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An analysis of T lymphocyte subsets in tumour-transplanted mice on the basis of Lyt antigenic markers and functions

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Summary. Small lymphocyte subsets were characterized radioautographically on the basis of several surface markers, *viz.* surface Ig (S-Ig), Thy-1 and Lyt (Ly-1, Ly-2 and 3) antigens in host lymphoid organs (thymus, spleen and blood) as well as at the tumour site at various stages of subcutaneous growth of two different syngeneic tumours—MPC-11 plasmacytoma and WEHI-164 fibrosarcoma in BALB/c mice. In both tumour-host combinations there was a rise in the levels of null (S-Ig$^-$, Thy-1$^-$) small lymphocytes as well as the Ly-23$^+$ subset of T small lymphocytes at all the sites examined. The absolute number of these two subsets also increased excepting the case of null cell rise in the thymus which was relative. The functional potentials of Lyt subsets were explored by employing *in vitro* and *in vivo* assays. While no appreciable levels of anti-tumour cytotoxic T cells (T_c) were detectable by a ^{51}Cr release assay in the host spleen or the tumour-draining lymph nodes at any stage of growth of MPC-11 tumour, such T_c was generated *in vitro* by a co-cultivation of unprimed spleen cells with irradiated MPC-11 cells. These T_c were Thy-1$^+$ and Ly-12$^+$, as noted from antibody+C′ mediated abrogation of cytotoxicity. These results suggested that the generation of anti-tumour T_c *in vivo* was suppressed in tumour-bearing hosts. The possibility of a cell-mediated suppression was tested by an adoptive transfer of thymocytes or splenocytes from tumour-bearing mice into naive or pre-immunized recipients which then received fresh tumour transplants. This procedure caused a *specific* enhancement of tumour growth in three tumour-host combinations: MPC-11 or WEHI-164 tumour in BALB/c mice and W-1 fibrosarcoma in CBA mice. The suppressor lineage lymphocytes appearing *in vivo* were found to be Thy-1$^+$ and Ly-1$^-$, 2$^+$, as noted from antibody +C′ mediated abrogation of their tumour-growth promoting ability. They appeared earlier (7 days) in the thymus and later (>2 weeks) in the spleen and then persisted during the tumour lifetime. The parallel kinetics of the increase in the overall level of Ly-23$^+$ cells and the appearance of Ly-2(3)$^+$ suppressor lineage T cells in tumour-bearing hosts may indicate that studies of T-cell surface markers may be useful in predicting changes in the functional lymphocyte subsets.

Abbreviations: DMEF, Dulbecco's modified Eagle's medium containing 10% FCS; FCS, foetal calf serum; HEM, HEPES-buffered minimal essential medium; HEPES, N-2-hydroxyethyl piperazine-N′-2-ethane sulphonic acid; MEM, minimal essential medium; RAMG, rabbit anti-mouse IgG; T_c, cytotoxic T cells; T_s, suppressor T cells; WEHI, Walter and Eliza Hall Institute of Medical Research.

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INTRODUCTION

Many tumours exhibit antigenic properties, and yet they continue to grow *in vivo* and kill the host. To understand the reasons for a failure on the part of the host to contain the tumour, one needs to have a clearer

