In vivo detection and partial characterization of effector and suppressor cell populations in spleens of mice with large metastatic fibrosarcomas

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Summary The MC-2 fibrosarcoma, which is a transplantable tumour syngeneic for BALB/c mice, metastasizes to lymph nodes draining subcutaneous inoculation sites, and also to the lungs. T cell-mediated immunity was detected in Winn assays using spleens from excision immunized mice. T cell-mediated antitumour immunity was also detected in spleens from mice with small tumours but disappeared as the tumour burden increased. Protective immune spleen cell activity in the Winn assay was inhibited by prior addition of spleen cells from mice with large tumours, causing increased tumour incidence. Splenic metastases occasionally occurred in the MC-2 model, but were not demonstrable by bioassay in any of the experiments detecting splenic suppressor cell activity. In vivo protective activity was restored to advanced-stage tumour-bearer spleens by whole-body ionizing irradiation (0.5 and 2.5 Gy) of donor mice 15h before sampling. Spleen cells from mice with small tumours remained protective after 1.5, 2.5 and 4.0 Gy of irradiation in vivo. These results are consistent with the properties of radiosensitive suppressor T cells. In contrast to reports in other tumour models, suppressor cells were not detected until late in the course of MC-2 development. This is surprising in view of the aggressively metastatic nature of MC-2. It is postulated that modulation of the antitumour immune response by the suppressor cells is associated with metastasis in this tumour model. The late appearance of both suppressor cells and metastatic cells in the spleen may reflect similar processes occurring earlier in regional lymph nodes.

The metastasis of malignant neoplasms is the major clinical problem in the treatment of cancer. More than 50% of cancer patients have metastases by the time their disease is detected (DeVita et al., 1975). Of deaths directly attributable to the tumour burden in cancer victims, the majority are caused by metastases rather than the primary tumour (Roos & Dingemans, 1979).

Metastasis is a multifactorial process in which both intrinsic (tumour) and extrinsic (host) factors contribute (Roos & Dingemans, 1979). Survival of tumour cells after invasion of tissue is at least partially under immunological control. The level of lymphoreticular infiltration into primary tumours has been inversely correlated with metastasis in some animal models (Eccles & Alexander, 1974) and human disease (Hamlin, 1968). This relationship is, however, not universally detected (Talmadge et al., 1981). Antitumour activity detected in lymph nodes draining primary tumours is often lost as tumour size increases (Flannery et al., 1973).

There would appear to be a link between systemic immunity manifested as concomitant tumour immunity (CTI) and metastasis (Gershon, 1974). CTI cannot be induced by all tumours, disappears with increasing tumour size (Sugarbaker et al., 1971) and, in contrast to non-metastasizing tumours (Kearney & Nelson, 1973), may be only transient in metastasizing tumour models (Finlay-Jones et al., 1980).

Antitumour immune responses may be suppressed by a variety of tumour-associated factors including tumour-bearer serum (Bartholomaeus et al., 1974), solubilized tumour cell components (Minami et al., 1979) and tumour cell culture supernatants (Hellström & Hellström, 1979). Extensive antigen shedding in vitro has been correlated with metastatic propensity in vivo (Currie & Alexander, 1974) and depression of macrophage activity in vivo, the latter resulting in enhanced tumour growth (Pike & Snyderman, 1976). Intravenous transfer of lymphoreticular cells from tumour-bearing animals can enhance tumour growth in immune animals simultaneously receiving tumour cells (Fujimoto et al., 1975) and can suppress the antitumour activity of transferred immune cells (Mills & North, 1983). Splenic suppressor cell activity appeared early in these tumour-bearing mice: Day 7 (Fujimoto et al., 1975) and Day 9 (North & Bursuker, 1984) after tumour inoculation. Similar results using Winn assays were obtained in another tumour system (Carter et al., 1984).
1983). Spleen cells from mice bearing the metastasizing Lewis lung carcinoma enhanced tumour growth in recipient mice but in contrast to the results above, this activity did not occur until late in tumour growth, after Day 18 (Treves et al., 1976).

