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NON-IMMUNOLOGICAL CELL DEATH OF INTRAVENOUSLY INJECTED MURINE TUMOUR CELLS

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Summary.—Most DBA mastocytoma and Sarcoma 180 cells trapped in the lungs of mice after i.v. injection died within 7 h. Rates of cell death were similar for both tumour cell lines. Rates of tumour cell death were unrelated to whether the cells were allogeneic or syngeneic, induced platelet aggregation or not, had different patterns of subsequent tumour growth, or were injected in varying numbers. Cell death was by coagulative necrosis, not apoptosis. Sarcoma 180 tumour cells were quickly localized in the lung and enclosed in platelet aggregates which remained, with degranulation, until the time of tumour cell death. However, platelet aggregation did not appear to play a role in tumour cell killing. The prevention of platelet aggregation by pre-treatment of mice with an anticoagulant had little effect on the rate of death of tumour cells in the lung. Mastocytoma tumour cells did not cause platelet aggregation, yet died in the lung at similar rates to Sarcoma 180 cells.

The killing of tumour cells in the lung did not appear to be cell-mediated. No mononuclear cells were seen in the vicinity of tumour cells and the type of cell death was not that associated with cell-mediated killing. The tumour cells did not die within 6 h of being injected into the peritoneal cavity.

It is suggested that a nonspecific non-immunological process results in the death of intravenously injected tumour cells in the lung. This process was not affected by differing oxygen levels in the inhaled gas.

IT has long been recognized that the majority of intravenously injected tumour cells fail to survive to form subsequent tumours even in those cancers which progress and kill the recipient animal (Iwasaki, 1915; Warren and Gates, 1936; Zeidman, McCutcheon and Loman, 1950; Baserga, Kisieleski and Halversen, 1960; Greene and Harvey, 1964). More recently the method of entrapment of circulating tumour cells has been studied in experiments with tumour cells labelled with radioisotopes (Fisher and Fisher, 1967*a*, *b*; Hofer, Prensky and Hughes, 1969; Fidler, 1970; Fisher and Fisher, 1976; Proctor, Auclair and Rudenstam, 1976; Sadler and Alexander, 1976; van den Brenk *et al.*, 1975) and in a number of ultrastructural studies (Jones, Wallace and Fraser, 1971; Vlaeminck *et al.*, 1972; Dingmans, 1973; Locker, Goldblatt and Leighton, 1970; Wallace *et al.*, 1973; Chew and Wallace, 1976; Chew, Josephson and Wallace, 1976; Warren, 1976). While the kinetics of tumour cell death after i.v. injection have been analysed in some detail using radioisotope-labelled tumour cells (van den Brenk *et al.*, 1975; Glaves and Weiss, 1976; Fidler, 1976), the cause and nature of this death have not been established.

The role of specific immunity in this process is not clear. It has been suggested that naturally occurring antibodies (Martin and Martin, 1975; Ménard, Colnaghi and Porta, 1977) or naturally occurring "killer cells" (Kiessling *et al.*, 1976) may be

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responsible. It has been shown in human studies that at least 50% of γG-globulin and 20% γM-globulin is present in the interstitial fluids (Ritzmann and Levin, 1969). If an antibody is responsible for the cell death in the animals used in the present studies, then this process would be likely to occur in the peritoneal cavity also.

It has been shown by Kerr, Wyllie and Currie (1972) that the 2 types of cell death, coagulative necrosis and apoptosis, have different basic mechanisms. Apoptosis is the mechanism of cell death in T-cell-mediated immunological killing (Don *et al.*, 1977; Sanderson and Glauert, 1977). A morphological study of the nature of tumour cell death after injection should therefore suggest whether cell-mediated immunity is involved.

There remains the possibility that the death of tumour cells in the lung is a nonspecific non-immunological process. Initial studies suggested that tumour cell lodgement in the lung was associated with platelet aggregation (Wood, 1971). Recent ultrastructural studies suggest that different lines of tumour cells may or may not induce platelet aggregation in the lung (Jones *et al.*, 1971; Locker *et al.*, 1970). The role of platelets in the lodgement of tumour cells in the lung is still not clear. There is a possibility that there is a relationship between the type of lodgement of tumour cells and their subsequent death.