knowledge of the immunobiology of the host-tumour relationship. With this objective, a number of studies were undertaken in our laboratory to characterize host cellular responses to tumour development in mice. We examined changes in the host lymphomyeloid organs during tumour development (Lala, 1976; Lala, Terrin, Lind & Kaizer, 1978), and characterized host lymphocyte subsets invading the tumour site as well as appearing in various lymphoid organs on the basis of their life history (Lala, 1974; Kaizer & Lala, 1977) and well recognized surface markers: IgM on B cells, Thy 1 antigen on T cells, and absence of either marker on 'null' cells (Lala & Kaizer, 1977; Garnis & Lala, 1978; Lala, Garnis, Kaizer, Jacobs & Santer, 1979a; Santer, Mastromarino & Lala, 1980). The latter studies revealed an increase in the null lymphocyte level at all sites during the earlier stages of development of transplanted tumours in a number of tumour-host combinations and also shortly before the clinical appearance of spontaneous mammary tumours in C3H/HeJ mice. While changes in the B-cell incidence at various sites were minimal and least consistent, T-cell incidence showed some minor decline in the peripheral lymphoid organs of tumour-transplanted mice, but an increase in hosts bearing spontaneous tumours. The absolute number of T cells infiltrating these tumours increased with increasing tumour mass in all host-tumour combinations. Since T cells represent a heterogeneous population, it was deemed necessary to characterize the T-cell subsets in tumour-bearing hosts further on the basis of additional surface markers and function. Lyt antigens (Ly-1, Ly-2 and 3) have now been recognized as important differentiation markers on T cells, also appearing on specific functional subsets (Cantor & Boyse, 1977). In the first part of this paper, we report our studies of changes in the host T, B and null lymphocyte subsets, as well as T-cell subsets expressing different Lyt antigens, identified radioautographically during the subcutaneous growth of two different syngeneic tumours—MPC-11 plasmacytoma and WEHI-164 fibrosarcoma in BALB/c mice. In the second part of this paper, we report the functional significance of these findings in the above host-tumour combinations as well as in CBA mice transplanted with syngeneic W-1 fibrosarcoma, by relating surface Lyt phenotypes on host T cells to two possible functional subsets: anti-tumour cytotoxic (T_c) cells and a suppressor T (T_s) cell lineage which may suppress the generation of T_c *in vivo*. We investigated (i) whether sufficient levels of T_c are generated *in vivo* within the host during the development of transplanted tumours; (b) whether significant levels of T_c can be generated *in vitro* from unprimed spleen cells co-cultivated with tumour cells; (c) the Lyt phenotype of the putative T_c; (d) whether a suppressor mechanism develops in the tumour-bearing hosts *in vivo* because of the generation of T_s, or their precursors, or their inducers and, if so, (e) what is the Lyt phenotype of such cell lineage.

These studies revealed an increase in the level of null lymphocytes as well as Ly-23$^+$ subset of T lymphocytes at the tumour site and host lymphoid organs. The Ly-23$^+$ subset appearing in the thymus and the spleen was capable of *specific* enhancement of tumour growth when transferred into fresh recipients at the time of tumour transplantation, indicating its ability to suppress the generation of T_c *in vivo*. Such T_c, although not detectable *in vivo* could be generated *in vitro* and expressed Ly-12$^+$ phenotype for the MPC-11 tumour.

MATERIALS AND METHODS

Animals

BALB/c (WEHI Stock, H-2^d; Lyt phenotype: Ly-1.2, 2.2 and 3.2) and CBA (WEHI Stock, H-2^k; Lyt phenotype Ly-1.1, 2.1 and 3.2) raised under specific pathogen-free conditions for 8 weeks and then conventionalized were used at 10–12 weeks for tumour transplantation or lymphocyte collection.

Tumours

MPC-11 is a well characterized plasmacytoma (Burton & Warner, 1977) induced by mineral oil and WEHI-164 (henceforth called W-164) is a well defined fibrosarcoma (Rollinghoff & Warner, 1973) induced by methylcolanthrene, both tumours induced in BALB/c mice. W-1 is a methylcholanthrene-induced fibrosarcoma in CBA/H strain mouse originally produced by Sir Michael Woodruff in Edinburgh. W-54 is a mammary adenocarcinoma which arose spontaneously in a CBA/H mouse in the same laboratory. Both these lines underwent serial passages in CBA mice and were brought to the Walter and Eliza Hall Institute by Dr Woodruff and since then have been maintained in CBA (WEHI Stock) mice. All tumour lines were maintained in tissue culture, as reported by Horibata & Harris (1970) and also by serial subcutaneous (s.c.) passage of 10^6 viable cells initially taken from exponentially growing tissue culture into syngeneic mice on the anteromedial aspect of the left

thigh. In the present experiments tumours in their third generation of s.c. passage were used. The resultant MPC-11 tumours showed visible necrotic foci at the centre at the end of 2 weeks, the necrotic centre further increasing in size with increasing tumour age. W-164 and W-1 tumours remained fleshy and devoid of any visible necrosis even at the end of 4 weeks, but haemorrhagic foci were commonly observed in all old tumours. W-54 tumours often showed surface ulceration after 2 weeks. The median animal survival time was 29 days for MPC-11, 40 days for W-164, 33 days for W-1 and 28 days for W-54 tumour bearing mice following s.c. transplantation of 10^6 tumour cells in the thigh of syngeneic hosts. Tumour growth curves were plotted by recording the average of the maximum and the minimum external tumour diameters measured with a pair of calipers usually at 2 day intervals.

