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COMPARISON OF *IN SITU* AND PERIPHERAL HOST IMMUNITY TO SYNGENEIC TUMOURS EMPLOYING THE MULTICELLULAR SPHEROID MODEL

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Summary.—The multicellular tumour spheroid was used as a model system to assess the *in situ* host immune response to the EMT6/Ro mammary tumour in syngeneic BALB/cKa mice. In sensitized mice the spheroids were rapidly infiltrated by host cells including macrophages, lymphocytes and granulocytes. Tumour cell killing was evident within 1 day and resulted in the eventual complete destruction of the spheroids. Host cells within the spheroids had a greater cytolytic capacity than the surrounding peritoneal cells and virtually no cytolytic activity was detectable in cells from the spleen. A similar discrepancy between *in situ* and peripheral immunity was found in mice bearing solid EMT6/Ro tumours.

THE INTERACTION of host immune cells and neoplastic cells *in vivo* is still poorly understood due to the complexities of both the immune system and the tumours themselves. Studies to assess the immunological response to tumours have mainly concentrated on the anti-tumour immunity of cells from peripheral lymphoid organs such as spleen or regional lymph nodes. Much less work has been done on *in situ* host immunity within the tumour, due largely to the difficulty in dissociating solid tumours and recovering high yields of both functionally viable neoplastic and host cells. Furthermore, the large number of neoplastic cells present in the dissociated cell suspensions may interfere with most *in vitro* assays of host cell immune responsiveness. Methods of separating host cells from tumour cells are thus essential for quantitative assessment of host cell immune reactivity. Alternatively, suitable model systems which accurately reflect the *in situ* environment have not been available. The multicellular tumour spheroid (MTS) (Sutherland *et al.*, 1971) which has many features found in solid tumours (reviewed in Sutherland & Durand, 1976) but is easily dissociated, appears to be a useful model system to study immunological responses within a tumour-like microenvironment. Using the MTS model system we have studied the kinetics of the *in situ* host immune responses to spheroids of EMT6 mammary tumour cells implanted into the peritoneal cavity of syngeneic BALB/cKa mice, and compared these responses to those of cells from the spleen, a peripheral lymphoid organ. In addition, we have recovered host cells from *in vivo* solid EMT6 tumours, separated them from the neoplastic cells by a centrifugal elutriation technique and compared the anti-tumour reactivity of these *in situ* cells to those of spleen cells from the tumour-bearing mice.

MATERIALS AND METHODS

The experimental techniques have previously been described in detail (Lord *et al.*, 1979). Briefly multicellular spheroids of EMT6/Ro (Rochester) tumour cells, a subline derived from the original tumour characterized by Rockwell *et al.* (1972) were grown *in vitro* and implanted into the peritoneal cavity of syngeneic BALB/cKa mice using an 18 gauge needle. These mice were either unsensitized or sensitized 15 days earlier with a single intraperitoneal injection of 1×10^7 lethally irradiated (5000 rad) EMT6/Ro cells. The spheroids as well as the peritoneal cells (PC) were recovered at various times later by

killing the mice and repeatedly flushing the peritoneal cavity with balanced salt solution (BSS). After washing, the spheroids were dissociated by trypsinization with 0·05% trypsin for 12 min at 37°C. Spleen cell suspensions were prepared by gentle teasing in BSS.

Killing of tumour cells in the spheroids was assessed with a colony-forming efficiency (CFE) assay of the spheroid associated cells (SAC) from the dissociated spheroids recovered from the peritoneal cavity. Host cell infiltration was determined morphologically from different counts on Wright's–Giemsa stained cytocentrifuge preparations of the SAC and PC. The cytolytic capacity of SAC, PC, and spleen cells was measured by using these cells as effector cells at several dilutions against ^{51}Cr labelled EMT6/Ro target cells (from *in vitro* monolayer cultures) in a 20 h ^{51}Cr release assay.

For the studies involving solid tumours, 2×10^5 EMT6/Ro cells were injected intramuscularly into the flanks of unsensitized BALB/cKa mice, and the resulting tumours used at 0·4–0·6 g. The tumours were dissociated using a modification (Siemann, personal communication) of the pronase, collagenase, DNAse cocktail described by Brown *et al.* (1979) and the host cells separated from the tumour cells by centrifugal elutriation. These *in situ* host cells as well as spleen cells from the same animals were then used as effector cells in the ^{51}Cr release assay.