This paper presents data from combined kinetic and morphological studies of the death of intravenously injected allogeneic and syngeneic tumour cells in the lungs of recipient animals. This was undertaken to clarify the role of immunity and platelets in this process. The effects of anticoagulants and of differing oxygen levels in the inhaled gas were also examined.

MATERIALS AND METHODS

Tumours.—A Sarcoma 180 (obtained from the Queensland Institute of Medical Research) was maintained by serial i.p. passage of approximately 10^7 cells every 10 days in an outbred strain of Quackenbush mice. The mastocytoma P-815 X-2 (obtained from the Walter and Eliza Hall Institute of Medical Research) was maintained by serial passage of approximately 10^7 cells every 8 days in syngeneic inbred DBA/2 mice.

Both tumours had a 100% take after as few as 10^4 cells were injected i.p. in their respective recipient strains. Both tumours were lethal to their hosts. In the present experiments tumour cells were injected i.v. into the same mouse strain as that in which they were maintained.

Tumour cell inoculation for morphological studies.—The tumour cells were harvested in the exponential growth phase 5–7 days after i.p. injection (Schiffer *et al.*, 1973), washed and resuspended in calcium and magnesium free Hanks' solution. Their concentration was measured with an electronic cell counter (Coulter, Model Z-F) and $2{\cdot}5 \times 10^6$ cells in 0·1 ml of Hanks' solution injected i.v. (into the dorsal vein of the penis) in mice lightly anaesthetized with sodium pentobarbitone. Mice were killed at various times after injection and tissues prepared for electron microscopy.

Electron microscopy.—Small blocks of tissue were fixed by immersion in 3% glutaraldehyde at 4° in evacuated vials for 1–2 h, post-fixed in OsO_4 for 1–2 h at room temperature, stained *en bloc* with uranyl acetate, dehydrated in ethanol and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HS7S electron microscope.

^{125}I-Iododeoxyuridine labelling of tumour cells.—The tumour cells from a single mouse were harvested in the exponential growth phase 5–7 days after i.p. injection, washed with RPMI 1640+10% FCS, then $5–10 \times 10^7$ cells were resuspended at 10^6 cells/ml RPMI 1640+10% FCS containing 0·5–1 μCi of ^{125}IUdR (S.A.= 5 Ci/mg; 1 mCi/ml; the Radiochemical Centre, Amersham, England) per ml of suspension. Because of their differing growth rates (Ablett *et al.*, 1978) the Sarcoma 180 tumour cells and mastocytoma tumour cells were labelled for 18 h and 3 h respectively. The cells were washed twice, suspended in calcium and magnesium free Hanks' solution and stored at room temperature until use (within 30 min).

Intravenous studies.—10^6 or $2{\cdot}5 \times 10^6$ labelled tumour cells prepared as above were injected i.v. *via* the dorsal vein of the penis in recipient mice. The number of mice injected at each interval with Sarcoma 180 cells or mastocytoma cells were 10 and 8 respectively. Mice were killed by cervical dislocation and whole organs were immediately removed, placed in individual vials and 70% ethanol added. The alcohol was changed daily for 3 days. This treatment has been found to remove approximately 99% of

acid-soluble ^{125}I from the tissue of small organs, leaving the remaining ^{125}I associated with whole DNA (Fidler, 1970). The whole organs were counted in a well-type Packard Gamma Counter.

The bladder with contained urine was taken and counted without further preparation. Comparisons were made with standard volumes of known concentrations of the original washed tumour-cell suspension that were also counted without further preparation.

Intact "killed" tumour cells were prepared by heating the tumour suspension at 60° for 10 min before injection.

Intraperitoneal studies.—10^6 labelled tumour cells prepared as above were injected i.p. into mice. The mice were killed by cervical dislocation and their peritoneal cavities were washed several times with a total of 50 ml of saline. Intact tumour cells were filtered from the saline with a glass fibre filter, washed with saline and counted without further treatment.

Anticoagulation studies.—15 min before the i.v. injection of tumour cells, the recipient mice were injected i.v. *via* the dorsal vein of the penis with 1000 u of heparin (Commonwealth Serum Laboratories).