Preparation of single cell suspensions for surface marker analysis of lymphocytes

Spleen, thymus and tumours (free from necrotic tissue) were minced with fine scissors, and single cell suspensions freed from clumps and debris were prepared as reported previously (Lala & Kaizer, 1977). Heparinized cardiac blood was subjected to red cell lysis by treatment for 15 min with buffered 0·168 M NH_4Cl at 4°. For surface labelling, all cells were finally suspended at a concentration of 10×10^6/ml of ice cold HEPES (Calbiochem, San Diego, Calif.) buffered minimal essential medium (HEM) supplemented with 10% foetal calf serum (FCS), both materials purchased from Grand Island Biological Co., New York. Individual tissues pooled from three animals per interval were subjected to surface marker studies.

Antisera

Details of the source, mode of production and cytotoxic titre of antisera used are provided in Table 1.

Surface immunolabelling

Protein A binds with the Fc portion of several IgG classes from a number of mammalian species (Goding, 1978). This fact has been exploited in our laboratories for an examination of a number of surface markers on mouse lymphocytes by sandwich labelling with Protein A, either using radioautographic (Lala, Johnson, Battye & Nossal, 1979b) or rosetting (Sandrin, Potter, Morgan & McKenzie, 1978) techniques. The former technique was employed here. In brief, 0·1 ml aliquots of single cell suspensions (10^7 cells/ml) were incubated at 4° with appropriate antisera (Table 1) or normal mouse serum (controls) at 1/20 final dilutions for 30 min followed by two washes through a discontinuous gradient of FCS (50, 75, 100%) in the HEM medium, and then incubated again with ^{125}I-labelled Protein A (sp. act. 40 Ci/g) at a final concentration of 1 μg/ml for 30 min at 4°. Staphylococcal Protein A (Pharmacia Fine Chemicals, Sweden) was radioiodinated with the chloramine T oxidation technique (Greenwood, Hunter & Glover, 1963) and used within 3 days of radioiodination. Following two further washes through FCS gradients after exposure to radiolabelled Protein A, cell pellets were resuspended in minute amounts of FCS, smeared on gelatin-coated microscope slides, and fixed in methanol for 40 min before processing for radioautography.

All antisera listed in Table 1 were spun in an air-driven ultracentrifuge at approximately 160,000 *g* for 30 min to remove any protein aggregate before they were employed for surface labelling. Their dilution levels (1/20) were based on pilot studies and were considerably lower than the dilutions needed for a plateau labelling incidence in the normal thymus and spleen, except in the case of anti-Ly-2.2 and 3.2 where any further dilution led to a small drop in the labelling incidence. The Thy-1.2 antiserum employed in this study contained adequate Protein A-binding IgG class antibody molecules in contrast to our experience with most other anti-Thy 1.2 sera in which the IgM class predominates.

Radioautography

This was performed according to the method developed by Kopriwa & Leblond (1962). The slides were dipped in Kodak NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, New York), diluted 1:1 with distilled water, exposed for 4 days, developed and stained with Giemsa. For each tissue at any time point a minimum of 500 small lymphocytes (nuclear diameter $\leqslant 8$ μm in smears) were scored except in the case of blood and tumour where the minimum number was 300. The number of silver grains were scored on each cell and a grain count distribution plotted. The threshold grain count for positive labelling, determined on the basis of background grain count distributions on cell-free areas (Lala & Patt, 1966), was taken as six grains in most cases and ten grains on a few occasions. Criteria used for identification of cells in smears have already been reported by us (Kaizer & Lala, 1977; Garnis & Lala, 1978; Santer *et al.*, 1980).

