Effects of Natural Toad Serum on Heterologous Vertebrate Cells

It has been shown that natural serum of the toad *Bufo marinus* will cause haemolysis of washed erythrocytes of several vertebrate species[1]. Recently, in unpublished experiments we attempted to transplant B16 mouse melanoma and Ehrlich Lettré ascites (ELD) tumours into adult toads (*Bufo marinus*) maintained at 37 °C. In spite of X-irradiation and/or cortisone conditioning, all attempts were totally unsuccessful. To discover whether these negative results could have been due primarily to the cytocidal action of toad body fluids rather than to a cell-mediated response, we exposed several types of mammalian tumour cells and normal vertebrate cells in vitro to freshly prepared, natural toad serum and plasma.

Material and methods. The tumour cells included those of in vitro-passaged B16 and ELD lines, permanent in vitro-adapted sublines SZ_4B16 and SZEL, and HeLa. Normal cells were those of *Bufo marinus* (heart and lung), mouse (embryonic and adult heart and lung), and goldfish (in vitro line of testis cells SZGT)[2]. None of the toads from which serum and plasma were collected had ever been injected with tumour cells. Blood was obtained by direct heart cannulation. Pooled serum was passed through a washed Millepore filter[3] to remove any residual cells. Serum and plasma were used within 3–4 h of preparation and were maintained before use in an ice bath. Part of each batch of pooled serum was heated in a water bath for 30 min at 58 °C. No anticoagulants were used during the preparation of plasma.

Eagle's minimum essential medium supplemented with 0.7% lactalbumin hydrolysate was used in all experiments. 20% heat-inactivated young calf serum was added to control cultures. 3 types of cultures were used for testing: a) Explants *in clots*. Chopped B16 explants taken directly from host mice were plated in firm clots of toad or avian plasma and filtered chick embryo extract. They were incubated for 48 h in serum-free medium. b) Monolayer cultures on glass. Cells plated at a standard concentration were incubated in ring chambers on coverslips for 24 h in standard medium. They were then examined, washed thoroughly and, except for the controls, provided with serum-free medium. 20% or 50% toad serum was added to the experimental cultures. Sample cultures were fixed for staining after 20–30 min. The rest were incubated for a further 19 h before fixation. c) Cell suspensions. Thoroughly-washed, dissociated cells were suspended, counted, and tested for viability in serum-free medium. Toad serum was added to small tubes containing 2×10^5 cells/ml. After 30 min at 37 °C, all samples were tested for viability with trypan blue.

Results and discussion. a) Explants. All 25 B16 explants incubated in avian plasma produced good outgrowth within 48 h (Figure 1). None of the 25 explants in toad plasma produced any outgrowth and all loose cells were dead (Figure 2). b) Monolayer cultures. All cell types except those of *Bufo* were severely damaged by unheated toad serum. To varying degree they showed immediate withdrawal of processes, frequent blebbing, rapid swelling presumably associated with changes in membrane permeability, and apparent clumping of cytoplasmic organelles. A characteristically ragged appearance was attained within 4–5 min (Figures 3 and 4). Cultures incubated overnight with 20% natural toad serum had few living cells next day. Short-term incubation (30 min) with heated toad serum had no apparent adverse effects. c) *Cell suspensions.* The results are summarised in Figure 5. The effect of toad serum appeared to be influenced by its concentration. Visible cell damage, including clumping and permeability to trypan blue, was obvious on haemocytometer examination.

Storage of serum at 5 °C reduced its lethal effects. Suspensions of HeLa and ELD cells tested with 50% toad serum refrigerated at 5 °C for 6 days recorded 53% and 80% viability, respectively. With 20 %serum, this figure rose to 73% and 87%.

LANDY et al.[4] showed that the normal blood serum of several avian and mammalian species had lethal effects in vitro on various mouse tumours, the agents responsible being regarded as natural antibody and complement. The serum of *Bufo marinus* contains a complement system apparently comparable in standard reactions to that of the

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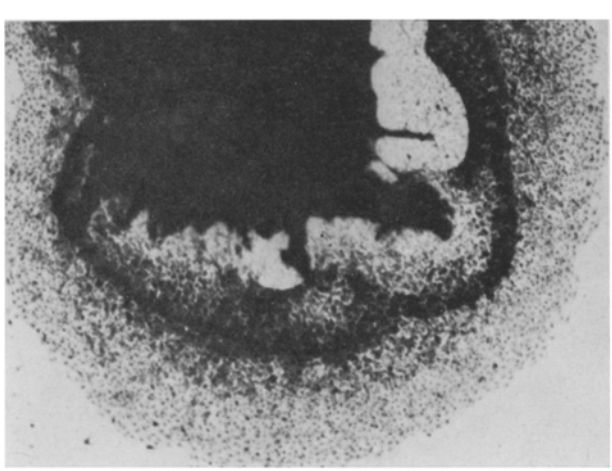

Fig. 1. Explant of B16 mouse melanoma after 48 h incubation in an avian plasma clot. Fixed and stained preparation.

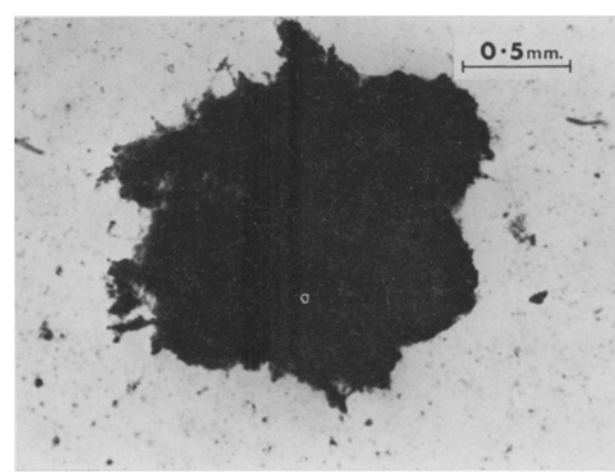

Fig. 2. Explant of B16 mouse melanoma after 48 h incubation in a toad plasma clot. Fixed and stained preparation.

