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Induction of tolerance to a murine fibrosarcoma in two zones of dosage – the involvement of suppressor cells

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Summary Small size inocula (10^1 – 10^3 cells) of cells from a syngeneic methylcholanthrene-induced fibrosarcoma (FSA) induced tolerance when injected s.c. into C3Hf mice. Mice were unable to respond to subsequent challenge with moderate, immunogenic doses of FSA. Tolerance was demonstrated in an *in vivo* transfer (Winn) assay and an *in vitro* tumour-specific T_H cell assay. Low zone tolerance was associated with the presence of tumour-specific T_S cells in the spleen.

Moderate size inocula (10^4 – 10^6 FSA cells) were immunogenic but larger cell doses ($>10^6$) were again tolerogenic. In the high zone, tolerance was associated with both tumour-specific T_S cells and non T suppressor cells that were not tumour-specific.

These results support the view that immunogenic tumours, as they grow from small cell numbers, might be able to escape host surveillance by specifically tolerizing the immune system. They also suggest that large tumour burdens can interfere with the host's immune response by inducing suppressor cells.

An important concept in tumour immunology is that tumours grow only if they can avoid host immune responses. An extension of this concept is that immune responses are selective forces during tumour progression. Numerous mechanisms have been envisaged and investigated by which tumours could escape immune defences. Early research revolved around lack of immunogenicity of tumours, shedding and modulation of cell surface antigens and blocking of tumour reactive lymphocytes by antigen-antibody complexes (Hellstrom & Hellstrom, 1969). More recent studies take into account our knowledge that the immune system is composed of complex interacting and self-regulating networks of cells and soluble factors and have focused on whether antigens on progressor tumours have properties that allow them to avoid protective immunity, for example by preferentially stimulating suppressor cell circuits (Moser *et al.*, 1983; Greene, 1980; Fujimoto *et al.*, 1976; Reinisch *et al.*, 1977; Frost *et al.*, 1982; Kolsch *et al.*, 1973; Mengersen *et al.*, 1975; North, 1984; Haubeck & Kolsch, 1982).

The ability of many tumours to stimulate T_S has been established although the conditions under which they are generated and the extent to which they facilitate growth of primary tumours still requires clarification. In our previous studies with a transplantable murine fibrosarcoma (FSA) we found both tumour-specific T_S and non-tumour-specific non-T suppressor cells in the spleens of

tumour-bearing mice in the later stages of tumour growth (Howie & McBride, 1982; McBride & Howie, 1984). The tumour grew initially in the face of developing systemic responses that were demonstrably protective. Concomitant immunity (Milas *et al.*, 1982) and tumour-specific responses could be demonstrated by both *in vitro* (Howie & McBride, 1982; McBride & Howie, 1984) and *in vivo* (Peters *et al.*, 1978; McBride *et al.*, 1980) assays. Suppressor cells did, however, appear to act in time to prevent tumour regression and to facilitate late metastatic spread. The development of T_S cells under such conditions has been described by many and most extensively investigated by North and colleagues (North, 1984). These studies, however, shed little light upon mechanisms operating during the initial stages of primary tumour growth which is presumably the most important period in terms of tumour escape.

In previous experiments we assessed only the response to FSA tumours growing from moderate-size inocula (4×10^5 cells). We subsequently varied the initial tumour load so as to build up a more complete picture of the host-tumour relationship. In this paper we show that both small (10^1 – 10^3 cells) and large (10^7 cells) size tumour inocula induce T_S cells and tolerance and only moderate size inocula induce immunity. This is therefore analogous to the classic two-zone tolerance phenomenon seen with certain soluble antigens (Mitchison, 1964).

