280 mm set up in sequence. Peaks were quantitated using a sigma 10 integrator while determination of pmol values and identification of peaks according to retention time and the 254/280 absorbance ratio were carried out using standard compounds [20].

RESULTS

Bioassay for [^{3}H]-*TdR uptake inhibition by MØ secretions*

Action on the [^{3}H]-TdR incorporation in HM6 melanoma cells by various cellular conditioned media shows that human alveolar macrophages as well as normal mouse PMØ secrete an inhibitor of thymidine incorporation into melanoma cells *in vitro* (Fig. 1). The media were conditioned by B16 cells, L cells, IC21 continuous macrophage cell line, C57BL, GIF and BDF1 mice peritoneal macrophages and a series of five human alveolar macrophages. The target cells were the HM6 human melanoma cell line. Only normal macrophages supernatants showed a [^{3}H]-TdR incorporation inhibitory effect. Our knowledge of the mouse peritoneal macrophage model prompted us to test the hypothesis of the thymidine secretion by human alveolar macrophages. This cell is a non-dividing cell which could have the same thymidine kinase deficiency as the other macrophages. A very convenient test to assay the phenomena of thymidine synthesis and release by macrophages is the transformation of [6-^{14}C]-orotic acid into [6-^{14}C]-TdR, which is then measured by thin-layer chromatography of nucleosides. The corresponding thymidine spot is then counted for ^{14}C B activity in a scintillation counter. This method is extremely precise (Fig. 2). Figure 2 shows the different peaks obtained by the

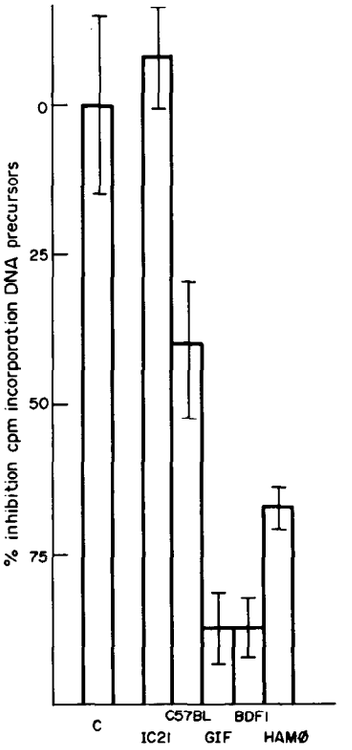

Fig. 1. Bioassay for % [^{3}H]-TdR uptake inhibition by MØ supernatants in media conditioned by various cell types. C576B, GIF, BDF1 and human alveolar macrophages. Controls are B16L cells and IC21 cell lines. Time of action of conditioned media on HM6 human melanoma target cells was 4 hr. *Time of medium conditioning by* 10^6 effector cells/ml *was* 72 hr.

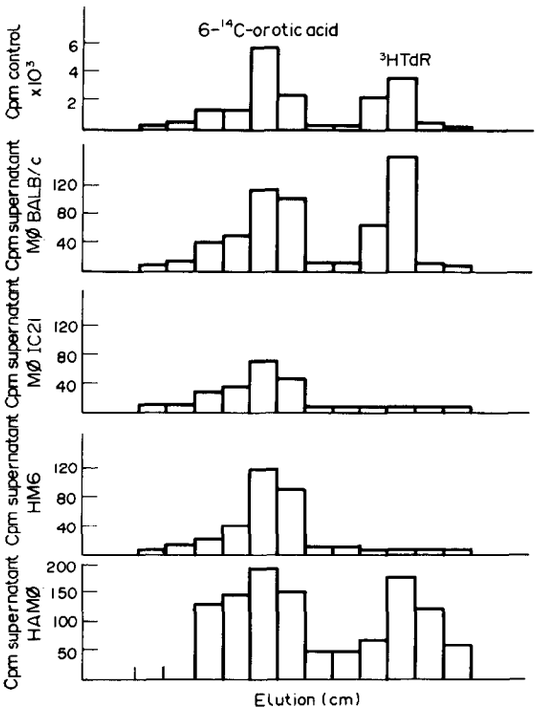

Fig. 2. Active synthesis of TdR by MØ. Thin-layer chromatography of effector cell supernatants. Human alveolar macrophages (10^6 cells/ml) *were incubated for* 72 hr *with* [6-^{14}C]*-orotic acid. Only normal macrophages show a* [^{14}C]*-TdR peak; transformed MØ and melanoma cells do not.*