We are investigating anti-tumour immune responses and their association with metastasis, using a methylcholanthrene-induced mouse fibrosarcoma designated MC-2. This tumour induces transient CTI in the syngeneic host, with CTI disappearing prior to the appearance of metastases in lymph nodes regional to s.c. inoculation sites (Finlay-Jones et al., 1980). The MC-2 tumour produces significantly larger metastases in immunosuppressed hosts (Finlay-Jones et al., 1980).

In this paper we describe the use of the Winn assay (Winn, 1961) to study the development and later suppression of splenic T cell-mediated immunity in animals inoculated with the MC-2 tumour. Anti-tumour activity was restored to the spleens of late-stage tumour-bearers by sublethal whole body irradiation of the donor animals.

Materials and methods

Animals

Inbred, age-matched, female BALB/c mice were used in all experiments. Host animals used in the Winn assays were 2–12 months old. Blood for serum complement was obtained by cardiac puncture from outbred male guinea pigs of 300–600 g body weight. All animals were supplied by the Department of Agriculture, South Australia.

Tumour

The metastasizing MC-2 fibrosarcoma which is syngeneic for BALB/c mice has been described previously (Finlay-Jones et al., 1980). The tumour was maintained in vitro by serial passage every 3 weeks. Single cell suspensions were prepared from tumour tissue by a combination of mechanical and enzymatic disaggregation (Sheridan & Finlay-Jones, 1977). Tumour cells used in Winn assays were grown in vitro in RPMI 1640 supplemented with 10% foetal calf serum and harvested with a 0.25% solution of hog pancreatic (Grade VI, Sigma, Mo., USA). New in vitro cultures were established from in vivo tumours at each passage. In the experiments described the tumour had been passaged 20–40 times in vivo. A dose of 10⁴ MC-2 cells was lethally tumorigenic in 100% of mice.

Immunization

Mice were inoculated with 10⁵ MC-2 cells s.c. on the ventral surface. Five to eight days later tumours of 30–90 mg weight were excised under pentobarbitone sodium (Nembutal, Abbott Labs., Sydney, Australia) anaesthesia. These mice were rechallenged s.c. with 10⁵ MC-2 cells 2–4 weeks later. In some experiments immunized mice were given a further challenge dose of 10⁴ MC-2 cells several months later. Mice rejecting challenge tumours were used as donors of immune spleen cells.

Winn assay (Winn, 1961)

Spleen cells from excision-immunized or tumour-bearing mice were mixed with MC-2 cells and inoculated s.c. into sublethally irradiated recipients at a final dose of 10⁵ spleen cells:10⁴ tumour cells/mouse. MC-2 cells were harvested from short-term tissue cultures. Spleen cells from age and sex-matched normal mice were assayed concurrently. As an additional control mice were also inoculated with 10⁴ MC-2 cells only. Each experimental group consisted of 7–15 mice. In the experiment reported in Table III spleen cells from immune, normal and advanced-stage tumour-bearing mouse were mixed together in various ratios immediately before addition to the tumour cell inoculum.

Assessment of treatment

Recipient mice were assessed for tumour incidence, primary tumour growth rate, primary site tumour and regional lymph node (axillary and inguinal) weight at autopsy. Recipients were autopsied when tumour growth was advanced, usually 20–28 days post-inoculation. Data indicating primary tumour incidence and size at autopsy have been presented. Primary tumour growth rates and the extent of lymph node metastases in Winn assay hosts provided no additional information and have been excluded for clarity.

Irradiation

Winn assay hosts were given 0.85 Gy min⁻¹ of whole-body ionizing radiation 6–48 h before inoculation. The total dose delivered was 4.0–4.5 Gy (Phillips, Holland, deep X-ray unit, 250 kV, 12 mA, nil added filter, half value layer 0.7 mm copper free control, source to box distance 68 cm). Early and advanced tumour-bearers used as spleen donors (Tables IV & V) were irradiated under the same conditions 10 and 24 days post-inoculation respectively. These mice were given 0.5, 2.5 or 4.0 Gy and spleens were sampled 15–23 h later. All mice were irradiated in compartmented perspex boxes.
T-cell depletion

Spleen cells were incubated for 70 min at 4°C with monoclonal anti-Thy 1.2 antibody (Ledbetter & Herzenberg, 1979; culture supernatant from cells obtained from the American Type Culture Collection, Rockville, Maryland, USA, ATCC No. TIB 107), or culture medium (RPMI 1640 + 10% foetal calf serum). Cells were washed once and incubated for 60 min at 37°C with either guinea pig serum diluted in RPMI 1640 or culture medium.