Induction of low zone tolerance by methylcholanthrene-induced cells confirms and extends the findings of Kolsch and coworkers (Kolsch *et al.*, 1973; Mengersen *et al.*, 1975; Haubeck & Kolsch, 1982) in other tumour systems. They have investigated in

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detail the Balb/c plasmacytoma ADJ-PC-5 and have shown that when irradiated plasmacytoma cells are injected in initially low but exponentially increasing doses, the first immunological reaction is T_S cell activation which can prevent *in vitro* T_C cell generation. This phenomenon could explain how immunogenic tumours grow in the first place. Tumours growing naturally from one or a few cells might, in the early stages of tumour growth, induce tumour-specific T_S which would facilitate escape from immune surveillance. This is a non-mutually exclusive alternative to the more common explanation that the agents used to induce such tumours are generally immunosuppressive (Stutman, 1975). As pointed out by Kolsch, it could also explain the propensity some tumours have for growing just as readily, if not more readily, from low as from moderate cell doses – the phenomenon of ‘sneaking through’ (Old *et al.*, 1962).

Materials and methods

Mice

$C_3Hf/Sed//Kam$ female mice were used. They were about 12 weeks of age at the start of the experiment.

Tumour

The methylcholanthrene-induced syngeneic fibrosarcoma (FSA) used in these experiments has been described in detail previously (Howie & McBride, 1982; McBride & Howie, 1984; Milas *et al.*, 1982; Peters *et al.*, 1978; McBride *et al.*, 1980). It had been transplanted 7–9 times when used. Tumour cell suspensions were made as described (Howie & McBride, 1982; McBride & Howie, 1984).

Experimental design

The aim of these experiments was to examine the effect of varying doses of FSA upon the immune response. Preliminary experiments established that 4×10^5 cells s.c. gave a strong response which peaked 7 days after challenge (Howie & McBride, 1982). Less than 10^4 cells gave no response while greater than 10^6 cells were also less effective. Varying doses of FSA were injected s.c. into the right flank followed 10 days later by 4×10^5 cells into the left flank. Spleens were removed one week later and their anti-tumour activity assayed.

Winn assay

T cells were enriched from spleen suspensions by passage over nylon wool columns (Howie & McBride, 1982). Cells were mixed with 2×10^4

viable FSA and injected s.c. into 40 sites in 20 recipient mice per treatment (Peters *et al.*, 1978; McBride, *et al.*, 1980). Sites were palpated for tumour growth. The day 21 results are reported which is the first day when all control sites were 100% positive. The results represent data from two separate experiments.

T_H and T_S cell assay

These assays have been published (Howie & McBride, 1982; McBride & Howie, 1984). In brief, $2.5 \times 10^6 \text{ ml}^{-1}$ spleen cells from mice primed with trinitrophenylated calf red cells were treated with anti-Thy 1.2 and complement and used as a B cell source in all cultures. Spleen (10^5 ml) cells from tumour-bearing mice were treated as described in the text and were the source of primed T cells. Lethally irradiated (50 Gy) TNP-FSA cells (10^4 ml^{-1}) were the source of antigen. Cultures of admixed T cells, B cells and antigen, with appropriate controls, were established in triplicate. The T_H cells are the limiting factor in these assays. Anti-TNP responses were measured on day 5 by indirect plaquing with TNP-SRBC as antigen.

Putative suppressor cells were added at 10^5 cells ml^{-1} to cell cultures known to be capable of responding i.e. containing splenic T cells from mice receiving 4×10^5 FSA s.c. 7 days previously. Specificity or non-specificity of suppression was assayed by examining the ability of the putative suppressors to inhibit responses of T cells taken from TNP-CRBC primed mice with TNP-CRBC as antigen (Howie & McBride, 1982; McBride & Howie, 1984).

The anti-Thy 1.2 used was monoclonal 30:H:12 which was a kind gift of Dr Micklem, Department of Zoology, Edinburgh University.

Results

We used two assays to measure tumour-specific responses. The first was a Winn assay in which nylon wool, non-adherent spleen cells from mice were mixed with viable tumour cells and injected s.c. into normal recipients (Peters *et al.*, 1978; McBride *et al.*, 1980). Immunity is dependent upon primed Lyl^+2^- cells and is immunologically specific (McBride & Howie, unpublished). The development of immunity in mice receiving standard inocula of tumour cells was prevented by prior inoculation of either low (10^1 – 10^3) or high (greater than 10^6) doses of the same tumour (Figure 1).

