dry weight of 413 mg/100 g, b.wt, 242 mg of which are lipids and 95 mg are proteins. Although not statistically significant, the increase in DNA (14%) parallels that of proteins. The glycogen content does not appear to be statistically modified. In hF-Cf diet fed rats, 76 mg of constituents other than lipids and proteins contribute to the increase in liver dry weight. Liver wet weight/dry weight ratios are decreased in this group of animals by about 16% which indicates that the increase in some constituents is not concomitant with an addition of water.

Discussion. We cannot at present advance hypotheses about the mechanism leading to the changes found in serum proteins under the present experimental conditions. The modifications in DNA, glycogen and protein content of the liver caused by CCl_4 are in agreement with previous findings[15,16]. From these results one may conclude that the accumulation of proteins has an important role in determining hepatomegaly both in CCl_4-treated and in hF-Cf fed-rats. Since DNA changes run parallel to those of proteins, one may think that the 2 modifications are causally connected. The DNA increase may be due to various causes, such as the presence of infiltrating cells, an augmentation in the number of binucleated cells or hepatic regeneration. CCl_4 could affect the hepatic glycogen level through an increased release of catecholamines by the adrenal medulla[17], since these substances reduce the hepatic glycogen level[18]. Another possibility is that, since CCl_4-treated rats probably suffer from malnutrition, as shown by their loss in b.wt, the reduction in liver glycogen may be due to a sort of starvation also capable of strongly decreasing the hepatic glycogen level[19]. The hypotheses on the pathogenesis of fatty liver have been discussed elsewhere[20].

1 E. Baraona, M.A. Leo, S.A. Borowski and C.S. Lieber, Science *190*, 794 (1975).
2 E. Baraona, M.A. Leo, S.A. Borowski and C.S. Lieber, J. clin. Invest. *60*, 546 (1977).
3 B. Lombardi, Lab. Invest. *15*, 1 (1966).
4 B. Lombardi, Fed. Proc. *30*, 139 (1971).
5 Th. Peters, Jr, Am. J. Physiol. *224*, 1363 (1973).
6 E.A. Smuckler, Pan. Med. *18*, 292 (1976).
7 N.D. Lee and R.H. Williams, J. biol. Chem. *200*, 451 (1953).
8 C. Agostini and P. Comi, Path. Eur. *7*, 151 (1972).
9 C. Agostini and A. Angelotti, Path. Eur. *8*, 105 (1973).
10 C. Agostini, A. Perin and A. Fonnesu, Br. J. exp. Path. *41*, 617 (1960).
11 C. Agostini and F. Muci, Experientia *35*, 518 (1979).
12 S. Lo, J.C. Russel and A.W. Taylor, J. appl. Physiol. *28*, 234 (1970).
13 F. Setaro and C.D.G. Morley, Analyt. Biochem. *81*, 467 (1977).
14 P. Armitage, Statistical methods in medical research. Blackwell Scientific Publications, Oxford and Edinburgh 1971.
15 R.M. Campbell and H.W. Kosterlitz, Br. J. exp. Path. *29*, 149 (1948).
16 U. Del Monte and A. Fonnesu, Sperimentale *109*, 459 (1959).
17 T.M. Brody and D.N. Calvert, Am. J. Physiol. *198*, 682 (1960).
18 G. Pogátsa, J. Tamás and E. Dubecz, Horm. Metab. Res. *10*, 378 (1978).
19 A.J. Parodi, Arch. Biochem. Biophys. *120*, 547 (1967).
20 M.U. Dianzani, Trattato di Patologia generale, 2nd ed. UTET, Torino 1972.

Collagenolytic activity of eosinophilic granuloma in vitro

M.F. Rousseau-Merck, S. Barbey, H. Mouly, S. Bazin and C. Nezelof[1]

Inserm U 77 et U 12, Hôpital Necker Enfants Malades, 149, rue de Sèvres, F-75730 Paris Cedex 15 (France), 22 November 1978

Summary. Lytic activity of eosinophilic granuloma and other tumours was studied in vitro on collagen substrate. Collagen degradation was measured through the release of hydroxyproline-rich peptides into the medium. The in vitro lytic action was at a maximum in the case of EG and was correlated with the presence of histiocytic cells.

Eosinophilic granuloma (EG), Hand-Schüller-Christian and Letterer-Siwe diseases are the 3 clinical forms of histiocytosis X[2]. Whatever the clinical form, histiocytosis X is related to a tumoral cellular proliferation probably of histiocytic origin and showing an ultra-structural marker called X body[3]. Biochemical[4] and membrane-receptor studies[5] were used to identify this tumoral histiocytic cell. Tissue culture is an useful tool in the study of histiocytosis X since the histiocytic cells (with their X body) can be maintained for several days and weeks in vitro, while neither neutrophil nor eosinophil polymorphonuclear cells are ever identified after 1 week in the same conditions[6].