Statistics

Tumour incidences between groups were compared using Tocher’s modification of Fisher’s Exact Probability test (Siegel, 1956). Differences between groups in the weights of primary tumours at autopsy were compared using Student’s t-test (Armitage, 1971).

Results

Anti-MC-2 activity of spleen cells from excision-immunized and tumour-bearing mice in vivo is T cell-dependent

Anti-MC-2 activity in the Winn assay was detected in spleen cells of excision-immunized (Table 1A) and early-stage tumour-bearing mice (Table 1B). This activity was depleted by pretreatment with monoclonal anti-Thy 1.2 antibody and complement. Immune and early-stage tumour-bearer spleen cells treated with complement only or growth medium

| Treatment of donor spleen cells | Winn assay hosts |
|-------------------------------|-----------------|
|                               | % Tumour incidence | Mean primary tumour weight ± s.e. (mg) |
| (A) Immune spleen              |                 |                                         |
| Anti-Thy 1.2 + C               | 100b            | 2770 ± 210                              |
| C only                         | 10c             | 1330 ± —                                 |
| Growth medium only             | 10c             | 30 ± —                                   |
| Normal spleen                  |                 |                                         |
| Anti-Thy 1.2 + C               | 90              | 1780 ± 320                              |
| C only                         | 100             | 2240 ± 280                              |
| Growth medium only             | 100             | 2160 ± 210                              |
| No spleen cells                | 100             | 2280 ± 180                              |
| (B) Day 10 tumour-bearer spleen |                 |                                         |
| Anti-Thy 1.2 + C               | 100             | 1590 ± 110                              |
| Growth medium only             | 50c             | 480 ± 200                               |
| Normal spleen                  |                 |                                         |
| Anti-Thy 1.2 + C               | 100             | 1230 ± 210                              |
| Growth medium only             | 100             | 1210 ± 170                              |
| No spleen cells                | 100             | 1150 ± 70                               |

*10 mice/group.

aTumour incidence significantly greater than complement or growth medium only treatments (P < 0.001, Fisher’s Exact test), but not significantly different from similarly treated normal spleen cells.

bTumour incidence significantly less than similarly treated normal spleen cells (P < 0.001, Fisher’s Exact test).

cPrimary tumour weights of spleen donors were 309 and 367 mg.

dSignificantly lower tumour incidence than T cell-depleted tumour-bearer spleen group, normal spleen groups and tumour cells only group (P < 0.05, Fisher’s Exact test). Of those mice developing tumours, mean primary tumour weights significantly less than all control groups (P < 0.02, Student’s t-test).
Table II Development and subsequent loss of detectable anti-tumour immunity in the spleens of mice with increasing tumour burden

| Days post-inoculation of spleen donors | Exp. No. | Donor primary tumour weight (mg) | Tumour bearer spleen | Normal spleen | Statistical significance |
|----------------------------------------|---------|---------------------------------|---------------------|--------------|-------------------------|
| 3                                      | 1       | 5                               | 82                  | 73           |                         |
| 6                                      | 1       | 25                              | 10                  | 100          |                         |
|                                        | 2       | 21                              | 20                  | 80           |                         |
| 13                                     | 1       | 560                             | 40                  | 100          |                         |
|                                        | 2       | 610                             | 20                  | 100          |                         |
| 20                                     | 1       | 2680                            | 100                 | 100          |                         |
|                                        | 2       | 2830                            | 100                 | 100          |                         |
| 27                                     | 1       | 5860                            | 100                 | 40           |                         |
|                                        | 2       | 6560                            | 78                  | 100          |                         |

*9–16 mice/group.
*10⁴ MC-2 only inocula also tested for each day/experiment: each produced 100% incidence.
*Fisher's exact test of tumour-bearer vs normal spleen cell group incidences.

only, conferred statistically significant protection (reduced tumour incidence and tumour weight at autopsy), when compared to recipients of antibody plus complement-treated immune and tumour-bearer spleen cells, normal spleen cells regardless of treatment, and control mice receiving MC-2 cells only.