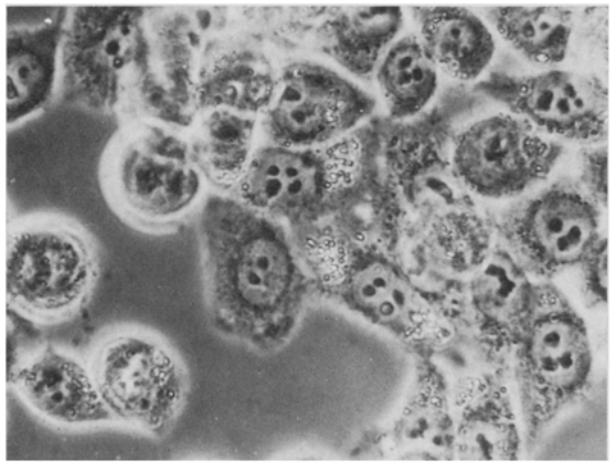

Fig. 3. Group of living HeLa cells immediately before treatment with toad serum. Phase contrast.

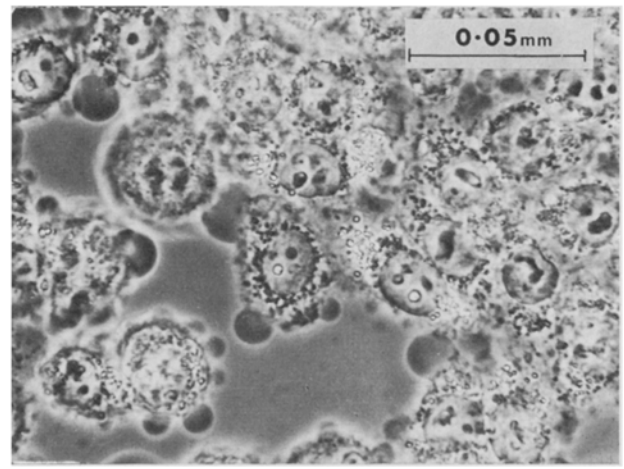

Fig. 4. Same group of HeLa cells as in Figure 3, photographed 20 min after addition of 50% toad serum to the medium. Phase contrast.

guinea-pig; its action against erythrocytes is selectively destructive[1].

Our results suggest that the destruction of mammalian tumour transplants in toads may have been due primarily to a naturally occurring, heat-labile, cytotoxic factor or factors in body fluids. They do not exclude the possibility of a concurrent, cell-mediated response in unconditioned toads. *Bufo marinus* can produce antibodies over a wide range of temperatures[5] including 37 °C[6].

Our results add to the available phylogenetic information regarding the natural toxicity of serum of specific animals for specific cell types. The role of the cytotoxicity of natural sera in the defence mechanisms of host animals will become apparent only as a greater range of data, particularly from in vitro studies, becomes available[7].

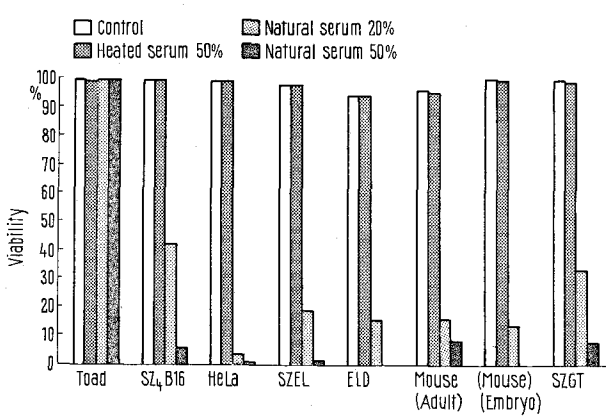

Fig. 5. Histogram showing percentage viability of dissociated normal and malignant cells exposed to toad serum for 30 min at 37°C. In all except mouse and SZGT, figures are based on means of at least 3 experiments with different batches of pooled serum.

Résumé. Le sérum et le plasma, nouvellement préparés, de l'Amphibien *Bufo marinus*, ont, in vitro, un effet rapidement cytotoxique sur des cellules normales et malignes des autres Vertébrés.

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[7] This work was supported by a grant from the University of Sydney Cancer Research Fund. We thank Miss Cynthia Batson for technical assistance.

Specific Stimulation of Mycobacteria Phagocytosis by Substances Liberated during the Cultivation of Lymph Node Cells from Tuberculin Hypersensitive Rabbits with the Specific Antigen

Macrophages play the crucial role in immunity against intracellular parasites[1]. They act, however, rather as pharmacologically active effector cells, whereas the proper immunologically specific process is triggered by the lymphocytes[2,3].

In a previous paper we have demonstrated the role of immune lymphocytes in the phagocytosis of foreign erythrocytes by peritoneal macrophages from normal non-sensitized animals[4]. In the present paper the stimulation of phagocytosis by mediators of delayed hypersensitivity[5] liberated during the interaction of hypersensitive lymphocytes with the specific antigen was studied.

For this purpose, live Bacillus Calmette-Guérin (BCG) vaccine (in homogenized suspension) was added to cultures of normal peritoneal macrophages cultured in Parker 199 (GIBCO) without or in the presence of supernatants prepared by the cultivation of lymph node cells from tuberculin hypersensitive rabbits with various doses or