HPLC OF TdR IN CONDITIONNED MEDIA

CELLS		THYMIDINE µM	THYMINE µM
B16 MELANOMA		—	—
C57BL MURINE MØ		2,3	—
BALB/C " "		1,5	—
GIF " "		0,9	—
P 388 D_1 TRANSFORMED		—	—
IC 21 MACROPHAGES		—	—
J 774 A "		—	—
HAMØ 1	HUMAN ALVEOLAR MACROPHAGES	0,5	—
HAMØ 2		0,3	—
HAMØ 3		0,3	—
HAMØ 4		0,3	—
HAMØ 5		0,3	—

Fig. 3. Levels of TdR produced by MØ. High-performance liquid chromatography shows the amount of TdR produced by different cell types at a concentration of 10^6 cells/ml *for* 72 hr *of incubation time.*

metabolization and release into the supernatant of [6-^{14}C]-orotic acid and its metabolite [6-^{14}C]-TdR in normal peritoneal macrophage and human alveolar macrophage supernatants.

Levels of TdR in mouse MØ and HAMØ supernatants

A second set of experiments on various conditioned media was done using the HPLC technique. The HPLC result for thymidine production by human alveolar macrophages was around 0.3 µm for all the HAMØ-conditioned media. This is very close to the threshold sensitivity of the method, as shown in Fig. 3, where we compare the production of TdR by various cell lines such as B16 melanoma, C57BL, BALB/c, GIF, mouse peritoneal MØ, P388D1, IC21 and J774A transformed macrophage cell lines; and five human alveolar macrophages in anaerobic growth conditions.

DISCUSSION

The use of three different techniques, bioassay, transformation of [6-^{14}C]-orotic acid into [6-^{14}C]-TdR and HPLC of nucleosides, have given us results that show that HAMØ produce a detectable amount of TdR *in vitro*. This amount has been found to be less than that of the mouse model. This could be due to the culture conditions used. Indeed, HAMØ are aerobic organisms *in vivo*. *In vitro* they are put in more anaerobic conditions, to which they have to adapt. This lowers their overall metabolic activities, which could explain the depression of TdR secretion.

The test of transformation of [6-^{14}C]-orotic acid into [6-^{14}C]-TdR gives us added precision on the synthesis. It is to be noted that this assay has a threshold sensitivity lower than 10^{-8} M, which is close to ten-fold that of HPLC. Of course, the end product has to be known or hypothesized and the precursor carefully chosen to meet the requirements. The separation technique used thereafter, be it chromatography, electrophoresis or any other procedure, depends on the molecule studied.

The biological significance of these results will depend on the availability and the concentration of TdR in the cellular microenvironment. A high concentration of thymidine—above 4×10^{-6} M—can produce a blockade of DNA synthesis called the 'thymidine blockade phenomenon' which is a reversible inhibition of ribonucleotide reductase by excess of TTP synthesized due to excess of Tdr in the pathway of DNA synthesis [21].

At lower concentrations TdR can be incorporated into proliferating cells without any

enhancement of growth. Thus the gradient of TdR around HAMØ modulates the growth of other cells in its vicinity. Indeed, HAMØ establish obligatory contacts with lung cells, including metastatic or primary tumour cells.

This effect may, however, be much too weak in its normal state to affect the growth of lung metastasis markedly, though sensitive cells may be more prone to this effector. We have engaged in further studies on the secretion by HAMØ of specific or non-specific effectors which may play a role, however discreet, in cellular interactions. This may prove to be useful in the manipulation of these intricate mechanisms, especially when tumour cells are involved.

Acknowledgement—We thank Dr A. Leyva, Department of Experimental Chemotherapy, Antoni van Leeuwenhoekhuis, Amsterdam, The Netherlands for his help in the HPLC procedure.