Because the Winn assay is not very well-suited to subpopulation analysis we turned to a sensitive *in vitro* assay for tumour specific T_H cell activity (Howie & McBride, 1982; McBride & Howie, 1984) to analyze this phenomenon further. The kinetics of

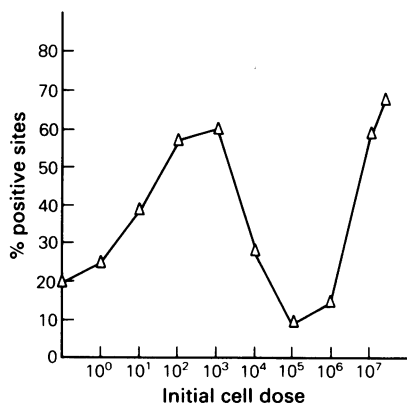


Figure 1 Two zone tolerance to FSA as measured by the Winn assay. Mice were pretreated with varying doses of FSA by injecting them into one flank. Ten days later 4×10^5 FSA cells were injected into the opposite flank. After a further 7 days, splenic T cells were isolated, admixed with viable FSA cells at a 33:1 ratio and recipient mice were injected with inocula containing 2×10^4 FSA per site. The percentage of positive sites on day 21 is shown, a time when all control sites receiving FSA alone or normal T cells plus FSA, were positive.

responses demonstrated using this assay have previously been shown to parallel closely those of the Winn assay (Howie & McBride, 1982; McBride & Howie, 1984). The T_H assay relies on the recognition *in vitro* of tumour-specific determinants on irradiated trinitrophenylated tumour cells by T_H cells and presentation of TNP to B cells to generate an anti-TNP response. As can be seen in Figure 2 as few as 10^1 – 10^3 viable FSA cells prevented the development of tumour-specific T_H cell activity in the spleens of mice subsequently challenged with 4×10^5 cells. Greater than 10^6 cells had a similar effect. Between these two zones immunity developed.

In both low and high dosage zones, suppressor cells developed (Figure 2). Spleen cells from these mice could inhibit anti-tumour responses of spleen cells from mice receiving only tumour challenge. Low zone suppressor cells were tumour-specific T_S cells in that they were Thy 1.2 positive (Figure 2) and did not suppress anti-CRBC T_H cell responses (Figure 3). High zone suppressor cells contained tumour-specific and non-tumour-specific cells in that whole spleen cells suppressed anti-CRBC and anti-FSA T_H cell responses (Figure 3) whereas nylon wool-passed cells only suppressed anti-tumour responses (Table I). This is a similar result to that already found in mice bearing large tumour burdens (Howie & McBride, 1982; McBride & Howie, 1984).

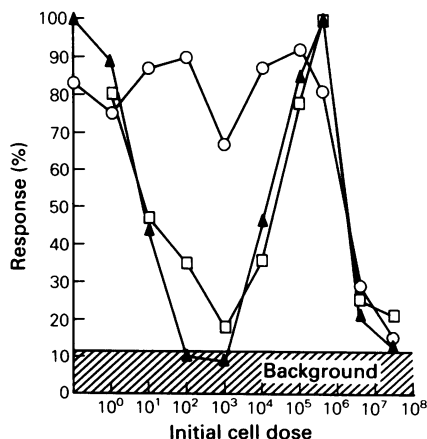


Figure 2 Two zone tolerance to FSA as measured by the T_H cell assay. Mice were treated in an identical fashion as for Figure 1 except the level of tumour-specific T cell help within the spleen cell populations was measured as described in Materials and methods. The control 100% value represents the PFC response generated by spleen cells from mice receiving 4×10^5 FSA cells only (no pretreatment). The effect of pretreatment with various doses of FSA is shown (▲—▲). The possibility that suppressor cells were responsible for the decreased responses by some of the pretreated groups was tested for by mixing spleen cells from these groups with spleen cells from control mice receiving only challenge with FSA (100% group). Suppressor cells were present where there was decreased responsiveness (□—□). Treatment with anti-Thy 1.2 plus complement abolished suppressor cells only in the low zone groups (○—○).

