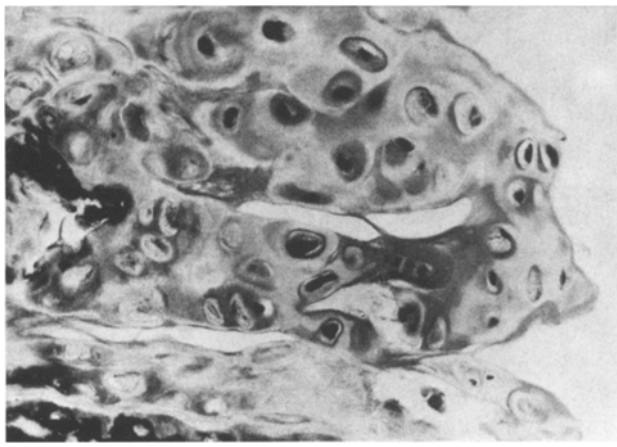

Figure 2. Cartilage bulging out of bone matrix. L6 cells cultured for 30 days on rat DBM pieces. The differentiated chondrocytes display a more or less spherical form; they are pink. The cartilage matrix, which is secreted by these cells in large amounts and separates them from one another, shows with variable intensity the bright blue which is characteristic for this substance after staining with Movat's pentachrome I (modified by Olah et al.[8]).

sterilization procedure causes a loss of osteogenic activity[6]. They were demineralized with two changes of HCl 0.6 N (70 vol./g) at 0 °C for 20 h. The bone pieces were then treated with phosphate buffer 0.2 M, pH 5.5, washed twice with water, and freeze-dried.

For each series of experiments, 12 petri dishes (diameter 5 cm) each containing 4–5 bone pieces were seeded with L6 cells suspended in the medium. Two thirds of the medium was changed twice a week. At first the bone pieces moved freely in the medium, but after 1 week they were held together by a multilayered cell sheet.

Half of the cultures were fixed at 20 days, the other half at 30 days; they were washed with Hank's solution and fixed with phosphate buffered formol 4%.

The bone pieces supporting the cells were dehydrated and embedded in methylmethacrylate. They were sectioned at 3µm with a rotation microtome type Biocut Jung No. 1130, and stained by a modification of Movat's pentachrom I stain[8].

Results. L6 cells, when incubated alone, behave essentially like myoblasts as described by Richler and Yaffe[7]; upon incubation some of the cells fuse into multinucleated myotubes. To observe the latter phenomenon in culture dishes, a disruption of the upper myoblastic sheet, leaving the myotubes intact, can be provoked by a 2-min treatment with Hank's solution containing 0.25% trypsin, followed by inactivation of the proteolytic enzyme with the normal incubation medium (fig. 1).

After 20 and 30 days incubation of the L6 cells with demineralized bone pieces, the cells show a high level of mitotic activity; they are piled up in many layers and invade the DBM pieces, lodging even in the lacunae formerly occupied by osteocytes. In one out of three culture vessels, formation of chondrocytes can be observed. These form rows of cells which are recognizable by their secretion of cartilage matrix brightly stained by alcian blue. The larger islets are bulging out of the bone pieces and the cells are separated by mucopolysaccharide in quantities as large as the diameter of the cells (fig. 2). The cells are pink and the bone pieces pinkish, in contrast to the blue stain of the cartilage matrix.

Discussion. L6 cells are thought to be cells of myoblastic origin, that can form muscular syncytia or myotubes[7]. After cellular differentiation the cells stop dividing. However mononucleated cells continue to proliferate. These cells, when cloned, again differentiate into myotubes or remain proliferating mononucleated cells. Thus, L6 cells appear to be myoblasts or committed muscle precursor cells. Nevertheless, it seems that the expression of the genome of the L6 cells can be modified by the presence of an active principle contained in the bone matrix in such a way that they differentiate into chondrocytes. This is relevant to the discussion on the origin of chondroblasts in ectopic bone formation[9]. L6 cells may be common precursor cells for both myoblasts and chondroblasts; the direction of the differentiation can be determined by exogenous factors and by the culture conditions.

In vivo, the cells that differentiate into chondrocytes in implants of bone matrix into muscle may also be of myoblastic origin by de-differentiation, or originate from a common precursor cell, rather than arising from non-differentiated mesenchymal cells.