**Development and subsequent loss of detectable anti-tumour immunity in vivo in mice with increasing tumour burden**

Immunity detectable in the Winn assay first appeared in the spleens of host animals approximately 6 days after inoculation of MC-2, and remained statistically significant for another 7 days (Table II). In other experiments (data not shown) weak antitumour activity has been detected in spleens of animals up to 23 days post inoculation of MC-2.

In one of the two experiments reported in Table II a dose of 10⁷ spleen cells from Day 27 tumour-bearers produced tumours in 3/3 immunosuppressed (4.5 Gy whole-body X-irradiation) recipients.

Splenic metastases were not detected in bioassays of other late-stage tumour-bearer spleen cell preparations used in the experiments described below.

**Suppression of immune spleen cell activity by spleen cells from mice with large tumours**

Since significant anti-tumour activity was generally absent from the spleens of mice with large tumours, experiments were performed to establish whether the immune response was modulated by the development of a suppressor cell population. Spleen cells from mice with large tumours were added to spleen cells from excision-immunized mice immediately prior to combination with MC-2 cells. In some cases spleen cells from normal mice were also added to this mixture (as indicated) to ensure that the total number of spleen cells inoculated into hosts was constant.

It can be seen in Table III that a mixture of immune and normal spleen cells (Group A) produced total protection against an otherwise 100% tumorigenic dose of MC-2 cells (Group G). In contrast, a mixture of late-stage tumour-bearer and normal spleen cells (Group B) was not protective. When 2.5 × 10⁵, 5 × 10⁵ or 5 × 10⁶ tumour-bearer spleen cells (Groups C, D and E respectively) were added to an otherwise protective dose of 5 × 10⁵ immune spleen cells (Group A) the protective response was significantly diminished. Splenic metastases were not detected in the late-stage tumour-bearer spleen cells when they were bioassayed in immunosuppressed mice.

This experiment has been repeated a number of times with similar results. Two variables were deemed critical to the demonstration of suppression: (i) a large tumour burden in the donor animal and (ii) the ratio of tumour-bearer spleen cells to immune cells, with the lower ratios showing marginally more suppression.
Table III Suppression of immune spleen cell activity in the Winn assay by addition of spleen cells from mice with large tumours*

| Group | 10^5 x [Tumour-bearer spleen : Normal spleen : Immune spleen] | Mean primary tumour weight ± s.e. (mg) | % Tumour incidencec |
|-------|--------------------------------------------------|---------------------------------|-------------------|
| A     | 0 : 5 : 5                                         | 0d                             | 2380 ± 340       |
| B     | 5 : 5 : 0                                         | 90                             | 2510 ± 340a      |
| C     | 2.5 : 2.5 : 5                                     | 70                             | 2220 ± 300       |
| D     | 5 : 0 : 5                                        | 56                             | 1460 ± 190a      |
| E     | 50 : 0 : 5                                       | 40                             | 1740 ± 300a      |
| F     | 50 : 5 : 0                                       | 100                            | 2380 ± 160       |
| G     | 0 : 0 : 0                                        | 100                            | —                |

*Advanced tumour-bearer spleens sampled 22 days after MC-2 tumour inoculation. Primary tumour weights of spleen donors 3293, 3924 mg.

Spleen cells from late-stage tumour-bearer, normal and excision immunized mice mixed in various ratios to final concentrations of 10^6 spleens cells/mouse. Spleen cells were then mixed with a 100% tumourigenic dose of 10^4 MC-2 cells and inoculated into Winn assay host animals.

9–10 mice/group.

Significantly lower tumour incidence than all other groups (0.0001 < P < 0.05, Fisher's Exact test) i.e. significant suppression of protective response when tumour-bearer spleen cells added.

Group E vs Group G, P < 0.001; Group F vs Group G, P < 0.01; Group C vs Group E, P < 0.05; Student's t-test.

Protective activity restored to spleens of mice with large tumours following whole-body irradiation

Animals with large primary tumours were given doses of 0.5, 2.5 or 4.0 Gy whole-body X-irradiation 15h prior to spleen sampling. Spleen cells from unirradiated mice with tumours of similar size were not protective in the Winn assay (Table IV).