Oxygen levels in inhaled gas.—Before an i.v. injection of ^{125}IUdR-labelled Sarcoma 180 cells, prepared as described, the mice were placed for 1 h in an airtight perspex box into which was bled either 10% oxygen in nitrogen or 40% oxygen in nitrogen. After the injection, which took place in room air, the mice were returned to the original oxygen/nitrogen mixtures until they were killed and whole organs taken.

Blood gas analysis (using a Radiometer Acid-base Laboratory ABL2) was carried out on 5 mice which had been inhaling either 10% or 40% oxygen in nitrogen for 1 h, or normal air.

RESULTS

Macroscopic studies

Table I shows the mortality in mice 2 months after an i.v. injection of various numbers of tumour cells. Usually only one tumour was found in mice that had died after an injection of Sarcoma 180 tumour cells. The tumours were large and were found mostly in extrapulmonary organs or in the subpleural lymphatics. Never more than one macroscopic parenchymal lung tumour was found in any group and few tumours occurred in the liver. In mice injected with mastocytoma cells, multiple tumours (30–50) were found in both the lungs and livers. Owing to their small size, tumours in other organs were difficult to find. The average survival time for mice injected with Sarcoma 180 was 5–7 weeks and for mice injected with mastocytoma 2–3 weeks. In both tumours a decrease in the cell numbers injected resulted in an increase in survival times of the mice.

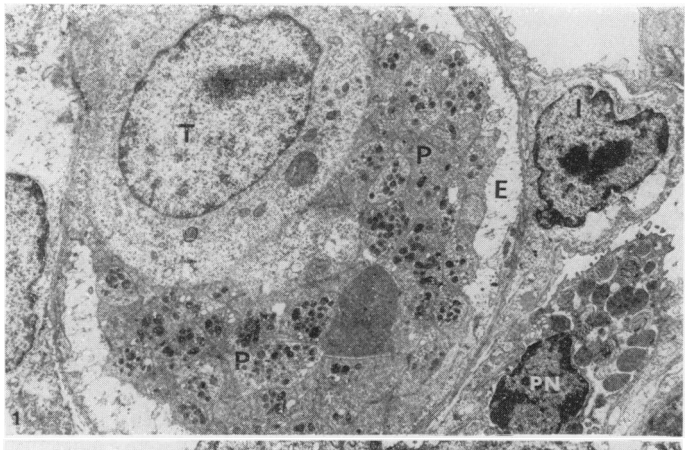
TABLE I.—*Mortality at 2 Months of Mice after Various Doses of Intravenously Injected Tumour Cells*

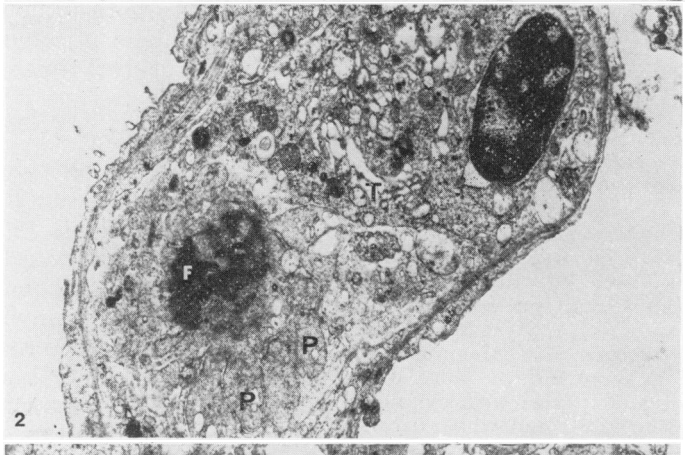
Tumour	Cell number	Mortality
Sarcoma 180	$4{\cdot}5\times10^6$	16/30 (53%)
	10^6	17/48 (35%)
	10^5	10/64 (16%)
	10^4	4/43 (9%)
Mastocytoma	10^6	29/29 (100%)
	10^5	27/27 (100%)

Electron microscopy

Sarcoma 180.—Individual tumour cells were identified in small arterioles and capillaries of animals killed 5 min after injection. The tumour cells were identifiable because of mitochondrial and nuclear abnormalities which previous studies had established as characteristic (Zuckerberg, 1973). Most tumour cells were surrounded by platelets. The surrounding platelets which were loose clusters at 5 min were by 10 min large compact masses (Fig. 1). Macrophages, lymphocytes or other normal circulating cells were rarely present in the aggregates. Endothelial cells adjacent to the aggregates sometimes showed extensive hydropic change (Fig. 1). The platelet masses remained for approximately 6 h with platelets in varying stages of degranulation. Material resembling fibrin was seen in some aggregates (Fig. 2). The tumour cells were still completely intravascular and showed no signs of endothelial breaching at 7 h.

