the preparation-dependent insufficient contrast is improved by using an accelerating voltage of only 16 kV and the possibility of increasing the primary current to 10^{-10}A without damage for the object (increase of the X-ray emission). With this acceleration voltage sufficient contrast is provided (fig. 1). A sample stage tilt for increasing the number of impulses is not necessary as in consequence of the higher specimen current in the present samples even shortening of the measuring time to 80 seconds yielded good results (table).

The comparison of the element peaks in the polaroid pictures shows that with a conventional TEM-X-ray microanalysis using 2000-Å tissue sections in several TEM systems (JEM 100B[5] and C, Elmiskop 2A[6], equipped with EDAX- and KEVEX-systems) in Szeged, Hungary; Brno, ČSSR; and Berlin, GDR as well as with the FESEM with field-emission gun and ultra-high vacuum (Brno, ČSSR) using 600-Å tissue sections typical and similar element spectra can be demonstrated, e.g. in the smooth muscle cell plasma of the arterial wall of different rats. So in rats with experimental arteriosclerosis, among others, the expected increase in elementary calcium could be proved even after conventional epon-embedding (fig. 2). Despite the fact that the time of measuring the same spot with a diameter of 300 nm has been experimentally prolonged up to 11 min, no tissue destruction or black contamination point was discernible (fig. 1).

Therefore FESEM with ultra-high vacuum - which, as far as we know, has not been applied before for X-ray microanalysis of biological and medical objects for a comparable purpose - shows a number of advantages:

a) ultra-thin section thickness of the object for analysis (between 500 and 2000 Å), b) shorter analysis time (between 80 and 320 sec) for 16 kV primary beam energy, c) higher beam current (about 10^{-10}A) and small analysis area (20-300 nm dependent on the biological problem). Moreover, d) the analysis is contamination-free (ultra-high vacuum), e) contrast in transmitted electrons is high also for conventional, i.e. relief-free ultra-thin sections.

The typical peaks of smooth muscle cell plasma of the arteriolar wall (Si, S, Cl) are recognizable already after 80 sec (left). Cu Lα, Fe and Cr derive from grid and instrument

A) 80 sec			B) 320 sec		
	0.645	7.		0.672	168.
	0.790	2.	Cu Lα	0.950	385.
Cu Lα	0.910	112.	Mg	1.260	19.
Al	1.470	9.	Si	1.745	591.
	1.510	20.	P	2.010	55.
Si	1.765	266.	S	2.322	482.
P	2.046	63.	Cl	2.634	4382.
S	2.322	213.		2.835	197.
	2.440	9.	K	3.310	6.
Cl	2.630	1525.	Ca	3.790	6.
	2.830	75.		4.270	6.
K	3.370	2.		4.300	9.
Ca	3.400	4.	Cr	5.440	42.
Fe	6.400	16.		5.640	18.
			Mn	5.775	7.
				5.950	2.
				6.030	6.
				6.120	1.
				6.270	2.
			Fe	6.415	158.

1 C.H. Becker and A. Jánossy, Acta biol. med. germ. *36*, 1413 (1977).
2 J.A. Chandler, in: X-Ray microanalysis in the electron microscope, p. 370. North-Holland Publ., Amsterdam 1977.
3 P. Echlin, Mikroskopie *35*, 45 (1979).
4 C.H. Becker, Experientia *35*, 1093 (1979).
5 C.H. Becker and A. Jánossy, Micron *10*, 267 (1979).
6 C.H. Becker, A. Jánossy and T.v. Zglinicki, Ul'trastrukt. osnovy patol. serdtsa i sosudov, p. 255. 'Metsniereba', Tbilissi 1981.

Incorporation of the antitumor alkaloid thaliblastine in liposomes enhances its cytotoxic activity in vitro[1]

D.K. Todorov and G. Deliconstantinos[2]

Oncological Research Institute, Medical Academy, Sofia 1156 (Bulgaria), and Department of Physiology, University of Athens Medical School, Athens 609 (Greece), 25 August 1981

Summary. The incorporation of the antitumor alkaloid thaliblastine in liposomes greatly enhances its cytotoxicity in Walker S and TLX-5 cell cultures.