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Membrane damage by coelomic fluid from *Holothuria polii* (Echinodermata)

C. Canicatti[1]

Institute of Zoology, via Archirafi 18, I-90123 Palermo (Italy), 19 September 1986

Summary. Rabbit erythrocyte membranes lyzed by *Holothuria polii* coelomic fluid, observed under the electron microscope, present lesions consisting of irregular holes which are heterogeneous in size (ranging from 50 Å to 250 Å) and ultrastructurally different from the ring-like structure produced by human complement. The protein pattern associated with the lyzed membrane was also examined.
Key words. Holothuria polii coelomic fluid; hemolysin; rabbit erythrocyte membranes; complement lesions.

The lysis of cells is one of the most important functions of the complement system. Observed using the electron microscope, this phenomenon is observed as the formation of holes in the damaged cells[2]. Typical ultrastructural complement lesions have

been described in mammals[2] and some lower vertebrates[3-6]. Nothing is known about lesions caused by hemolysins of invertebrates. These naturally-occurring molecules display a lytic activity against vertebrate erythrocytes used as target cells[7-12]. In some cases the hemolysins are thought to be complement-like[8,13,14], and in others they may cause lysis by a completely different, not specified mechanism[15,16]. In neither case is there any ultrastructural study of the lesions caused by invertebrate hemolysins.