After 5–7 h, tumour cells that were visualized showed either hydropic change or coagulative necrosis (Figs 2 and 3). Only rare fragments of condensed cytoplasm consistent with apoptosis (Kerr *et al.*, 1972) were seen, which suggested that this was not a significant process in this system.





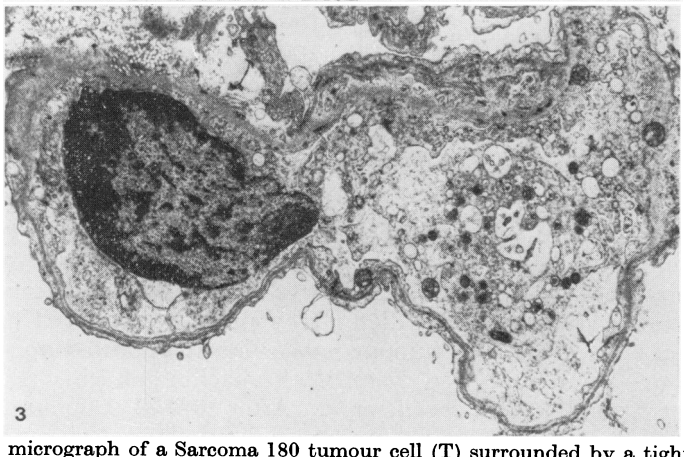


FIG 1.—Electron micrograph of a Sarcoma 180 tumour cell (T) surrounded by a tight aggregate of platelets (P) in a lung arteriole 15 min after injection. The endothelial cells (E) show hydropic change. PN is a Type II pneumocyte. I is an interstitial cell. ×4150.

FIG. 2.—Electron micrograph of a Sarcoma 180 tumour cell (T), showing early changes of coagulative necrosis, in a lung capillary 6 h after injection, with an associated clot of fibrin (F) and degranulated platelets (P). ×9630.

FIG. 3.—Electron micrograph of a Sarcoma 180 tumour cell showing coagulative necrosis and disruption of cytoplasm in a lung capillary 7 h after injection. Platelets appear to be absent. ×7550.

After 7 h very few tumour cells were seen and platelet clumps were absent.

Tumour cells entrapped in the lungs after being injected i.v, into mice that had been pretreated with heparin did not have any platelets associated with them, but when visualized 5–7 h after injection showed hydropic change or coagulative necrosis.

Mastocytoma.—We were unable to identify these tumour cells conclusively by electron microscopy. However, no preferential collections of platelets or other circulating cell types were observed.

Oxygen levels in blood

The oxygen content of the blood of mice which had been inhaling either 10% or 40% oxygen in nitrogen for 1 h or normal air is shown in Table II. The PO_2

TABLE II.—*Oxygen Content in the Blood of Quackenbush Mice after at least 1 h in Atmospheres of Various Oxygen Content*

% Oxygen in inhaled gas	PO_2 of blood
10	$25{\cdot}5 \pm 2{\cdot}7$
20 (normal air)	$49{\cdot}1 \pm 4{\cdot}8$
40	$88{\cdot}7 \pm 3{\cdot}9$

of the blood of mice inhaling either 10% or 40% oxygen were respectively approximately a half or double that of mice inhaling normal air.