The antitumor agent thaliblastine (USA, thalicarpine) derived from the roots of several species of thalictrum (Ranunculaceae), is a dimeric aporphine benzylisoquinoline alkaloid with N-O-O triangulation in its molecule[3,4].

Thaliblastine exhibits cytotoxic activity in vitro on KB cells, L_{1210} cells, human embryonal fibroblasts, and SAg cells[4-6], and also antitumor action in vivo on several transplanted tumors[7,8]. The absence of myelosuppressive and immunosuppressive actions have allowed thaliblastine to enter phase I clinical trials in the USA[9] and more recently in Bulgaria.

Liposomes, phospholipid vesicles, have gained recognition as promising drug carriers, firstly because of their composition and secondly because the flexibility of their composition may offer the possibility of selective cellular interactions[10,11]. Liposome-incorporated antitumor drugs are more efficient in prolonging the survival time of tumor-bearing animals[12,13], escape premature inactivation[14], prevent cardiotoxicity[15] and act on cells resistant to the free drug[16,17].

This communication describes the interaction of liposome-incorporated thaliblastine with tumor cells, and the resulting effect on the ability of these cells to grow in an in vitro tissue culture assay system.

Material and methods. Thalicarpine (National Cancer Institute, Bethesda, MD, USA) and the identical Thaliblastine (IOC, Bulgarian Acad. Sciences, Sofia) is a white crystalline powder with a m.p. 158-161 °C, $(a)_D^{23} + 79.1°$ (1% in chloroform) UV absolute maximum at 282 nm, 23,400 and at 302 nm, 18,400. Tritium (^{3}H)-labeled thaliblastine (sp. act. 4.8 mCi/mmole) was prepared by refluxing thaliblastine with tritiated water, resulting in the exchange of hydrogen with tritium. The labeled compound was tested chromatographically (R_f-values: 0.46 in benzene-methanol 7:3, 0.42 in chloroform-methanol 9:1, on fluorescent silica plates)

and no detectable decomposition was found. A further sample was dissolved in isotonic saline and extracted after 5 min with chloroform only a very slight change in specific activity (+6%) was observed.

Phosphatidylcholine (egg) and phosphatidic acid were obtained from Lipid Products, South Nutfield, Surrey, U.K. N-Octadecylamine (stearylamine) was obtained from ICN, K and K Laboratories, Plainview, N.Y., USA and cholesterol from Sigma Chem. Co. Liposomes composed of egg phosphatidylcholine (PC), cholesterol (C) and stearylamine (S) (positively charged) or phosphatidic acid (PA), (negatively charged), in a molar ratio (7:2:1,40 μmoles PC) were prepared essentially as previously described[18]. Briefly, ^{3}H-labeled thaliblastine was incorporated into liposomes by adding an aqueous solution of the drug in phosphate buffered saline, pH 7.4 (PBS) to the dried lipids. This material was then sonicated under a nitrogen atmosphere using a titanium probe (1.9 cm tip diameter) for a total of 5 min (1 min sonication with 30 sec cooling periods) at 4 °C. Unincorporated thaliblastine was separated from the liposomes by gel filtration on a Sepharose 6B (Pharmacia) column and was then exhaustively dialyzed against phosphate-buffered saline pH 7.4 (PBS). The extent of thaliblastine incorporation within the liposomes was calculated by relating the specific activity of liposome-incorporated ^{3}H-thaliblastine to its initial total specific activity in the original lipid suspension in the buffer. Radioactivity was determined in a Packard Tri-carb 3380 Scintillation counter by dissolving the liposomes in 250 μl of 10% sodium dodecyl sulfate and counting in a dioxane based scintillation fluid (Bray's solution).