As the destructive effect of EG upon bone tissue suggests a cellular release of active proteolytic substances, we intended to demonstrate and to quantify in vitro the lytic activity of the EG cellular population using a collagen film as the substrate for culture. Tumour fragments of various type and origin were used as controls of the lytic activity.

Material and methods. Tumoral material. Fragments of 5 clinically and histologically demonstrated EG originating from bone lesions of children were taken and put on the collagen film in Leighton tubes. Explants of various tumours from both children and adults (table 1) were placed under the same conditions.

Collagen substrate. The collagen substrate was prepared according to the recommendations of Ehrman and Gey[7] and Bornstein[8]. The rat tail tendons were cut free and immersed in a solution of 0.1 M acetic acid. After a gauze filtration, the acetic acid collagen solutions was dialysed against distilled water until the desired viscosity was obtained. The dialysed collagen solution was spread on the surfaces of coverslips. Exposure to ammonia vapour gelled the solution. The coverslips were washed with distilled water and immersed in the nutritive medium (MEM + 10% calf serum) with antibiotics (penicillin, 200 units/ml and streptomycin 0.1 mg/ml). These coverslips can be kept for at least 1 month under refrigeration.

Culture. 6–8 explants (0.1 mm^3) were distributed on each coverslip in a sterile Leighton tube. 4–12 collagen coverslips were used for each tumour. The explants were put on the collagen film in a minimum of medium (0.2 ml) to assure their adhesion on the coverslip. Medium (1.8 ml) was added the day after and its renewal occurred once a week. When the cultures were terminated, the coverslips were fixed with Bouin solution and stained with hemalum eosin.

Enzymology. 1. Acid phosphatase[9] and leucyl aminopeptidase[10] activities were tested on the coverslips following the growth of the culture. 2. Collagenase activity. Collagen degradation was determined by the release of hydroxyproline-rich peptides into the medium. After acid hydrolysis of the peptides, hydroxyproline was separated by chromatography[11] and determined by Stegemann's method[12]. The

Table 1. Lytic effects of tumour cultures

Number	Tumours	Explants with lysis/total number of explants	Percentage of explants with lysis (%)	Beginning of lysis (day)	Cells in the area of lysis	Growth of cultures
1	EG	90/152	60	7	Round + pc[a] fibroblastic	Excellent
2	EG	15/48	30	9	Round fibroblastic	Average
3	EG	16/48	33	7	Round + pc fibroblastic	Excellent
4	EG	28/48	58	12	Round + pc fibroblastic	Excellent
5	EG	23/32	71	10	Round + pc fibroblastic	Excellent
6	Malignant(?) histiocytosis	6/32	19	21	Large, round fibroblastic	Average
7	Lymphoma	28/48	56	3	Small, round	Excellent
8	Embryonic sarcoma	4/32	12	21	Fibroblastic	Excellent
9	Neuroblastoma	14/48	29	10	Neuroblastlike fibroblast	Average
10	Ovarian dysgerminoma	0/48	0	–	–	Necrotic
11	Seminoma	4/32	12	20	Round + pc fibroblastic	Average
12	Neuroepithelioma	0/32	0	–	–	Necrotic
13	Giant cell tumour of bone	6/32	19	15	Round + pc fibroblastic	Excellent
14	Burkitt lymphoma	0/48	0	–	–	Excellent
15	Osteosarcoma	19/48	40	8	Large	Average

[a] pc, polycaryocytes.

technique needed a minimum of 10 µg/ml hydroxyproline to obtain a result at the point where only half the collagen substrate remains.

Results. Though all 12 tumours had lysed collagen in vitro, the 5 EG preparations demonstrated the highest level of lysis.

Eosinophilic granulomas. Half or more of the explants of EG lysed the collagen substrate. Lysis appeared during the 2nd week of culture (table 1). In 2 of 4 cases, the collagen degradation by the cultures was strong enough to be detected by the measurement of hydroxyproline released in the medium (table 2). Collagenolytic activity was always present in the EG but showed a different intensity from one tumour to another. During the period of tissue culture, collagenolytic activity sometimes diminished and sometimes increased. Fibroblastic overgrowth occurring in culture 5 could explain the decrease of hydroxyproline after 22 days in culture.

In the areas of lysis, the cells were mostly round with lightly eosinophilic cytoplasm and a folded nucleus. Fibroblast-like cells were always present but polycaryocytes were only found sometimes. The cellular population maintained the same pattern of growth in the area of collagen lysis as it did without collagen substrate[6]. The leucyl aminopeptidase and acid phosphatase activities were positive. There was a preferential print of acid phosphatase on both the round cells and the giant ones, as previously observed[4].