I^{125} UdR-labelled tumour cells

Fig. 4 shows that the majority of i.v. injected Sarcoma 180 tumour cells were initially trapped in the lungs. These tumour cells rapidly disappeared from the lungs. This was closely followed by an increase in the radioactivity of the urine. The increase in radioactivity in the urine shown here is representative of all experiments involving the i.v. injection of labelled Sarcoma 180 tumour cells. The rate of tumour-cell loss from the lungs was independent of the number of tumour cells initially injected. Few tumour cells remained in the lungs after 7 h. Radioactivity of the liver was low 10 min after injection (<2% of total radioactivity) but

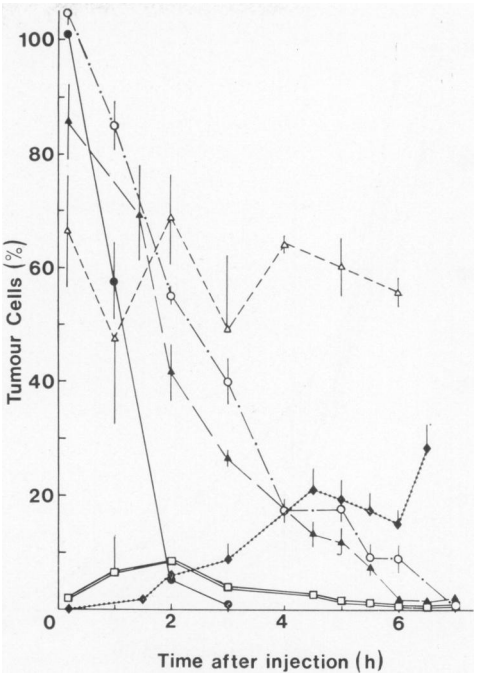


FIG. 4.—The percentage of i.v. or i.p. injected ^{125}I-labelled Sarcoma 180 cells remaining at various times: in the peritoneal cavity after an i.p. injection of 10^6 cells (△ – – – △); in the lungs after i.v. injection of 10^6 cells (▲ – – – ▲), $2{\cdot}5 \times 10^6$ cells (○ – · – · – ○) or 10^6 "heat killed" cells (●——●); in the liver after i.v. injection of 10^6 cells (□ == □); in the urine after i.v. injection of 10^6 cells (◆ ····· ◆). Percentages of cells remaining were determined from the ratio of radioactivity to that of the initial inoculum. Radioactivity in urine is expressed as if representing whole cells. Bar represents ±1 s.e. mean.

increased slightly to peak at 2 h. The radioactivity of the liver was low again by 6 h (<1% of total radioactivity and was at background level after 24 h. The radioactivity of the kidneys, spleen and thymus showed a similar pattern to that of the liver.

The majority of intact heat killed tumour cells that were injected were also initially trapped in the lungs. These cells disappeared more rapidly from the lungs than live cells, few remaining after 3 h.

The number of live Sarcoma 180 tumour cells recoverable after being injected i.p. did not decrease significantly after 6 h.

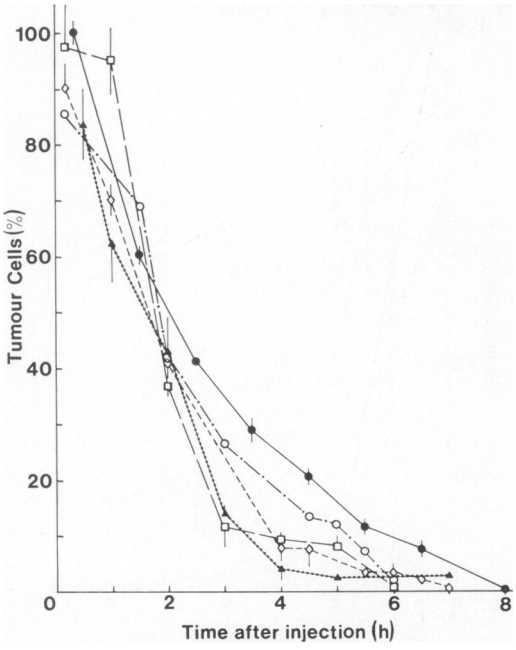


FIG. 5.—The percentage of i.v. injected ^{125}I-labelled Sarcoma 180 cells remaining at various times in the lungs: of mice treated with heparin and injected with 10^6 cells (◇ – – – ◇) or $2{\cdot}5\times10^6$ cells (●——●); of mice injected with 10^6 cells and kept in atmospheres containing 10% oxygen (□ – – – □) or 40% oxygen (▲·····▲); of untreated mice injected with 10^6 cells (○ – – – ○). Bars represent ±1 s.e. mean.