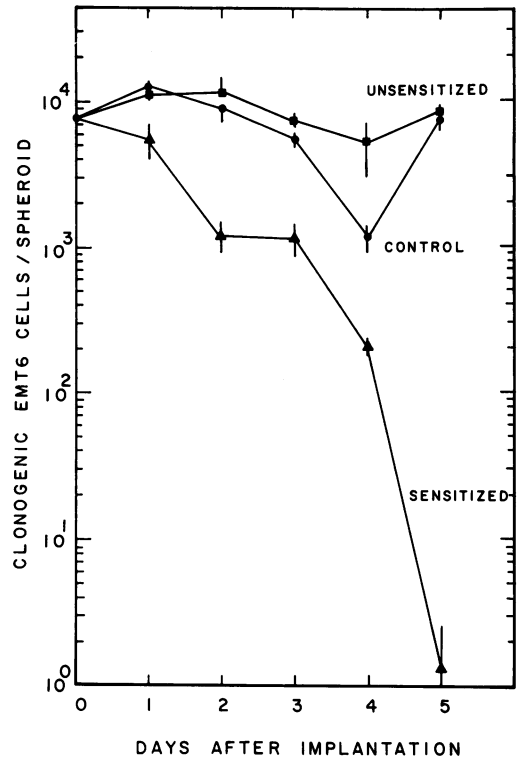

FIG. 1.—The kinetics of spheroid tumour cell kill following implantation of 60 spheroids/mouse (732 $\mu m \pm 60$ diameter) into sensitized or unsensitized syngeneic mice. Control spheroids were maintained *in vitro*.

RESULTS

Tumour cell killing

A distinct advantage of the EMT6 MTS model is that the damage mediated by the immune system can be quantitatively determined by a CFE assay on the dissociated spheroid cells. In these experiments the absolute number of clonogenic cells/recovered spheroid was calculated by multiplying the CFE by the number of cells/spheroid to eliminate error in the determination of CFE due to the presence of large numbers of host cells. Killing of tumour cells is quickly evident when spheroids are implanted into mice previously sensitized to EMT6 cells (Fig. 1). A decrease in clonogenic cells/spheroid is seen within one day and by 5 days after implantation virtually no clonogenic cells are detectable. In contrast, the clonogenicity of tumour cells recovered from spheroids implanted into unsensitized mice does not differ from that of control spheroids maintained *in vitro*.

Immunological specificity

The specificity of the immunological response to the EMT6 spheroids was determined by implanting them into groups of BALB/cKa ($H\text{-}2^d$) mice which were either unsensitized (control), sensitized specifically with EMT6/Ro cells or sensitized with an allogeneic cell line, the EL4 leukaemia (C57BL/6 origin, $H\text{-}2^b$), or another cell of the same H-2 type, the P815 mastocytoma (DBA/2 origin, $H\text{-}2^d$). The number of clonogenic cells recovered

TABLE.—*Immunological specificity of EMT6 tumour cell destruction*

Mice	Cells used for sensitizing	Percent of control* clonogenic cells/spheroids
BALB/c (H-2^d)	None	100·0
	EL4 (H-2^b)	90·8
	P815 (H-2^d)	89·5
	EMT6 (H-2^d)	0·15

* EMT6 spheroids were recovered 5 days after mplantation.

from the EMT6/Ro spheroids implanted into either of the groups of nonspecifically (EL4 or P815) sensitized mice was only slightly less than that in unsensitized control mice (the Table). In marked contrast, the spheroids recovered from mice specifically sensitized to the EMT6/Ro cells had only 0·15% of the clonogenic cells of controls. The killing of EMT6/Ro cells in spheroids is thus due to a specific immunological response to EMT6/Ro tumour antigens.

Host cell infiltration

Spheroids implanted into either unsensitized or sensitized mice were rapidly infiltrated by host lymphoid cells. In sensitized mice, granulocytes (primarily neutrophils) were initially present in large numbers but then declined, while macrophages which comprised only 12% of the cells at Day 1, steadily increased, becoming the predominant host cell type (Fig. 2, upper panel). The percentage of lymphocytes present was much lower, but like the macrophages, they increased as tumour cell killing progressed.