In this study I investigated the ultrastructure of the lesions pro-

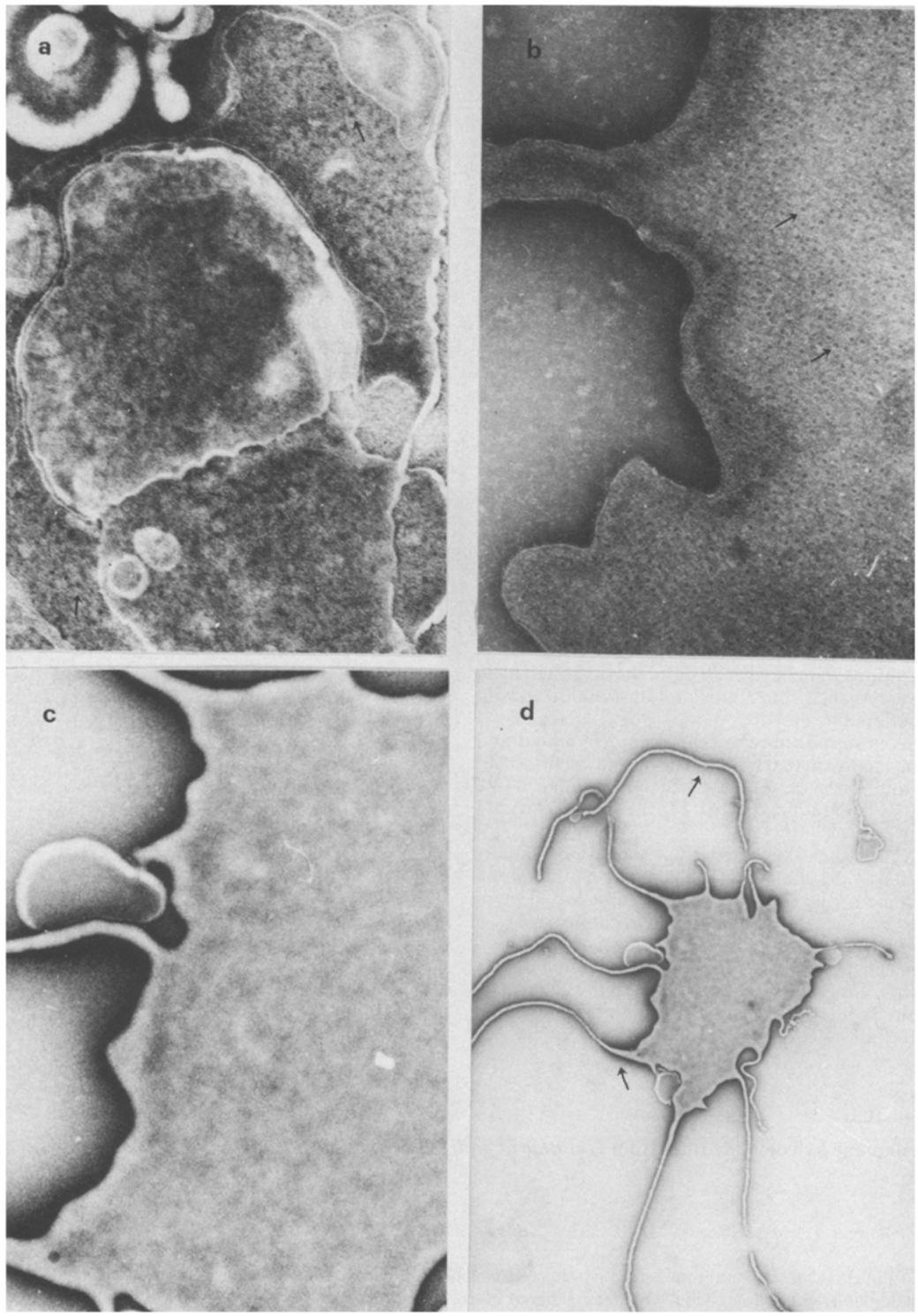

Figure 1. Rabbit red cell membranes lyzed by *Holothuria polii* coelomic fluid (panel *a*). The arrows indicate the irregular holes that cover the surfaces of membrane fragments. Magnification ×42,000.
Panel *b* shows an image of the rabbit erythrocyte membrane lyzed, for comparison, with human serum. The arrows point to typical ring-like complement lesions. ×70,000.
In panel *c* a rabbit erythrocyte membrane fragment lyzed by osmotic lysis is shown. ×42,000. At a lower magnification (8400×) of the same membrane fragment (panel *d*) the arrows show the long tubular projections emanating from the edge.

duced on rabbit erythrocyte membranes by the coelomic fluid of *Holothuria polii,* which possesses a naturally-occurring lytic factor[10]. Moreover, the molecular composition of the *Holothuria* proteins associated with lyzed membranes was analyzed.

Material and methods. Adult specimens of *Holothuria polii* D. Ch. were gathered from the gulf of Trapani.

The coelomic fluid (CF) of many animals was pooled. After removing hemocytes by centrifugation at 4000 × g for 30 min at 4 °C, the CF was divided into 20-ml aliquots and stored at −70 °C.

Before use, the 20-ml CF aliquots were concentrated by ultrafiltration in a Diaflo equipped with UM2 membrane (Amicon Corp. Lexington, Mass.) to 2 ml (protein concentration of about 1.2 mg/ml) and then extensively dialyzed against Tris-NaCl 0.05 M $CaCl_2$ 0.02 M pH 8.0 (CF^{++}). The CF^{++} samples were used throughout the experiments. Aliquots of total CF were labeled with ^{125}I (Amersham, England) according to Mac Conahey and Dixon[17] and then extensively dialyzed in Tris-NaCl 0.05 M, $CaCl_2$ 0.02 M, pH 8.0.

Human serum was obtained from a normal individual by cubital vein puncture. Rabbit erythrocytes (RRBC) were purchased from Sclavo-Firenze; before use, they were washed in isotonic Tris-NaCl 0.05 M pH 8.0 (Tris) or isotonic veronal-buffered saline pH 7.5 containing 0.5 mM $MgCl_2$, 0.15 mM CaCl and 0.1% gelatin (VBS^{++}).

RRBC membranes were prepared by incubating 40 λ of packed RRBC washed with Tris and/or VBS^{++} with, respectively, 0.5 ml of *H. polii* CF^{++} and 0.5 ml human serum at 37 °C in a water bath until complete hemolysis was apparent. Control RRBC membranes were obtained by osmotic lysis in distilled water. The ghosts were pelleted in a microfuge (Beckman model B) and washed three times in ethylene diamine tetraacetic acid Na_2-salt (EDTA) pH 8.0.

Electron microscopic examinations were made at once by drying a drop of erythrocyte membranes on Colloidion-Carbon coated 200 mesh copper grids, and negatively staining with 2% sodium phosphotungstate pH 7.5. A Philips electron microscope was used and operated at 60 kV. Micrographs were taken at magnifications of 12,000-, 60,000-, and 99,000-fold.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Laemmli buffer system[18]. 40 λ of RRBC membranes lyzed by ^{125}I-labeled CF^{++} were washed three times in EDTA and then dissolved in 100 λ of sample buffer containing 3% SDS and boiled for 5 min at 100 °C. 10–30 λ of these preparations were analyzed on 10% SDS-PAGE under non-reducing and reducing conditions. Gels were dried and exposed to Kodak X-OMAT AR films for different periods of time.

Lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,00), bovine serum albumin (66,200), phosphorylase B (92,500) (BIO-RAD) were used as molecular weight markers.