Table 1. Antisera used for lymphocyte labelling or C′ mediated cytotoxicity

Antiserum	Method of immunization	Source	Cytotoxic titre
RAMG	Rabbit anti-mouse IgG, having substantial anti-κ chain activity	WEHI*	1/200
Anti-Thy 1.2	AKR anti-C3H, nude spleen absorbed	WEHI†	1/200
Anti-Ia^k	ATH anti-ATL	WEHI*	1/200
Anti-Ly 1.2	C3H anti-CE	Austin Hosp.‡	1/128
Anti-Ly 2.2§	(C3H × BDP)F_1 anti-B10.BR	Austin Hosp.	1/256
Anti-Ly 3.2	[BL.PL(75NS) × C58]F_1 anti-CE	Austin Hosp.	1/128

* Courtesy of Dr J. Goding.
† Courtesy of J. Gamble.
‡ Raised by I. F. C. McKenzie.
§ Contains also anti-Ly 4.2 not recognized by BALB/c cells which carry Ly 4.1.

Computation of surface marker bearing cells
The incidences of surface Ig$^+$ (s-Ig$^+$) and thy-1$^+$ small lymphocytes were determined directly, and the incidence of null (S-Ig$^-$, Thy-1$^-$) small lymphocytes was estimated both directly and indirectly. In the former case, cells went through two successive binding steps, first with anti-Thy-1 serum and then with rabbit anti-mouse globulin (RAMG, 2 mg/ml) with intervening washes, followed by exposure to radiolabelled Protein A. This labelled all B and T cells, and the small lymphocytes remaining unlabelled with this protocol provided a direct measure of null cells. In the second case, null cell percentage was computed as 100 − %SIg$^+$ − % Thy-1$^+$ cells. Since there was excellent agreement between the two estimates (within 1%–2%), direct estimation was abandoned in later experiments. For an identification of Lyt surface markers, cell aliquots were exposed to (i) anti-Ly-1.2, (ii) anti-Ly-2.2 plus anti-Ly-3.2 and (iii) anti-Ly-1.2 plus anti-Ly-2.2 plus anti-Ly-3.2. Small lymphocyte labelling indices in sample (i) provided the incidence of Ly-1$^+$ + Ly-123$^+$ cells, (ii) Ly-23$^+$ + Ly-123$^+$ cells and (iii) Ly-1$^+$ + Ly-23$^+$ + Ly-123$^+$ cells. Thus, the incidence of Ly-1$^+$ cells was given by (iii) − (ii), Ly-23$^+$ cells by (iii) − (i), Ly-123$^+$ cells by (i) + (ii) − (iii) and all Lyt$^+$ cells by (iii).

In vitro *generation of T_c against syngeneic tumour cells*
This was done according to the method described by Burton, Thompson & Warner (1975). In brief, normal BALB/c spleens were minced and teased through a sieve (80 mesh/inch^2) in Eagle's minimal essential medium (MEM) with non-essential amino acids (Grand Island Biological Co., New York) buffered with sodium bicarbonate and supplemented with 10% FCS, Penicillin (100 u./ml), streptomycin (100 μg/ml) and 10^{-4} M 2-mercaptoethanol. This medium will be referred to as MEMF. Cell clumps were removed by layering on FCS (Lala & Kaizer, 1977) and a viable cell count performed with eosin exclusion method. To generate anti-tumour cytotoxic cells *in vitro*, viable spleen cells pooled from five mice were co-cultured with irradiated (5000 rad X-ray) MPC-11 tumour cells (derived from tissue culture) using 100:1 responder: stimulator cell ratio in compartmentalized tissue culture trays (25 wells, Sterilin Ltd, Richmond, Surrey), each well containing 15×10^6 viable spleen cells in 4 ml MEMF. Anti-tumour cytotoxic activity was assayed after 5 days of culture period.