Tumour incidence was significantly less in the 0.5 Gy- and 2.5 Gy-treated tumour-bearer spleen donor groups than that seen in groups receiving tumour cells only, or tumour cells mixed with spleen cells from normal or irradiated naive mice, or unirradiated tumour-bearers. Primary tumours which developed in Winn assay host mice receiving spleen cells from 2.5 Gy treated tumour-bearing animals were also significantly smaller at autopsy than those in the various control treatment groups. Protective activity was reduced to statistically insignificant levels in spleen cells from late-stage tumour-bearers pretreated with the highest dose of radiation (4.0 Gy).

Radiosensitivity of protective splenic effector cells from early-stage tumour-bearing donor mice

Doses of 1.5 and 2.5 Gy of whole-body X-irradiation 23h prior to spleen sampling did not reduce protective activity conferred by spleen cells from mice with small tumours (Table V). Anti-tumour effector cells were partially susceptible to 4.0 Gy of whole-body irradiation. Tumour incidence in mice receiving spleen cells from 4.0 Gy-pretreated early-stage tumour-bearing donors was not significantly different from the control group receiving MC-2 cells only. However, the autopsy weights of primary-site tumours of the former group were significantly less than those in the control group, indicating some residual protective activity.

Discussion

In investigating factors influencing the metastasis of a murine fibrosarcoma, we (Finlay-Jones et al.,
1980) have found several associations between antitumour immunity and metastasis. Firstly, the MC-2 fibrosarcoma induces transient CTI in the syngeneic host, with CTI developing then disappearing prior to the growth of metastases. Secondly, metastasis of MC-2 is enhanced in immunosuppressed hosts. We have also found that tissue-culture-derived sublines with reduced metastatic propensity induce a prolonged CTI and show increased metastasis in immunosuppressed hosts (Finlay-Jones & Dent, in preparation). With the use of the Winn assay, we have documented in this paper the acquisition then loss of T-lymphocyte dependent immunity in spleens of tumour-bearing hosts, and present evidence which suggests that this modulation was due to splenic suppressor cells.

*In vivo* immunity to the metastasizing murine fibrosarcoma MC-2 was dependent on T cells (Table I). Spleen cells from normal animals usually showed little protective activity. Similar findings using Winn assays have been reported for other chemically-induced, but non-metastasizing sarcomas (Shimizu & Shen, 1979; Carter *et al.*, 1983).

Anti-tumour immunity as detected in the Winn assay emerged in, and was then lost from spleens of tumour-bearing animals (Table II). As with CTI (Finlay-Jones *et al.*, 1980), immunity detectable in the Winn assay first appeared in the spleens of host animals approximately 6 days after inoculation of MC-2. However, in contrast to CTI, statistically significant protection was detectable in the Winn assay for at least another 7 days (Table II). Significant protection was detectable in the spleens of animals with primary tumour burdens of >500 mg, whereas CTI in this model could only be demonstrated over an approximate tumour weight range of 5–80 mg (Finlay-Jones *et al.*, 1980). In separate experiments (not shown) partial protection was present up to 23 days after inoculation of MC-2 into spleen donors. However, the Winn assay has been used in this model to test systemic (splenic) immunity, whereas CTI may reflect local immunity (i.e. regional lymph node immunity). It should be noted that in both cases anti-tumour activity disappeared from animals as their tumour burden increased.

Artificial increases in tumour load produced by injection of soluble tumour antigens (Gatenby *et al.*, 1981) or irradiated tumour cells (Hellström & Hellström, 1978; North, 1982) induce cell populations capable of suppressing anti-tumour immune responses. In the MC-2 model, loss of protective activity from spleens of mice with large primary tumours and metastases, was coincident with the development *in vivo* of suppressive activity against otherwise protective immune spleen cells (Table III). Others have reported the development of a suppressor cell population in the spleens of tumour-bearing mice but, in contrast to our findings, this appeared early (Days 7–9) in tumour growth (Fujimoto *et al.*, 1975; Carter *et al.*, 1983; North & Bursuker, 1984). In contrast, Treves *et al.*
(1976) described tumour-enhancing cells developing in spleens of mice late (Day 19+) in the growth of the metastasizing Lewis lung carcinoma. It may be that differences in assays used could account for the differences in kinetics. However, the ratios of tumour cells to spleen cells (immune and suppressor) used in our experiments were similar to those of Carter et al. (1983). An alternative possibility is that the differences in kinetics may relate to biological differences in the tumour systems used. The metastatic propensities of the tumour models may influence the type of suppressor cells arising and the timing of their expression.