Other tumours. 6 tumours from various origins degraded collagen to a lesser extent than the EG and, in 3 other cases, not at all. Only the lymphoma (No 7) showed an early lysis quite comparable in its extent with that of the EG. Nevertheless, cells inside the area of lysis were small and had a regular nucleus with a big nucleolus. These cells belonged to the homogeneous cellular population identifiable in histological studies.

Explants of the 6 tumours degraded collagen; the rates varied from 1 in 5 to 1 in 3. The morphology of cells in the area of lysis of 4 tumours (Nos 6, 7, 9, 15) remained comparable to the initial tumour. Only 2 tumours had, both in the area of degraded collagen and in initial histology, histiocytic and giant cells similar to the ones described in the cultures of EG (Nos 11 and 13).

Discussion. The in vitro behaviour of EG on a collagen substrate confirms its in vitro bone lysis ability. However, the other tumours with the exception of cases 7 and 15 degraded the same substrate to a much lesser extent.

Numerous tissues are able to degrade collagen[13–15]. The cells which provoke this process are either mobile cells such as leucocytes[16,17] and macrophages[18,19] or tumour cells [15,20].

Cellular populations in the lysis area of 4 tumours (cases 6, 7, 9 and 15) had a tumoral morphology correlated with initial histology. In 2 tumours, macrophages and polycaryocytes were present. These cells were part of the inflammatory process found in the seminoma (No 11) and have been identified by Wood et al.[21] concerning giant cell bone tumours (case 13).

For EG the cells in the lysis area had the same morphological characteristics and demonstrated the same behaviour as histiocytic cells[5]. Neither neutrophils nor eosinophils were identified in the lysis area. The acid phosphatase and leucyl aminopeptidase activities of the histiocytic cells and the polycaryocytes make it possible once more to associate their proteolytic and collagenolytic activities[19].

Table 2. Amount of collagen degradation in the supernatants of eosinophilic granuloma cultures

Number	Day of determination	Concentration of hydroxyproline (µg/ml medium)
Medium	–	Not detectable
1	7	18,2
	21	73,6
2	Not done	Not done
3	21	Not detectable
4	21	13,3
5	12	10
	22	Not detectable

In conclusion, without considering their pathological or non-pathological nature, histiocytic cells have an in vitro collagenolytic activity in the EG as well as in other tumours.

1 Acknowledgments. The authors wish to express their gratitude to Dr F. Jaubert for his comments on the manuscript and to Mrs M.P. Gassama and M. Leborgne for their technical assistance.

2 L. Lichtenstein, Arch. Path. *56*, 84 (1953).
3 F. Basset and J. Turiaf, C.r. Acad. Sci. (Paris) *261*, 3701 (1965).
4 C. Nezelof, S. Després, S. Barbey and M.F. Rousseau, Biomedicine *20*, 414 (1974).
5 C. Nezelof, N. Diebold and M.F. Rousseau-Merck, J. Path. *122*, 105 (1977).
6 F. Basset and C. Nezelof, Revue fr. Étud. clin. biol. *14*, 31 (1969).
7 R.L. Ehrmann and G.O. Gey, J. natl Cancer Inst. *16*, 1375 (1956).
8 M.B. Bornstein, Lab. Invest. *7*, 134 (1958).
9 A.G.E. Pearse, in: Histochemistry, Theoretical and applied, vol. 1, p. 732. Churchill, London 1968.
10 T. Barka and P.J. Anderson, in: Histochemistry, practice and bibliography, p. 289. Harper and Row, New York 1963.
11 J.M. Partridge and D.T. Elsden, Biochem. J. *80*, 34 (1961).
12 H. Stegemann and K. Stalderk, Clin. chim. Acta *18*, 267 (1967).
13 S. Bazin, M. Le Lous and A. Delaunay, Front. Matrix Biol. *3*, 234 (1976).
14 A. Z. Eisen, E.A. Bauer and J.J. Jeffrey, J. invest. Derm. *55*, 359 (1970).
15 A.C. Taylor, B.M. Levy and J.W. Simpson, Nature *228*, 366 (1970).
16 G.S. Lazarus, J.R. Daniels, J. Lian and M.C. Burleigh, Am. J. Path. *68*, 565 (1972).
17 E.G. Bassett, J.R. Baker, P.A. Baker and D.B. Myers, Aust. J. exp. Biol. med. Sci. *54*, 459 (1976).
18 S.E. Mergenhagen, S.M. Wahl, L.M. Wahl, J.E. Horton and G. Raisz, Annl N.Y. Acad. Sci. *256*, 132 (1975).
19 T.N. Salthouse and B.F. Matlaga, Experientia *28*, 326 (1972).
20 K. Hashimoto, Y. Yamanishi, E. Maeyens, M.K. Dabbous and T. Kanzaki, Cancer Res. *33*, 2790 (1973).
21 G.W. Wood, J.R. Neff, K.A. Gollahon and W.K. Gourley, J. Path. *125*, 53 (1978).