In unsensitized mice, a different pattern of host cell infiltration occurred (Fig. 2, lower panel). At Day 1, the percentages of each of the host cell types were similar to those in spheroids in sensitized mice. Granulocytes declined slightly during the first few days but were always the predominant host cell present. From Day 2 onward, macrophages comprised 20–30% of the SAC. In marked contrast to the sensitized situation, lymphocytes never

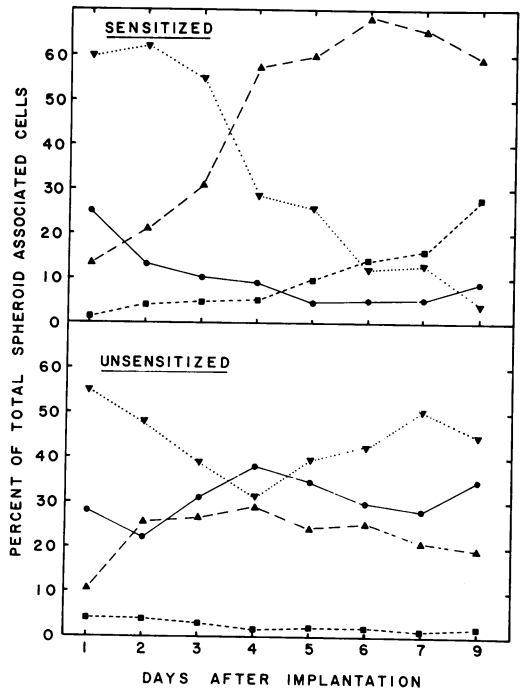

FIG. 2.—The kinetics of host cell infiltration into spheroids (848 μm $\pm$ 45 diameter) implanted into sensitized (upper panel) or unsensitized (lower panel) syngeneic mice. Macrophages, ▲; lymphocytes, ■; granulocytes, ▼; tumour cells, ●.

increased, remaining at less than 5% of the total SAC.

Host cell cytotoxic capacity

To confirm the functional capability of the host lymphoid cells the SAC (infiltrating cells), the PC (non-infiltrating cells) or cells from the spleen were used as effector cells in a cytolytic *in vitro* assay against ^{51}Cr labelled EMT6/Ro target cells. Cytolytic activity was low in both the SAC and PC during the first 3 days following implantation but increased to substantial levels at later times (Fig. 3). The activity of the SAC continued to increase until Day 6, while that of the non-infiltrating PC peaked at Day 5 and then decreased to low levels. In marked contrast to the high levels of cytolytic activity of *in situ* cells, virtually no activity was detectable in the spleen cells at the times

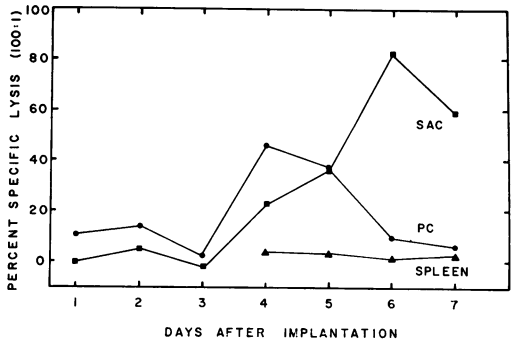

FIG. 3.—The kinetics of host cell cytolytic activity following implantation of spheroids into sensitized syngeneic mice. Specific ^{51}Cr release was measured at an effector: target cell ratio of 100:1.

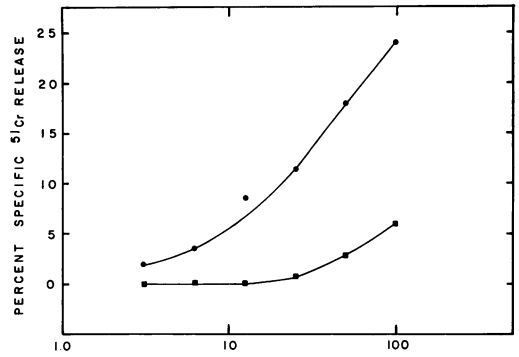

FIG. 4.—Comparison of the cytolytic activity of host cells derived from 0·5 g *in vivo* solid EMT6/Ro tumours and spleen cells from the same tumor-bearing mice. Tumour derived host cells, ●; Spleen cells, ■.

tested. No cytolytic activity was detectable in SAC, PC, or spleen cells following implantation of spheroids into unsensitized BALB/cKa mice.

In addition to the studies with the MTS system, *in vivo* solid EMT6/Ro tumours were also examined for host cell infiltration and immune reactivity. Based on 10 separate determinations, dissociated EMT6/Ro tumours (0·4–0·6 g) were found to contain 60–70% (63·3 ± 4·8) host cells including macrophages (38·8% ± 10·6), granulocytes (14·0% ± 5·3) and lymphocytes (10·9% ± 5·7). Thus, the ratio of total host cells to tumour cells was fairly constant for tumours in this size range but the cell types comprising the host cell population were more variable. These percentages were similar to those from spheroids recovered 4–5 days after implantation in sensitized mice (Fig. 2). In order to assess the immune reactivity of these *in situ* host cells it was essential to remove the tumour cells, which may act as unlabelled inhibitors in the ^{51}Cr release assay. The large size difference between the tumour cells and the majority of the host cells allowed us to use the centrifugal elutriation technique to accomplish this separation. Populations containing greater than 95% host cells were then used as effector cells in the ^{51}Cr release assay. As with the MTS system, the tumour-infiltrating host cells had significant cytolytic activity, while only minimal activity was detectable in the spleen cells from the same tumour-bearing animals (Fig. 4).