The eventual loss of immunity and development of suppressor cell activity in the spleens of late-stage MC-2 bearing mice may be associated with onset of metastases in the spleens of some mice, and may reflect a similar process occurring much earlier in lymph nodes draining the primary tumour inoculation site. Such a process may then facilitate the development of lymph node metastases which are histologically observable only 8 days after s.c. inoculation of MC-2 (Finlay-Jones et al., 1980). The immune responsiveness of lymph node cells from mice with MC-2 tumours, and its relationship to the appearance of lymph node metastases, is currently under investigation.

Information concerning the nature of the suppressor cells was obtained in experiments in which sublethal whole-body irradiation of mice with large tumours restored anti-tumour protective activity to their spleens (Table IV). In contrast, anti-tumour effector cells present in the spleens of mice with small tumours were resistant to 2.5 Gy or less of whole-body irradiation (Table V). Residual protective activity was also detected in the spleens of early-stage tumour-bearing mice given 4.0 Gy of whole-body irradiation. It would seem that the radiation-induced restoration of protection to the spleens of mice with large tumours (Table IV), was due either to a direct stimulatory influence on inactive effector cells, or elimination of a cell subpopulation modulating effector cell activity. However, the former is unlikely in that the activity of effector cells was not directly enhanced by low dose irradiation (Table V).

The activity of suppressor cells can be ablated experimentally using immunosuppressive treatments which appear to leave cytotoxic T cell activity, mitogen responsiveness and delayed-type hypersensitivity intact (Röllinghoff et al., 1977; Glaser, 1979; Minami et al., 1979; North, 1982). Suppressor T cells are known to be more radiosensitive than other T cell subpopulations (Basten et al., 1975; Dutton, 1975) and this is thought to explain observed augmentation of immune responses following low-dose ionizing radiation (Anderson et al., 1981; Doria et al., 1982). Radiation may also enhance antigen processing by macrophages, leaving their phagocytic and migratory behaviour intact (Anderson & Warner, 1976).

Sublethal whole-body irradiation of mice with small tumours can reduce tumour incidence or size (Hellström et al., 1978). This effect can be duplicated by radiation restricted to the spleen region (Enker & Jacobitz, 1980) and is probably due to elimination of suppressor T cells, since reconstitution with T cell-enriched normal spleen cells soon after irradiation can result in tumour growth comparable with that in unirradiated controls (Hellström et al., 1978). It is possible that irradiation of tumour-bearers early in tumour development has direct cytocidal activity on the tumour. Radiation-induced gut damage may reduce the nutritional status of the host and therefore indirectly inhibit tumour growth. Leakage of gut contents may also serve to non-specifically stimulate the host's immune system. Artificial lung metastases may be reduced by high dose (12.0 Gy) irradiation of recipient gut tissue a week prior to i.v. tumour challenge (Ando et al., 1980). Some of these technical drawbacks concerning the use of irradiation in directly affecting cells of the immune system have been overcome in the experiments reported in Tables IV and V.

Splenic metastases are occasionally found in mice bearing late-stage MC-2 tumours. However, suppression detected by mixing tumour-bearer and immune spleen cells in the Winn assay was probably not due to simple addition of putative splenic tumour metastases to the tumour load. Tumours did not arise in bioassays of the tumour-bearer spleen cells which showed suppression. Further, the radiosensitivity of the suppressor cells is at variance with the relative radioresistance of MC-2 tumour cells (Dent & Finlay-Jones, unpublished observations).

Development of suppressor cell activity and loss of protection may be reversed by manipulation of the cellular immune response. An understanding of the changes in cellular immune responses, and the potential for manipulating them, for example with low doses of radiation, may lead to more effective cancer therapy.

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