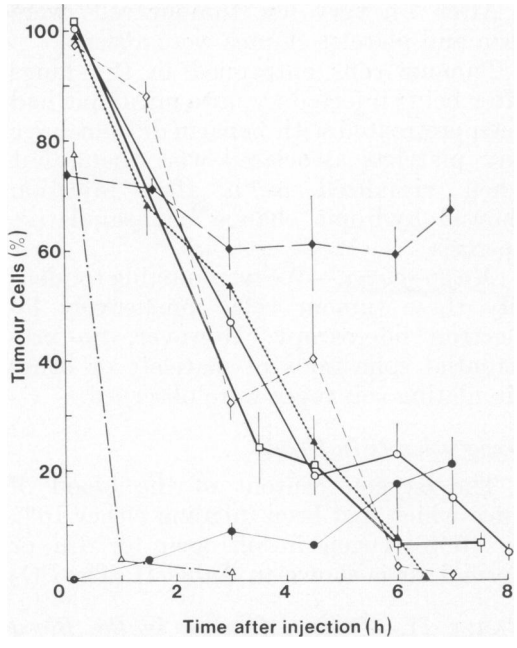


FIG. 6.—The percentage of i.v. or i.p. injected ^{125}I-labelled mastocytoma cells remaining at various times: in the peritoneal cavity of untreated mice after an i.p. injection of 10^6 cells (◆ – – – ◆); in the lungs of untreated mice after an i.v. injection of 10^6 cells (◇ – – – ◇), $2{\cdot}5\times10^6$ cells (○——○) or 10^6 "heat-killed" cells (△ – · – △); in the lung of heparin-treated mice after an i.v. injection of 10^6 cells (▲·····▲) or $2{\cdot}5\times10^6$ cells (□ == □); in the urine of untreated mice after an i.v. injection of 10^6 cells (●——●). Radioactivity in urine is expressed as if representing whole cells. Bar represents ±1 s.e. mean.

Fig. 5 shows that pretreatment of the mice with heparin before an i.v. injection of Sarcoma 180 tumour cells did not significantly alter the loss of tumour cells from the lungs. Similarly changes in the percentage of oxygen in the inhaled gas did not have a significant effect.

Fig. 6 shows the loss of mastocytoma tumour cells from the lungs after i.v. injection, with the subsequent increase in radioactivity in the urine. Initially the rate of the loss of tumour cells from the lungs of animals after an i.v. injection of 10^6 or $2{\cdot}5\times10^6$ cells was similar, but 7 h after the i.v. injection animals that had been injected with $2{\cdot}5\times10^6$ cells had a higher percentage of cells remaining in their lungs than mice injected with 10^6 cells ($P<0{\cdot}01$). The rate of the loss of tumour cells from the lungs was not significantly altered by pretreatment of the mice with heparin. The radioactivity in the urine is representative of all experiments involving the i.v. injection of radioactively labelled mastocytoma tumour cells.

The number of live mastocytoma cells recoverable after being injected i.p. did not decrease significantly after 7 h.

DISCUSSION

These results show that the bulk of the tumour cells entrapped in the lungs dis-

appeared within 7 h after i.v. injection. This is consistent with the observations of Glaves and Weiss (1976) and Fidler (1976). It appeared that the tumour cells in the lungs were dying. The disappearance of tumour cells from the lungs was associated with the appearance of radioactivity in the urine, although it was difficult to make quantitative comparisons. It is possible that some tumour cells were being redistributed from the lungs into other organs, as appears to be the case with i.v. injected syngeneic lymphocytes (Rannie and Donald, 1977). However, in the experiments described in the present paper, the radioactivity of other organs of the body peaked between 2 and 4 h. The radioactivity of each organ was $<1\%$ of the total radioactivity by 7 h and at background levels by 24 h.

The electron microscope studies showed that the majority of Sarcoma 180 tumour cells visualized between 5 and 7 h were undergoing coagulative necrosis. This was reflected in the development of pulmonary oedema in some of the animals at 6 h. This form of cell death is not associated with T-cell killing, which has the morphological features characteristic of apoptosis (Kerr *et al.*, 1972; Russell, Rosenau and Lee, 1972; Don *et al.*, 1977; Sanderson and Glauert, 1977). Neither lymphocytes nor macrophages were seen in close association with the tumour cells before their death. While naturally occurring "killer cells" do not appear to be T-cell in nature (Kiessling *et al.*, 1976), their action is cell-mediated and likely to induce apoptosis.