Ultrastructural localization of vanadium in the blood cells of Ascidiacea

L. Botte, S. Scippa and M. de Vincentiis

II Chair of Histology and Embryology, Faculty of Sciences, Via Mezzocannone, 8 Naples (Italy) and Zoological Station, Villa Comunale, I-80134 Naples (Italy), 4 October 1978

Summary. X-ray histospectrographic analysis at the scanning and transmission electron microscope (STEM) are made on the blood cells of *Phallusia mamillata* Cuvier and *Ciona intestinalis*, to study the 'direct' intracellular sites of accumulation of vanadium. The results show a clear accumulation of vanadium on the membrane and in the granules of vacuoles of amebocytes, signet ring cell, compartment cell and traces of metal in the 'vanadophores' of vanadocytes.

It has been reported that certain species of Tunicata manifest the faculty of absorbing great quantities of vanadium from the surrounding sea-water[2]. According to the majority of authors, this metal is selectively localized in a particular type of blood cell named 'vanadocyte'[3-5]. A vanadocyte measures 8-13 µm. Its cytoplasm contains voluminous acidophilic globules which, under the electron microscope, appear as more or less compact electron-dense masses, denominated vanadophores.

Vanadium, according to several authors, is found in the form of a tripositive valency and thus constitutes the native haemovanadin. This in turn has remarkable reducing influence on osmic acid[6]. This property has been utilized to asses indirectly the ultrastructural localization of vanadium and its compound in the blood cells during histogenesis[7,8]. Thus, in order to furnish information on sites of retainment of vanadium in the blood cells of the Ascidiacea, we have undertaken an ultrastructural histospectrographic microanalysis, by energy-dispersive X-ray analysis of different types of blood corpuscles of *Phallusia mamillata* Cuvier and *Ciona intestinalis*.

Materials and methods. Blood, collected through a puncture in the cardiac vessel, was centrifuged at 3000 rpm for 10 min. The pellet so obtained and preparation of the branchial epithelial cells were fixed in 10% neutral formalin in sea-water pH 6.9. The fixed material was dehydrated and embedded in epon[9]. 2-µm sections, obtained on a Ultrotome III, were examined by scanning transmission electron microscope Elmiscope ST 100 F Siemens (STEM) in combination with an energy dispersive X-ray detector. Attention was focused mainly on the microanalysis of the following categories of blood cells: amebocytes, signet ring cell, compartment cells and vanadocytes which are mainly involved in the accumulation and synthesis of vanadium complex.

Results and discussion. Data obtained from both species showed that: a) the vanadium is selectively concentrated in the vacuole membrane of amebocytes, signet ring cell and compartment cell; b) vanadium granules are present inside the vacuoles too; c) vanadium is very scanty in the vanadocytes. In fact the spectra, obtained by scanning of vacuole membrane and inside of the vacuole, showed peaks well corresponding to vanadium (figure A and B). On the other hand, spectrum of vanadophores showed a peak corresponding to the base line of the vanadium spectrum (figure C) which means just at the limit of the sensibility of the method employed (e.g. 1×10^{-19} g absolute weight of the element examined). Simultaneous control scanning of the blood cell cytoplasm without vacuoles and those of the resing used for embedding gave negative results. Besides

→

A Scanning transmission electron micrograph of a 'amebocyte' of the blood of *Phallusia mamillata* Cuvier. Presence of large vacuole with numerous clustered granules. The vacuolar membrane and the clustered granules inside of the vacuole are electron dense. - Energy dispersive X-ray spectra from the vacuolar membrane (1) and from the clustered granules inside the vacuole of the 'amebocyte'(2).
B Scanning transmission electron micrograph of a 'signet ring cell' of *Phallusia mamillata* Cuvier. The peculiarity of this cell is the presence of a large vacuole pressing upon the cytoplasm and nucleus in a polar cap. The vacuolar membrane and clustered granules are electron dense. - Energy dispersive X-ray spectra taken on the vacuolar membrane (1) and the clustered granules inside the vacuole of the 'signet ring cell' (2).
C Scanning transmission electron micrograph of a 'vanadocyte' of *Phallusia mamillata* Cuvier. Note the numerous electron dense globules 'vanadophore' around the nucleus. - Energy dispersive X-ray spectra of a tipical 'vanadophore' (2) and a control zone on the resin (1).