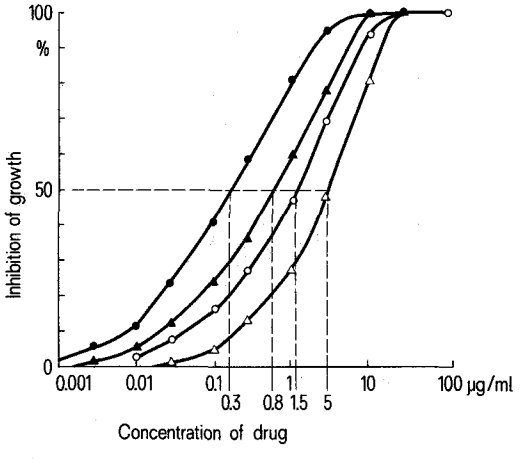
The cytotoxic activity of the free thaliblastine and of thaliblastine incorporated in liposomes was estimated on established suspension cell cultures grown asynchronously in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum, 50 μg/ml streptomycin and 100 IU/ml of penicillin, under an atmosphere of 10% CO_2 in air[19].

Two cell lines; Walker S (sensitive) and TLX5-highly sensitive to thaliblastine, were derived from ascitic Walker 256 carcinosarcoma in Wistar CB rats, and solid TLX5 lymphoma in CBA/LAC mice, respectively. The drug was added to cells suspended at 10^5 per ml in culture medium in 200 μl amounts into the wells of a 96-well plastic plate. The effect of the drug on cell growth was evaluated by taking cell counts at 24 h intervals from several wells of each series, using a Coulter counter. The mean inhibitory dose, ID_{50} (50% inhibitory dose) was estimated from growth curves[19]. Cell viability was assessed by trypan blue exclusion 1 min after the addition of 1% solution of the dye.

Results and discussion. Liposomes prepared with phosphatidylcholine: phosphatidic acid (7:1) have been shown to have a net negative charge at pH 7.4[20]. Since thaliblastine would be slightly positively charged at this pH, due to its 2 heterocyclic nitrogen groups, this should lead to a high degree of incorporation. Indeed, 20–25% of the added thaliblastine was incorporated in negatively charged liposomes. On the other hand, incorporation of thaliblastine in positively charged liposomes, composed of phosphatidylcholine: stearylamine (7:1) resulted in a 10-fold decrease of incorporation as compared to that of the negatively charged liposomes. Cholesterol was added to increase the liposome stability as well as to minimize the permeability changes that may be caused by interactions with serum proteins[21].

The cytotoxic activity of thaliblastine incorporated in negatively charged liposomes was several times higher than that of the free alkaloid. In Walker S cells, after 72 h of incubation at 37 °C the mean inhibitory dose (ID_{50}) was found to be 5 μg/ml for the free and 1.5 μg/ml for the liposome-incorporated thaliblastine; in TLX5 cells the ID_{50} was found to be 0.8 μg/ml and 0.3 μg/ml respectively (see fig.). In control experiments Walker S and TLX5 cells were incubated with 'empty' liposomes (without thaliblastine). Incubation of phospholipid vesicles with cells can result in lipid exchange between cell and vesicle[22,23]; however, this exchange had no detectable effect on the cell growth. Cell permeability was not affected by incubation with liposomes; the cells remained intact and impermeable to trypan blue. This impermeability to the dye was maintained despite evidence that vesicular lipids (e.g., cholesterol, phosphatidylcholine) are transferable to the cells[22], causing extensive functional changes of the enzymes of the membrane[24,25]. No difference in the ID_{50} was found for cells treated with the free alkaloid alone or mixed with 'empty' liposomes.

In conclusion, the use of liposomes as carrier of the antitumor alkaloid thaliblastine enhances the cytotoxicity of the drug. This approach may prove useful in the attempt to increase the cytotoxic activity of the drug, in vivo, in tumor-bearing animals. Further experiments are required to verify the validity of these results in vivo on tumor-bearing animals where the problem of 'targeting' is crucial.



Dose response curves for free thaliblastine (TBL) ▲—▲ and liposomal TBL ●—● against TLX5 cells; and for free TBL ○—○ and liposomal TBL △—△ against Walker sensitive cells.