A naturally occurring circulating antibody (Martin and Martin, 1975, Ménard *et al.*, 1977) appears to be unlikely to be involved, as in the allogeneic system 100% of the mice injected i.p., and up to 53% of the mice injected i.v. died from the tumour, and in the syngeneic system 100% of the mice died whether injected i.p. or i.v. Antibodies are present in the peritoneal cavity (Ritzmann and Levin, 1969), yet the majority of S180 tumour cells injected i.p. did not die within 6 h.

Sarcoma 180, which is allogeneic in the Quackenbush mice, after i.v. injection gave rise to a single extrapulmonary tumour in 47% of the mice, but rarely to a pulmonary tumour. The mastocytoma, which is syngeneic in the DBA/2 mice, gave rise to multiple tumours in the lungs and liver in 100% of the mice injected. It might be expected that the different patterns of pulmonary tumour growth in these systems would be reflected in different rates of tumour-cell death. This was not the case. Mastocytoma cells died at the same rate in the lungs as the S180 cells, although obviously more mastocytoma cells survived to produce tumours.

The fact that an i.v. injection of Sarcoma 180 cells generally gave rise to a single tumour, irrespective of the number of cells injected, requires some interpretation and may be due to an immunological process (such as "concomitant immunity"; Gershon, Carter and Kondo, 1967) occurring much later than the process being discussed here.

Morphologically the factor most conspicuous before tumour cell death in the Sarcoma 180 system was the massive platelet aggregation which appeared to enclose the tumour cells. It seems possible that these enclosing platelet aggregates could be lethal for these tumour cells. This aggregation of platelets and associated fibrin appears to be a common feature in the entrapment of circulating tumour cells of many types (Wood, 1971; Jones *et al.*, 1971; Chew and Wallace, 1976; Chew *et al.*, 1976; Warren, 1976). No preferential collections of other circulating cell types were found by us. Platelets making up the aggregates degranulated and disappeared when the tumour cells died. Chew and his co-workers have made a similar observation. They noticed that platelet aggregates which initially enclosed the injected tumour cells disappeared at 6 h. The tumour cells remaining showed signs of disintegration, but these workers did not comment further on the mechanism of cell death.

However, it appears that platelet aggregation is not essential for the death of the majority of tumour cells entrapped in the

lung capillaries. The mastocytoma tumour cells do not appear to induce platelet aggregation after i.v. injection. This lack of platelet aggregation has been observed in other tumour systems (Vlaeminck *et al.*, 1972; Locker *et al.*, 1970; Dingmans, 1973). The loss of mastocytoma tumour cells from the lungs was similar to that of S180 tumour cells despite the lack of platelet aggregation around the tumour cells. Further, as shown by other workers (Fisher and Fisher, 1967*a*; Hilgard *et al.*, 1972; Brown, 1973), the pretreatment of the mice with an anticoagulant does not significantly influence the subsequent disappearance of the majority of the tumour cells initially entrapped in the lungs, although it prevents platelet aggregation. It has been suggested that the aggregation of platelets around a tumour cell could protect the tumour cell from cell-mediated defence mechanisms in the animal (Brown, 1973). While anticoagulants appear to reduce metastasis formation (Brown, 1973), the possible role of platelets in metastasis formation is likely to be influenced by a number of other factors including the nature of the tumour cell and the integrity of the endothelial cells in vessel walls (Warren, 1976).

The above arguments suggest that a non-immunological, nonspecific mechanism is involved in the death of tumour cells in the lung. This has been proposed for some time by a number of authors and together with them we are attempting to elucidate this mechanism, both *in vivo* and *in vitro*. Our *in vivo* experiments have begun with a study of the effect of varying atmospheric oxygen levels on the pattern of tumour-cell death. It seemed possible that the oxygen levels present in the lungs might not have been conducive to tumour-cell survival. This was suggested by *in vitro* studies in which it was shown that Sarcoma 180 tumour cells had a higher colony-forming efficiency in low oxygen tensions (Ablett *et al.*, 1978). This finding was not reflected in any changes in the viability of the tumour cells in the lungs of mice breathing gas mixtures of differing oxygen content, that resulted in wide variations of the PO_2 of their blood.

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