Anti-tumour cytotoxicity assay by ^{51}Cr release
This was done as reported by Burton *et al.* (1975). MPC-11 target cells obtained from culture were labelled with ^{51}Cr (sodium chromate) by incubating 5×10^6 target cells with 100 μCi ^{51}Cr in 0·5 ml medium for 30 min at 37°, followed by washing. Labelled target cells were adjusted to 25×10^4 cells/ml of Dulbecco's modified Eagle's medium (Commonwealth Serum Laboratories, Melbourne), supplemented with 10% FCS (henceforth called DMEF). This medium was employed for all cytotoxicity assays performed in microtitre trays (Microtest II, Falcon Plastic, Oxnard, Calif.), using 25×10^3 labelled target cells per well (flat bottom) to provide attacker:target ratios of 25:1,

50:1 and 100:1. They were set up in 200 μl volumes in quadruplicates. These trays were incubated for 4 hr at 37° and then 1 hr at 45° to allow maximal release of ^{51}Cr from the dead cells. Radioactivity present in 100 μl volume of the supernatant was counted in a gamma counter. Background (BG) spontaneous release was provided by the supernatant in wells containing target cells alone, and the maximum release by the counts in 100 μl volume obtained after lysing the labelled targets with zaponin (Coulter Electronics, Florida) in a final volume of 200 μl. Percentage specific lysis was given by: (test counts − BG counts) × 100/(maximum release − BG counts). To identify the surface phenotypes of T_c, attacker cell populations were sometimes subjected to appropriate antibody and complement-mediated killing and then the residual T_c activity was measured. In such cases, the attacker:target ratio was always based on the viable attacker cell number immediately before the antibody treatment, so that a possible enrichment of T_c resistant to such treatment amongst the attacker cell population used in the ^{51}Cr release assay was avoided.

Identification of surface phenotype of functional lymphocyte subsets

To identify the surface phenotype of some lymphocyte subsets (killer cells generated *in vitro* or subset with tumour-enhancing ability generated *in vivo*), cells were subjected to an antibody plus complement-mediated killing before functional assays. Lymphoid cells (25×10^6 cells/ml of DMEF) were treated with appropriate antisera (final dilutions: 1/8 for Ly-1 and Ly-2, and 1/10 for Thy-1 and Ia^k) at 4° for 30 min, washed twice by spinning through excess of DME and then incubated in the presence of rabbit complement (agarose absorbed; pretested for negligible toxicity and high activity, final dilution 1/6 in DME) at 37° for 30 min. Following two washes, cells were used for functional assays. To avoid cell loss by transfer from one tube to another, cells were handled in the same tube during the whole procedure.

Adoptive transfer of lymphoid cells

The *in vivo* effects of adoptive intravenous transfer of $1–2 \times 10^7$ lymphoid cells taken from the thymus or the spleen of mice bearing 21 day old subcutaneous tumours were tested in fresh mice which received subcutaneous transplants shortly thereafter. Effects on the growth rate of subcutaneous tumour development were noted by recording the external tumour diameters in a minimum of six experimental animals per group, as compared with those in control mice. Controls were provided by three groups of animals: (i) those receiving no lymphoid cells; (ii) those receiving lymphoid cells from healthy normal mice; or (iii) those receiving lymphoid cells from mice bearing an unrelated syngenic tumour. In some cases where lymphoid cells were treated with antibody plus complement before an adoptive transfer, the inoculum dose was based on the viable cell number immediately before this treatment and was not readjusted following the treatment, so that a possible selective enrichment of a surviving cell type in the inoculum was avoided.

Sensitization of hosts to tumours

In the adoptive transfer assay, some recipient mice were presensitized to the tumour with the hope of inducing partial or complete tumour immunity. In the case of MPC-11 plasmacytoma, BALB/c mice were injected i.p. with 5×10^6 live mitomycin C treated MPC-11 tumour cells (taken from tissue culture). These animals were used for experiments 3 weeks later. As would be seen from the results, this protocol failed to result in an effective and lasting immunity against MPC-11 tumour growth by the subcutaneous route. In the case of W-1 fibrosarcoma in CBA mice, a surgical procedure was adopted in which 10^6 viable W-1 cells were injected subcutaneously in the right calf region. One week later the right leg was amputated by disarticulation at the knee. These animals were used 2 weeks later for experiments and proved to be immune to a subcutaneous challenge with 10^6 W-1 cells.