1 Acknowledgment. We are most greatful to Drs T. Connors, for providing thalicarpine and P. Farmer, for preparing the radioactive drug, MRC Toxicology Unit, Carshalton, Surrey, U.K. We are also greatfully indepted to Dr G. Gregoriadis, Clinical Research Centre, Harrow, Middlesex, U.K. and Professor S.G.A. Alivisatos, Univ. of Athens Med. School, Greece, for help and advice during the preparation of this manuscript. D.K.T. was supported financially by the American Cancer Society - Eleanor Roosevelt - International Cancer Fellowship awarded by the International Union against cancer.

2 Reprint requests to G.D., Department of Physiology, University of Athens Medical School, Athens 609 (Greece).

3 K.Y. Zee-Cheng and C.C. Cheng, Cancer Chemother. Rep. *3*, 49 (1972).
4 L.M. Allen and P.J. Greaven, Cancer Res. *33*, 3112 (1973).
5 D.K. Todorov and M.S. Damyonova, C. r. bulg. Acad. Sci. *28*, 709 (1975).
6 I.E. Broder and S.K. Carter, Thalicarpine (NSC-68075) Clinical Brochure, NCI, Bethesda, MD, USA 1971.
7 A. Maleev, J. Stoychkov, D. Todorov, K. Mihaleva, N. Mollov and H. Dutschewska, C. r. bulg. Acad. Sci. *28*, 713 (1975).
8 J.N. Stoychkov and A.S. Miloushev, Biomedicine (Express) *33*, 42 (1980).

9 P.J. Creaven, M.H. Cohen, O.S. Selawry, F. Tejada and L.E. Broder, Cancer Chemother. Rep. *59*, 1001 (1975).
10 G. Gregoriadis, in: Drug carriers in biology and medicine, p. 287. Ed. G. Gregoriadis. Academic Press, New York 1979.
11 B.E. Ryman and D.A. Tyrell, in: Essays in biochemistry, vol. 16, p. 49. Eds P.N. Campbell and R.D. Marshall. Academic Press, London 1980.
12 J. Fruhling, W. Penasse, G. Laurent, C. Brassin, J. Hildebra, M. Vanhaele, R. Vanhaele, M. Deleers and J.M. Ruysscha, Eur. J. Cancer *16*, 1409 (1980).
13 J.W. Weinstein, R.L. Magin, R.L. Cysyk and D.S. Taharko, Cancer Res. *40*, 1388 (1980).
14 R.L. Juliano and H.N. McCullough, J. Pharmac. exp. Ther. *214*, 381 (1980).
15 E.A. Forssen and Z.A. Tokes, Proc. natl Acad. Sci. USA *78*, 1973 (1981).
16 D. Papahadjopoulos, G. Poste, W.J. Vail and J.L. Biedler, Cancer Res. *36*, 2988 (1976).
17 S.B. Kaye, J.A. Boten and B.E. Ryman, Eur. J. Cancer *17*, 279 (1981).
18 G. Deliconstantinos, G. Gregoriadis, G. Abel, M. Jones and D. Robertson, Biochem. Soc. Trans. *5*, 1326 (1977).
19 B.J. Phillips, Biochem. Pharmac. *23*, 131 (1974).
20 A.D. Bangham, M.M. Standish and J.C. Watkins, J. molec. Biol. *13*, 238 (1965).
21 C. Kirby, J. Clarke and G. Gregoriadis, Biochem. J. *186*, 591 (1980).
22 D. Papahadjopoulos, E. Mayhew, G. Poste and S. Smith, Nature *252*, 163 (1974).
23 G. Deliconstantinos, IRCS med. Sci. *8*, 493 (1980).
24 T.R. Tritton, S.A. Murphree and A.C. Sartorelli, Biochem. Pharmac. *26*, 2319 (1977).
25 S.G.A. Alivisatos, G. Deliconstantinos, A. Papaphilis and G. Theodosiadis, Biochim. biophys. Acta *643*, 642 (1981).
26 G. Deliconstantinos, Experientia *37*, 81 (1981).