Results and discussion. As previously demonstrated[10], normal coelomic fluid of *Holothuria polii* was able to lyze rabbit erythrocytes in an alkaline medium supplemented with calcium. This property is lost after heat-inactivation (temperature exceeding 50 °C) suggesting that a heat-sensitive lytic factor (hemolysin) is involved in the hemolytic reaction.

To evaluate the damage produced by *H. polii* hemolysin, the membrane of lyzed rabbit erythrocytes was negatively stained with 2% sodium phosphotungstate pH 7.5 and subjected to electron microscope observation. As shown in figure 1 (panel a), not one of the erythrocyte membrane fragments contained the typical complement lesions produced by human serum (panel b) used for comparison. These lesions present the same characteristics as the ones previously described; they consist of regular holes with a dark central portion surrounded by a clear ring with an inner diameter of 100–110 Å[19]. The membrane lyzed by *H. polii* coelomic fluid appeared to be covered by irregular holes, heterogeneous in size, ranging from 50 Å to 250 Å but never surrounded by a ring-like structure (fig. 1, panel a). On the other hand, the membrane fragments obtained by osmotic lysis (panel c) present a finely granular surface without holes and very often with long tubular projections emanating from the edge (panel d) like the stromalytic forms described by Baker[20].

The analysis conducted with 10% SDS-PAGE of the proteins associated with the membrane of rabbit erythrocytes, after lysis by *H. polii* coelomic fluid, is shown in figure 2. By using ^{125}I-labeled coelomic fluid, so as to exclude the membrane proteins of the rabbit erythrocytes, I could detect (track B) two strongly radioactive bands: a high molecular weight band which did not penetrate into 10% gels and a band with a mol.wt. of about 27,000. A weak band with a mol.wt. of 31,000 was also present. Under reducing conditions (track C) the high mol.wt. band disappeared and two strongly radioactive bands with mol.wt. of about 80,500 and 64,000, appeared instead. The 31,000 band had a stronger intensity, while the 27,000 one was lost, probably as a result of reduction. These proteins could be responsible for the membrane damage observed under the electron microscope even if there is not, at present, any evidence to establish the possible mechanism of action. On the basis of the ultrastructural data, a complement-like cytolytic mechanism can probably be excluded.

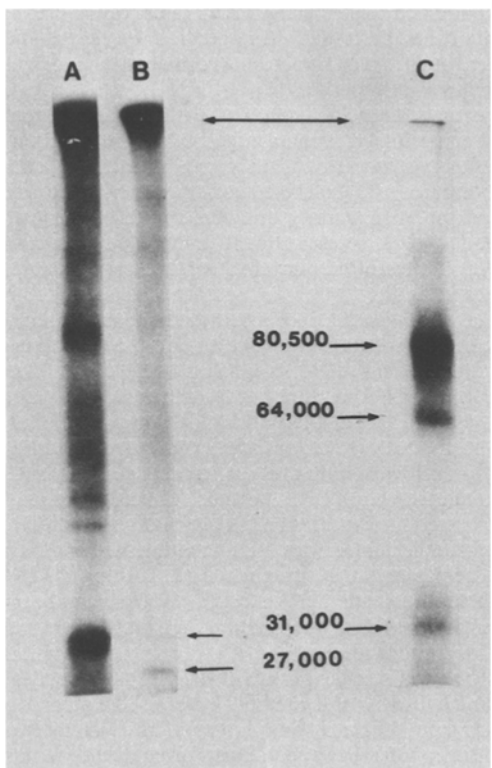

Figure 2. Figure 2 depicts the electrophoretic pattern in 10% SDS-PAGE of SDS-dissociated rabbit erythrocyte membranes lyzed by ^{125}I-labeled *Holothuria polii* coelomic fluid under non-reducing (track B) and reducing (track C) conditions. The arrow on the top indicates the high molecular weight protein which does not penetrate the 10% gel. Track A represents the non-reducing patterns of whole ^{125}I-labeled coelomic fluid.
The calculated molecular weights of each band are indicated by arrows.

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Enhancement of fibronectin expression by herbimycin A[1]

K. Umezawa, S. Atsumi, T. Matsushima and T. Takeuchi

Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108 (Japan), and Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141 (Japan), 8 August 1986

Summary. Herbimycin A specifically increased the level of fibronectin mRNA in Rous sarcoma virus-infected rat kidney cells, and the time course of fibronectin expression was found to be closely related to that of morphological change induced by herbimycin A.
Key words. Herbimycin A; fibronectin; β-actin; rat kidney cells.