RESULTS

Surface marker analysis of host lymphocytes

The incidence of various small lymphocyte subsets in the lymphoid organs of normal young adult BALB/c mice, based on identification of surface markers by ^{125}I-Protein A radioautography, is presented in Table 2. The estimates of T, B and null small lymphocyte levels are in general agreement with our previously published data in other mouse strains (Garnis & Lala, 1978; Chatterjee-Hasrouni, Santer & Lala, 1980). The incidences of Thy-1^+ cells in the present study (Table 2) are in excellent agreement with the incidences of Lyt^+ cells (inclusive of Ly-1^+, 23^+ and 123^+ subsets) confirming the notion that these antigens are exclusive T-cell markers. Incidences of different Lyt bearing subsets are in general accord with data reported on the basis of cytotoxicity tests (Cantor & Boyse, 1975a,

Table 2. Incidence (%) of small lymphocyte subsets in normal BALB/c mice*

Tissue	Thy-1$^+$	S-Ig$^+$	Null	Lyt$^+$	Ly-1$^+$	Ly 23$^+$	Ly 123$^+$
Thymus	97	1·5	1·5	98	18	6	74
Spleen	42	48	10	41	17	4	20
Blood	51	40	9	49	21	4	24

* Pooled from three animals.

1975b) with one exception, *viz.* a somewhat higher value for Ly-1$^+$ cells in the thymus. However, the incidence of a significant level of Ly-1$^+$ thymocytes capable of emigrating from the thymus has been reported recently by others (Scolly, Kochen, Burcher & Weissman, 1978; Mathieson, Sharrow, Campbell & Asofsky, 1979; Betel, Mathieson, Sharrow & Asofsky, 1980) using fluoresceinated antibodies. Finally, the frequency of Ly-23$^+$ cells was low (4%–6%) in all lymphoid organs (Table 2) of normal mice. These results are in good agreement with our data (Chatterjee-Hasrouni, Hogarth and Lala, unpublished) in CBA mice obtained with the use of monoclonal anti-Ly antibodies raised in one of our laboratories (Hogarth, Potter, Cornell, McLachlan & McKenzie, 1980).

Temporal changes in the incidence of splenic small lymphocyte subsets during subcutaneous tumour development is shown in Fig. 1 for BALB/c mice transplanted with 10^6 MPC-11 cells, or W-164 cells. The levels of Thy-1$^+$ cells again matched closely with those of total Lyt$^+$ cells in all tumour-bearing animals. While splenic T or B small lymphocyte levels showed only minor changes, null small lymphocyte levels showed some increase in both host-tumour combinations (from 10% at day 0 to 16% at 24–28 days). Of the T small lymphocytes, changes were noted in the levels of all Lyt subsets during tumour growth. Of these, a rise in the level of Ly-23 subset at some time point was the most noteworthy feature. For example, there was a four-fold increase at day 4 in MPC-11 bearing mice, and two-and-a-half-fold increase at day 24 in W-164 bearing mice. Tumour-transplanted mice showed splenomegaly which was in part due to an increment in the lymphoid elements. The rest of the increase in splenic cellularity was primarily due to erythroid

