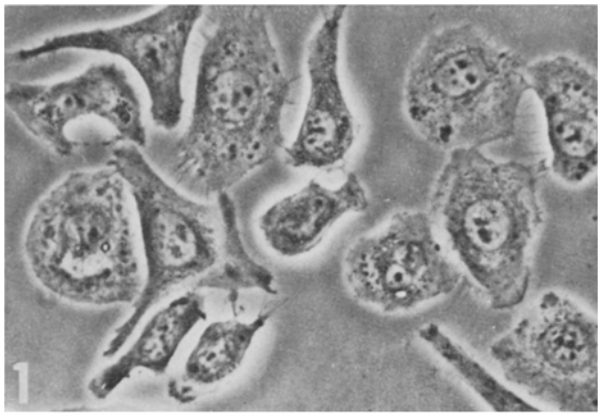
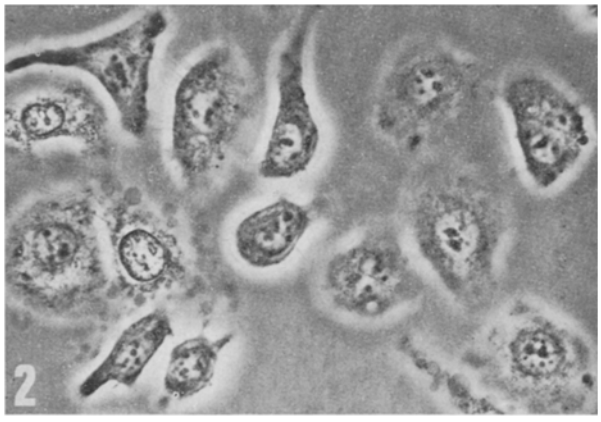
Cytotoxic Effects of an Alcoholic Extract of a Sponge, *Suberites inconstans*

Many toxic and pharmacologically active substances have been isolated from marine invertebrates[1–4]. Among marine sponges investigated, some have been found to contain antiviral substances[5], antibiotics and antitumour substances[2]. In our investigation of marine sponges in Singapore waters, we found that an alcoholic extract of the sponge, *Suberites inconstans*, was cytotoxic to HeLa cells.

Suberites inconstans were collected from a local reef, drained of sea water and cut into small pieces. Non-sponge materials were carefully removed and discarded. The sponge was immersed in equal volumes of 95% ethanol and shaken for a week. The alcoholic extract was then filtered and concentrated by flash evaporation before freeze-drying and storage. The freeze-dried material was redissolved in 50% ethanol, centrifuged to remove any insoluble particles and sterilized by membrane filtration. The concentration of sponge material was determined by taking aliquouts of the extract and evaporating to complete dryness, and weighed.





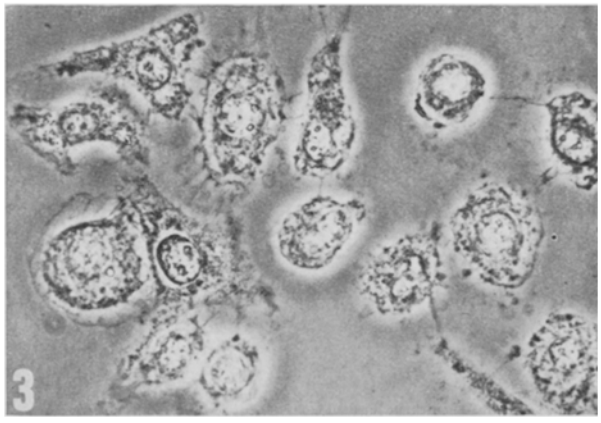


Fig. 1–3. Phase contrast photographs of HeLa cells which were exposed to the sponge extract for 0, 10 and 60 min respectively. ×400.

Stock cultures of HeLa cells (Strain E, Commonwealth Serum Laboratories, Australia) were grown as monolayers in McCoy 5a medium (modified, GIBCO) containing 10% ox serum and antibiotics as described previously[6].

The effect of the sponge extract on HeLa cell growth over 4 days was investigated using DNA as an index of growth. DNA was estimated according to the method of BURTON[7] using calf thymus DNA as standard. HeLa cells (approximately 5×10^4 cells/ml) were grown at 37 °C for 24 h in roller tubes containing McCoy medium with 10% ox serum and antibiotics, after which the medium was replaced with fresh media containing sponge materials ranging from 0 to 1.2 mg/ml. After another 48 h of incubation, the media were renewed with fresh solutions containing the same concentrations of sponge extract. At the end of the next 48 h of incubation, the DNA content for each tube was analysed.

The results are summarized in the Table. The DNA content per tube of HeLa cells at the beginning of the experiment was 4.5 µg. After 4 days' growth in the absence of sponge extract, this was increased to 35.4 µg, an 8-fold increase. The effect of the sponge extract varied with its concentration. At concentrations up to 6 µg/ml the extract had no inhibitory effect on cell growth. Above this concentration, the inhibition was partial while at concentrations of 60 µg/ml and above, growth was totally inhibited with concomitant reduction in DNA content. The latter was a result of cell lysis. At concentrations of 24 µg/ml and below, the cytotoxic effect could be reversed even after 4 days of exposure.