Common antigenicity between a human thymic epithelial cell product and a thymus-dependent serum factor[1]

E.F. Potworowski, Danuta Kowalczyk, V.V. Micusan and T. Seemayer

Immunology Research Center, Armand-Frappier Institute, P.O. Box 100, Laval (Québec, Canada H7N 4Z3), and Department of Pathology, Montreal Children's Hospital, Montreal (Canada), 30 November 1981

Summary. Specific immunofluorescence of human thymic epithelial cytoplasm was obtained with antibodies to supernatant of thymic epithelial cultures, and with anti-prealbumin antibodies. These antibodies also reacted with normal serum but not with serum from Di George patients. The data indicates that thymic epithelium and a component of the prealbumin fraction of normal serum share a common antigen believed to be thymic hormone.

Thymic hormone activity has been described in a number of thymic extracts, in the supernatant of cultured thymic epithelial cells (thymic epithelial supernatant or TES)[2,3], in the prealbumin fraction of serum[4] and in several synthetic polypeptides[5,6]. Recent efforts by various groups have shown that antibodies to the synthetic polypeptide FTS (facteur thymique sérique) bind thymic epithelial cells[7,8]. In this work, we present evidence in support of a thymic epithelial origin of the factor bound to prealbumin in normal individuals. Antibodies to prealbumin were obtained from Behring diagnostic. The preparation of TES has previously been described[9]. Antibodies to TES were prepared in rabbits according to a standard protocol[10]. Normal human serum was obtained from healthy volunteers and sera from Di George patients, devoid of a functional thymus, were a gift of Drs C. Griscelli and E. Reece.

Anti-TES serum applied to frozen sections of fresh human thymus with a fluorescein conjugated goat anti-rabbit serum stained strongly and exclusively the cytoplasm of reticuloepithelial cells. While positively staining individual epithelial cells were found both in the medulla and in the cortex, an accumulation of these cells were found to line the outer cortical layer, immediately adjacent to the connective tissue capsule or the interlobular septum (fig). Thymic epithelial cells grown in culture were also examined in the same way. While not all cells were found to be positively stained, those that were displayed a spotty cytoplasmic pattern often extending into the cytoplasmic processes. Cultured human fibroblasts treated in the same fashion did not display any fluorescence.

Anti-prealbumin serum was used without any adsorption in the same way as the anti-TES serum. Whether tested on frozen sections of human thymus, on cultured thymic epithelium, or on cultured human fibroblasts, the reaction was in all cases identical to that obtained with the anti-TES serum.

The presence of TES antigen in human serum was tested by immunofluorescence on thymic sections using anti-TES serum after various absorptions. Anti-TES serum was absorbed for 2 h at 37 °C with normal human serum, with Di George serum, with TES or with phosphate buffered saline (PBS) in various ratios and applied to serial frozen sections of human thymus, using in all cases a conjugate dilution of 1:8. The results (table 1) show that normal human serum and TES abolished the reaction of anti-TES with thymic epithelial cells, while Di George serum and PBS were unable to do so.

Similarly anti-prealbumin serum was absorbed with normal human serum, with Di George serum, with TES or with PBS and applied to serial frozen sections of human thymus. Here again (table 2), normal human serum and TES completely absorbed out the antibodies in anti-prealbumin directed against thymic epithelial cells, while Di George serum and PBS had no such effect.

In order to determine whether anti-prealbumin serum had a single antibody directed against an antigen shared by

Table 1. Intensity of immunofluorescence staining of epithelial cells in thymic sections treated with an antiserum to thymic epithelial supernatant[a] (TES), after various absorptions

Anti-TES serum/ absorbant ratio	Absorbants Normal human serum	Di George serum	TES	PBS
3:1	++(+)/+++[b]	++(+)	(+)	++(+)/+++
2:1	++	++(+)	-	+++
1:1	-	++	-	+++

[a] Anti-TES prepared in rabbits was used with goat anti-rabbit fluorescein conjugate. [b] Fluorescence intensity was evaluated in arbitrary units ranging from - (negative) to ++++ (strongly positive).