Herbimycin A was isolated from the culture filtrate of *Streptomyces* in 1979 as an ansamycin antibiotic with herbicidal activity[2]. Recently, it was again isolated from the culture broth of *Streptomyces* sp. MH237-CF8 as an active substance that causes reversion of the transformed morphology of Rous sarcoma virus-infected rat kidney cells to the normal morphology[3]: herbimycin A-treated cells infected with a temperature-sensitive mutant of Rous sarcoma virus became flattened and more elongated and showed similar morphology to that of cells at the non-permissive temperature.

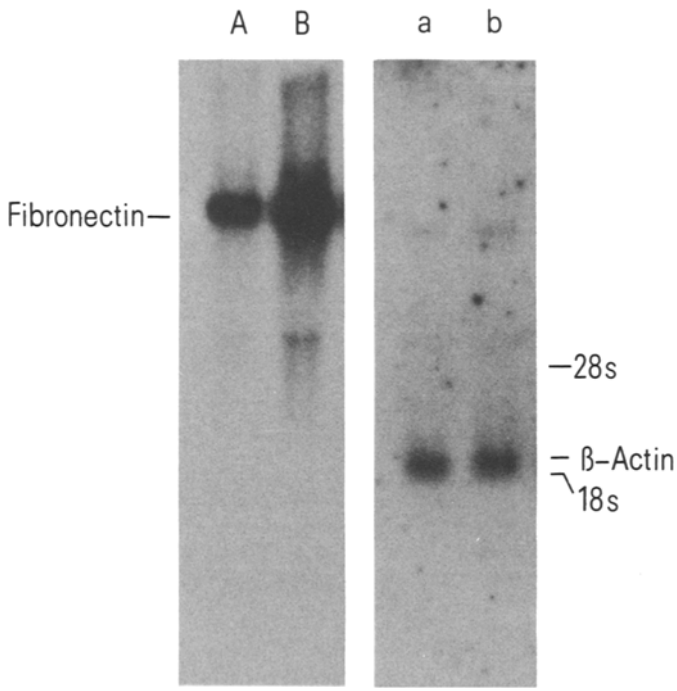

Figure 1. Fibronectin expression in ts/NRK cells. The cells were incubated at 33 °C (*A*) or 39 °C (*B*) for 2 days and harvested for RNA extraction. 10 μg of total RNA was applied on agarose gel electrophoresis and after Northern blotting, the blotted filter was hybridized with fibronectin cDNA (*A* and *B*) or β-actin gene (*a* and *b*).

Fibronectin is a large glycoprotein that plays an essential role in cell-cell and cell-basal lamina adhesion[4]. It is located on the cell surface and in plasma, and its primary structure has recently been elucidated[5]. Transformed cells have a reduced level of fibronectin on their surface and this decrease is known to be regulated at the transcriptional level[6]. Therefore, we have studied the effect of herbimycin A on fibronectin expression in tumor cells.

Materials and methods. Herbimycin A was kindly supplied by Dr Y. Uehara, National Institute of Health, Tokyo. A human fibronectin cDNA clone, pFH1[7], was a gift from Dr A.R. Kornblihtt, INGEBI, Buenos Ayres, and the β-actin gene, pRβAc3'ut[8], was kindly supplied by Dr K. Tokunaga, Chiba Cancer Center Research Institute. Normal rat kidney cells infected with the temperature-sensitive mutant of Rous sarcoma virus (ts/NRK cells)[9] were obtained from Dr M. Yoshida, Cancer Institute, Tokyo. The cells were grown at 33 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G and streptomycin. Approximately 2×10^6 cells were seeded into 40 ml of medium in a Nunc 175-cm^2 flask and harvested in the sub-confluent state after culture for 54 h. Herbimycin A was added for the indicated times.

Total RNA was extracted from sub-confluent monolayers by the guanidine hydrochloride method[10]. About 300 μg of RNA was extracted from cells in 5 Nunc 175-cm^2 flasks. Transfer of RNA from electrophoresis gels containing formaldehyde to nitrocellulose membranes and hybridization to the pFH1 probe were carried out as described in the manual of T. Maniatis et al.[11].

Results and discussion. ts/NRK cells show the morphology of tumor cells with criss-crossing and piling up at the permissive temperature (33 °C) and flattened normal morphology with contact inhibition at the non-permissive temperature (39 °C)[9]. As shown in figure 1, expression of fibronectin was markedly in-