Effect of *Suberites inconstans* extract on HeLa cell growth

Concentration of *Suberites inconstans* extract (µg/ml)	HeLa cell DNA content per tube	
	After 4 days growth	2 days after removal of sponge material
1,200	0	0
120	0.2	0
60	1.5	0.5
24	14.7	25.2
12	27.8	45.8
6	35.9	44.8
2	34.4	46.1
0	35.4	

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Observations were also made under phase contrast microscopy using a Leitz Orthoplan microscope fitted with planar objectives and Heine phase contrast condenser. For this purpose, the extract was diluted with serum-free McCoy medium to give a concentration of 10 µl/ml of medium. HeLa cells grown on coverslips were inverted over depression slides containing sponge extract in medium and observations were made over a 2-h period. Cells exposed to medium containing an equivalent amount of ethanol but without sponge extract were used as controls.

Figure 1 shows a population of HeLa cells immediately after a coverslip culture was inverted over medium containing sponge extract. After 10 min of exposure to the extract, spherical globules were observed near the periphery of the cells (Figure 2). In a few cells, the cytoplasm appeared to have fragmented and the nuclei showed prominent nuclear membranes with little nuclear detail. Marked changes were seen 1 h later (Figure 3). Cytoplasmic material appeared to be fragmented and were found to aggreagate around the nucleus in some cells, leaving a clear zone adjacent to the cell membrane. In some cells, the cell membrane appeared to have ruptured. The nuclear membrane was more prominent in some cells while in other cells it appeared to have ruptured also. Nuclear detail was lacking in most of the cells and the nucleoli were either not seen or appeared as small dark clumps. These features were also observed under the electron microscope, and a more detailed ultrastructural study is now underway[8]. No recovery was obtained when this coverslip culture was placed in sponge-free medium after the experiment.

The experiments have shown that an alcoholic extract of the sponge, *Suberites inconstans*, was cytotoxic to HeLa cells; recovery was possible only when the cells were exposed to low concentrations of the extract.

Zusammenfassung. Ein alkoholischer Extrakt des Schwammes *Suberites inconstans* erweist sich als ein Zellgift gegenüber Hela-Zellen. Wachstumsstörungen treten auf, und mikroskopisch sichtbare Veränderungen an Kernen und Membranen werden festgestellt.

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Closed Circulation in the Rat Spleen as Evidenced by Scanning Electron Microscopy of Vascular Casts

No conclusive evidence has been available to settle the question whether the arterial blood of the spleen is directly drained into the venous sinuses or flows into the spaces of the cords of Billroth before being received by the sinuses[1]. The former 'closed' theory was originated by WEIDENREICH[2] in 1901, and the latter, 'open' theory by HELLY[3] in the next year. Although the 'closed' theory was strengthened in the 1930's by some light microscopists such as BJÖRKMAN[4], who injected various matters into splenic vessels, and KNISELY[5], who observed the spleen in living animals, the general view of modern histologists seems inclined to the 'open' theory[1]. Recent electron microscope studies on ultrathin sections of the organ have also failed to demonstrate a closed circulation in the spleen[6].

The present study was undertaken to shed light on this field of study by observing directly and 3-dimensionally the vascular casts under the scanning electron microscope.

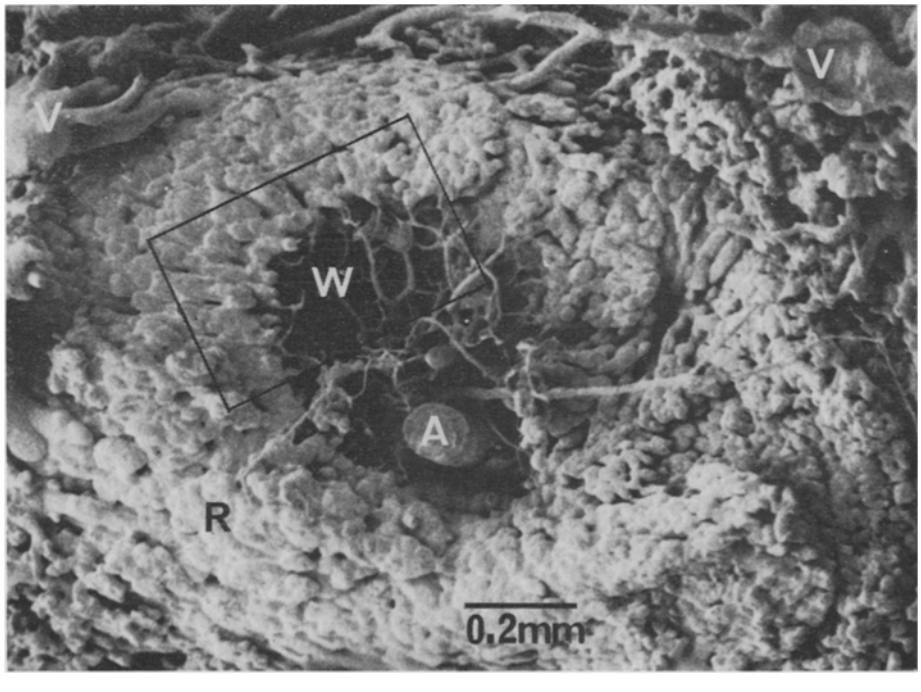

Fig. 1. A low power scanning electron micrograph showing the vascular arrangement in the rat spleen. Red pulp (R), white pulp (W), a central artery (A) and trabecular veins (V) are seen.