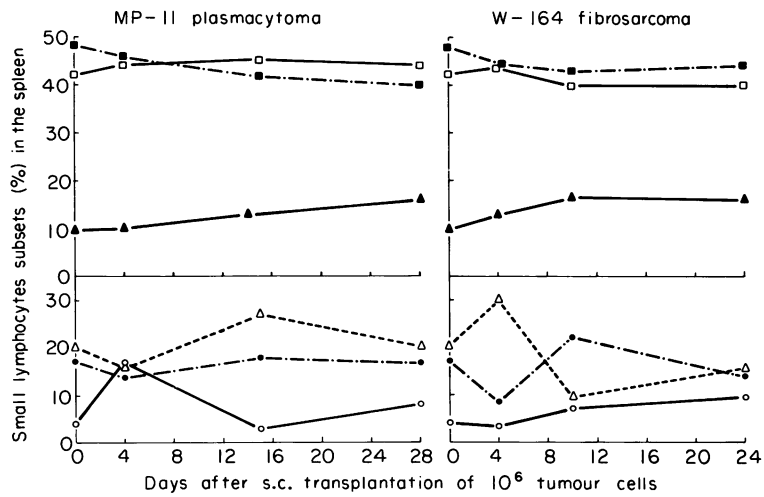

Figure 1. Temporal changes in the incidence of splenic small lymphocyte subsets in MPC-11 or W-164 transplanted BALB/c mice. Spleens are pooled from three mice per interval. (■) Surface Ig$^+$; (□) Thy-1$^+$; (▲) null; (△) Ly-123$^+$; (●) Ly-1$^+$; (○) Ly-23$^+$.

elements, and to a minor extent to other cells such as granulocytes, monocytes and macrophages. The absolute number of small lymphocytes in the spleen (data not shown) increased two–three-fold normal level at 3–4 weeks of tumour age in both tumour-host combinations. Thus, there was an increase in the absolute number of all small lymphocyte subsets in the spleen during tumour development.

Temporal changes in the incidence of thymic small lymphocyte subsets are shown in Fig. 2 for MPC-11 or W-164 transplanted mice. In both cases values for the incidence of Thy-1$^+$ small thymocytes matched closely with those for Lyt$^+$ cells. T-cell incidence measured in either way showed some decline with increasing tumour age in both cases, due primarily to a substantial increase in the null cell incidence. For example, null small lymphocytes increased from a control level of 1·5% to 19%–20% at 3–4 weeks of tumour growth. B small lymphocytes remained unchanged at a low level of 1%–2%. Of the T small lymphocytes, the incidence of Ly-123$^+$ cells showed a marked decline in both tumour-bearing hosts from 74% at day 0 to 28%–31% at day 4, followed by some further minor decline at later intervals. The incidence of Ly-1$^+$ small lymphocytes showed either a minor rise (from 18% at day 0 to 25% at day 24 in W-164 bearing mice) or some substantial increase (to 35% at day 28 in MPC-11 bearing mice).

Tumour-transplanted mice showed a rapid decline in thymic weight and cellularity possibly due to the disappearance of cortisone sensitive thymocytes because of stress. This may have been responsible for a rapid decrease in the incidence of Ly-123$^+$ cells. To test whether the rises in the levels of thymic Ly-1$^+$ or Ly-23$^+$ cells in tumour-transplanted mice were relative or absolute, the total numbers of thymic T small lymphocyte subsets were computed and the data are presented in Fig. 3. The total number of small lymphocytes dropped most rapidly between 0 and 4 days and then less rapidly at later intervals to about one-half the original value at day 24 in W-164 bearing mice, and to one-third of the original value at day 28 in MPC-11 bearing mice. The number of Ly-123$^+$ cells declined somewhat more rapidly at 0–4 days, and less rapidly at later intervals in both cases. The number of Ly-1$^+$ cells showed either a small decline (MPC-11 hosts) or no change (W-164 hosts). The total number of Ly-23$^+$ cells, in contrast, showed a four and a half to five-fold increase at 4 days followed by either no change (W-164 hosts) or some secondary decline (MPC-11 hosts still retaining two and a half times control value at 28 days).

The incidence of small lymphocyte subsets in the blood of tumour-bearing hosts as well as at the tumour site could be determined only at certain tumour ages, since materials from other intervals were either techni-