DISCUSSION

These experiments demonstrate the usefulness of the multicellular tumour spheroid model for the study of *in situ* tumour immunity and the importance of examining *in situ* rather than just peripheral immune responses in determining the animal's state of anti-tumour immune reactivity. The spheroid which has many of the features of *in vivo* solid tumours (Sutherland & Durand, 1976) and has previously been used to study alloimmune reactions (MacDonald & Howell, 1978; Lord *et al.*, 1979) has many favourable attributes for the study of immune mechanisms in solid tumours. Most notably, it is easily dissociated into single cells without apparent loss of cells so that the possibility of selective recovery of cell populations is avoided. The use of clonogenic cell lines such as the EMT6 grown either as spheroids or solid tumours allows the quantitative assessment of immunological effects of the tumour. This model system thus makes the study of *in situ* immunity technically feasible and, with the use of cell lines such as the EMT6, allows the direct comparison between a model and the *in vivo* situation.

The implantation of multicellular spheroids of EMT6/Ro tumour cells into the peritoneal cavities of syngeneic BALB/cKa mice resulted in a rapid and marked infiltration by host lymphoid cells. In unsensitized mice, this host cell infiltrate, which was composed mainly of granulocytes (with fewer macrophages and only a minimal number of lymphocytes) did not result in detectable tumour cell killing as measured by the CFE of cells from the recovered spheroids.

An entirely different state of immune reactivity was observed when spheroids were implanted into previously sensitized mice. Granulocytes, although present initially, were replaced by macrophages and a significant number of lymphocytes. The marked killing of tumour cells, which was evident within a few days, was immunologically specific and eventually resulted in the complete destruction of the spheroids. The host cells recovered from the spheroids had marked cytolytic activity toward EMT6/Ro cells while the surrounding peritoneal cells had lower levels of cytotoxicity. However, spleen cells from these same animals had no detectable activity. The cells comprising *in vivo* EMT6/Ro intramuscular tumours were also studied and thus the results with the MT model system could be directly compared to the *in vivo* situation. As with implanted spheroids, intramuscular EMT6/Ro tumours were infiltrated with large numbers of host macrophages, granulocytes and lymphocytes. When these *in situ* host cells were separated from the neoplastic cells, they were shown to have cytolytic activity toward EMT6/Ro cells. Although the tumours were implanted in the leg while the spheroids were placed in the peritoneal cavity, spleens from both the tumour-bearing and the spheroid implanted animals had little or no detectable cytolytic activity. These similarities between MTS and solid tumours indicate that the MTS is a model system which is both practical for the study of *in situ* anti-tumour immune mechanisms, and for the EMT6/Ro tumour studied, it was predictive of *in vivo* solid tumour responses. The disparity found in this study between *in situ* and peripheral immune reactivity provides some indication of both the complexity of the host–tumour interaction and the necessity of examining several parameters before statements can be made regarding the immunogenicity of a tumour cell line or the state of immune reactivity to a given tumour.

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REFERENCES

BROWN, J. M., YU, N. Y. & WORKMAN, P. (1979) Pharmacokinetic considerations in testing hypoxic cell radiosensitizers in mouse tumours. *Br. J. Cancer*, **39**, 310.

LORD, E. M., NARDELLA, G. B. & SUTHERLAND, R. M. (1979) The multicellular tumor spheroid model. I. Characterization of the allograft response in sensitized allogeneic mice. *Cell. Immunol.*, in press.

MACDONALD, H. R. & HOWELL, R. L. (1978) The multicellular spheroid as a model tumor allograft. I. Quantitative assessment of spheroid destruction in alloimmune mice. *Transplant.*, **25**, 136.

ROCKWELL, S. C., KALLMAN, R. F. & FAJARDO, L. F. (1972) Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture adopted derivative. *J. Natl Cancer Inst.*, **40**, 735.

SUTHERLAND, R. M. & DURAND, R. E. (1976) Radiation response of multicell spheroids—an *in vitro* tumor model. *Curr. Top. Rad. Res. Quart.*, **11**, 87.

SUTHERLAND, R. M., MCCREDIE, J. A. & INCH, W. R. (1971) Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J. Natl Cancer Inst.*, **46**, 113.