Table I Suppression of anti-FSA and anti-CRBC responses by T cells from high zone tolerant mice.

	Percent suppression	
	FSA response	CRBC response
Whole spleen	98	95
Nylon wool nonadherent cells	96	25

Nylon wool nonadherent spleen cells and non-separated cells from mice receiving 10^7 FSA s.c. followed by challenge with 4×10^5 FSA as in Figure 1 were tested for their ability to suppress anti-FSA and anti-CRBC responses as in Figures 2 and 3.

Discussion

We have shown that this immunogenic fibrosarcoma can induce two zones of immunological tolerance in normal mice. As few as 10^1 – 10^3 viable cells can induce low zone tolerance while 10^5 – 10^6

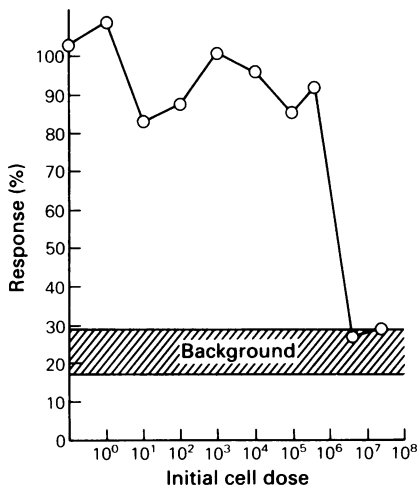


Figure 3 The specificity of suppressor cells generated by low and high doses of FSA. The ability of spleen cells from mice pretreated with varying doses of FSA and challenged with 4×10^5 FSA cells as in Figure 1 to suppress the generation of TNP-CRBC responses was tested. The 100% value represents responses of separated T and B cell populations from TNP-CRBC primed mice with TNP-CRBC as antigen. Non-specific suppressor cells were present only in the high dose groups.

cells stimulate powerful responses. Larger cell doses induce high zone tolerance which is associated with a more complex and more generalized state of immunosuppression. This last state is probably responsible for the marked loss in immunity when this tumour grows large and may allow metastases to develop (Milas *et al.*, 1974).

Low zone tolerance may account for several aspects of tumour behaviour. One of these is 'sneaking through' (Kolsch *et al.*, 1973; Mengersen *et al.*, 1975; Haubeck & Kolsch, 1982). One would predict that for 'sneaking through' to be explained on this basis there would have to be a dose window where tumour take is inhibited by the development of immunity but this manifestation of immunity can be masked by larger tumour cell numbers. Below this window tolerance would be induced. Further-

more to see 'sneaking through', the transplanted tumour must be sufficiently resistant to natural immune mechanisms and sufficiently clonogenic to grow from cell doses that induce tolerance. These requirements would explain why 'sneaking through' is not seen with all tumours and opens up the possibility that low zone T_S cell induction may be a more general phenomenon.

It is possible that tumours, even ones capable of inducing immunity, might have initially escaped the attentions of the host immune system by inducing tolerance. It would be interesting to study the highly immunogenic UV-induced tumours in this regard. It should be noted that, with the possible exception of certain virus-coded products, there is no compelling reason to consider tumour antigens as being anything other than self or minimally altered self components, perhaps exceptional only in the amount and timing of their expression. One might expect responses to such antigens to be under close suppressor cell control. Under natural conditions anti-tumour responses might therefore require breakage of a tolerant state and could be considered as largely autoimmune in nature.

Finally, we previously noted that immunotherapy of this tumour with *C. parvum* was only effective when inocula of moderate size were used (Peters *et al.*, 1978). Not only were small-size inocula not rejected but tumour take was actually enhanced. We can now explain the lack of effect of *C. parvum* on small size inocula as being due to the presence of a tolerant state.

These studies reemphasize the need for extreme care when drawing conclusions from experiments where single doses of transplanted tumours are used and suggest that tolerant mice might be a useful tool for studying the effects of the immune system and immuno-therapy on tumour behaviour.

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