REFERENCES

1. Arnould R, Libert A, Vercammen-Grandjean A, Lejeune FJ. Mechanisms of thymidine synthesis and release by macrophages. Effect on malignant melanoma. *Anticancer Res* 1981, **1**, 25–30.
2. Lejeune FJ, Vercammen-Grandjean A. Secretory activity of macrophages in relation to cytotoxicity and modulation of cell function. In: Dingle JT, Jacques PJ, Shaw IH, eds. *Lysosomes in Applied Biology and Therapeutics 6*. Amsterdam, North-Holland, 1979, 425–445.
3. Vercammen-Grandjean AF, Lejeune FJ. Production and mode of action of macrophage secretions that interfere with the *in vitro* incorporation of radioactive DNA precursors by tumor cells. In: James K, McBride JK, Stuart A, eds. *The Proceedings of the EURES Symposium on the Macrophage and Cancer*. Edinburgh, Univerity Medical School, 1977, 50–59.
4. Stadecker MJ, Calderon J, Karnovsky ML, Unanue ER. Synthesis and release of thymidine by macrophages. *J Immunol* 1977, **119**, 1738–1743.
5. Zaharlo DS, Ramonas LM. Comparison of *in vitro* and *in vivo* effects of thymidine on L1210 leukemia in mice. *JNCI* 1982, **68**, 875–880.
6. Stadecker MJ, Unanue ER. The regulation of thymidine secretion by macrophages. *J Immunol* 1979, **123**, 568–571.
7. Ellims PH. Thymidine as an anticancer agent, alone or in combination. A biochemical appraisal. *Cancer Chemother Pharmacol* 1982, **10**, 1–6.
8. Schornagel JH, Leyva A, Pinedo HM. Is there a role for thymidine in cancer chemotherapy? *Cancer Treat Rev* 1982, **9**, 331–352.
9. Lin TS, Fischer PG, Marsh JC, Prusoff WH. Antitumor activity of the 3'-chloroethylnitrosourea analog of thymidine and the prevention by co-administrated thymidine of lethality but not of anticancer activity. *Cancer Res* 1982, **42**, 1624–1629.
10. Young CW, Woodcock TM, Martin DS. Modulation of 5-FU action by thymidine in murine and human tumors. *Cancer Treat Rep*, 1981, **65** (Suppl. 3), 83–87.
11. Au JL, Rustum YM, Ledesma EJ, Mittelman A, Creaven PJ. Clinical pharmacological studies of concurrent infusion of 5-fluorouracil and thymidine in treatment of colorectal carcinomas. *Cancer Res* 1982, **42**, 2930–2937.
12. Breitman TR, Keene BR. Synergistic cytotoxicity to melanomas and leukemias *in vitro* with thymidine (NSG-21548) and arabinosylcytosine (NSG-63878). *Proc AACR* 1979, **20**, 89 (abstract).
13. Lejeune FJ, Regnier R. Studies on a non specific and non cytotoxic factor produced by macrophages. *Br J Cancer* 1974, **30**, 181 (abstract).
14. Lejeune FJ, Regnier R. Foetal bovine serum (FBS) induced cytostatic effect of peritoneal macrophages against mouse melanoma *Bering Inst Mitt* 1975, **56**, 28–34.
15. Lejeune FJ. Macrophage secretions affecting the growth of other cells. In: Wybran J, Staquet MJ, eds. *Clinical Tumour Immunology*. Oxford, Pergamon Press, 1976, 9–18.
16. Mauel J, Defendi V. Infection and transformation of mouse peritoneal macrophages by Simian Virus 40. *J Exp Med* 1971, **134**, 335–350.
17. Finley TN, Swenson EW, Huger GL, Ladman AJ. Bronchopulmonary lavage in normal subjects and patients with obstructive lung disease. *Ann Intern Med* 1967, **66**, 651–658.
18. Cohen AB, Cline MJ. The human alveolar macrophage: isolation cultivation *in vitro* and studies of morphologic and functional characteristics. *J Clin Invest* 1971, **50**, 1390–1398.
19. Randerath K, Randerath E. Thin layer chromatography. *Methods Enzymol* 1967, **12**, 323–347.

20. Leyva A, Appel H, Pinedo HM. Purine modulation of thymidine activity in L1210 leukemia cells *in vitro*. *Leuk Res.* Submitted.
21. Martin DS, Stolfi RL, Sawyer RC *et al.* An overview of thymidine. *Cancer* 1980, **45**, 1117–1128.