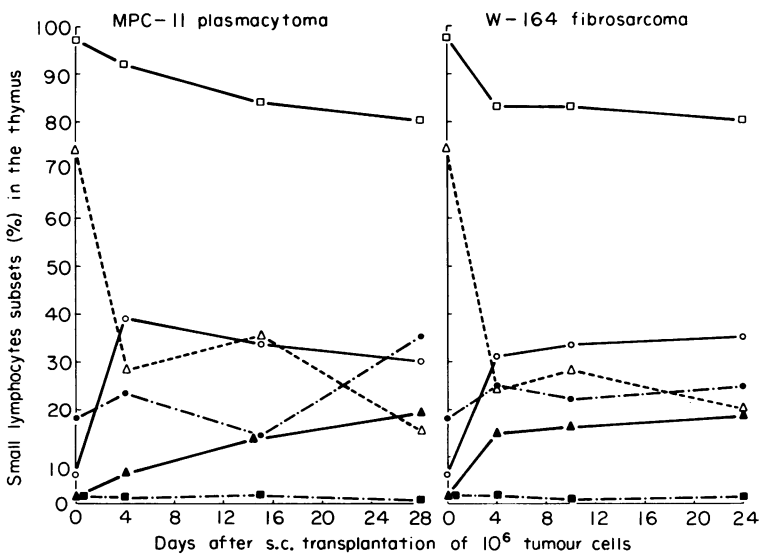

Figure 2. Temporal changes in the incidence of thymic small lymphocyte subsets in MPC-11 or W-164 transplanted BALB/c mice. Thymuses are pooled from three mice per interval. (■) Surface Ig$^+$; (□) Thy-1$^+$; (▲) null; (△) Ly-123$^+$; (●) Ly-1$^+$; (○) Ly-23$^+$.

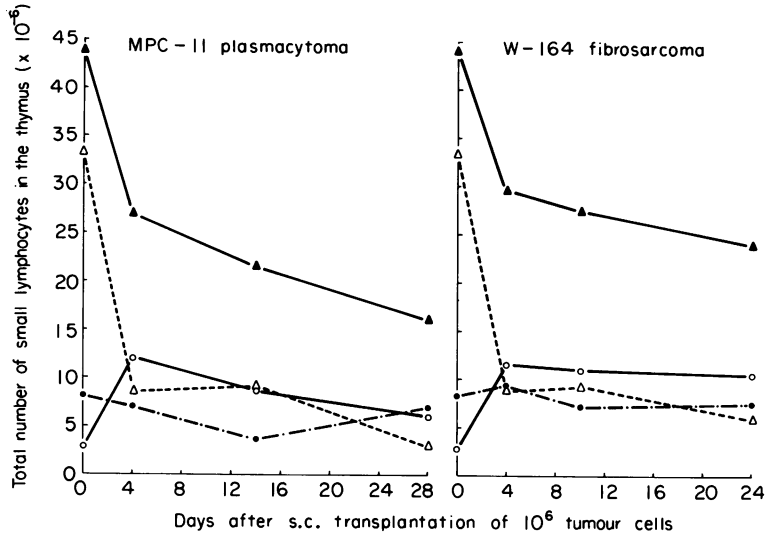

Figure 3. Temporal changes in the absolute number of thymic small lymphocyte subsets in MPC-11 or W-164 transplanted hosts. (▲) Lyt$^+$; (△) Ly-123$^+$; (●) Ly-1$^+$; (○) Ly-23$^+$.

cally unacceptable or yielded too few lymphocytes for a dependable analysis. The results are presented in Table 3. Most noteworthy findings are as follows: (i) there was a high frequency of null small lymphocytes both in the blood of the tumour-bearing hosts as well as within the tumour; (ii) the frequency of Ly-123$^+$ cells was low at both sites, but that of Ly-23$^+$ cells was high at both sites (14%–15% in the blood, and 24%–32% within the tumour). Lymphocytes, however, constituted only 3·4%–6·5% of all cells within these tumours (data not presented).

Functional potential of host lymphocytes

Evaluation of the presence of anti-tumour T_c in vivo. Table 4 shows the results of percentage specific lysis of MPC-11 cells by spleen cells or draining (inguinal) lymph node cells isolated from MPC-11 tumour-bearing mice at different days of tumour growth. No appreciable enhancement of killing above the control level was seen at any stage of tumour development. A minor rise on day 3 was not significant at $P = 0{\cdot}05$. The small amount of killing exhibited by cells from normal as well as tumour-bearing mice may represent NK-type killing, although MPC-11 is known to be a poor NK target. This view is supported by the fact that treatment of the attacker cells with anti-Thy-1 + C′ did not abrogate this killing.

T_c induction in vitro. Co-cultivation of normal BALB/c spleen cells with MPC-11 cells generated a substantial amount of killing ability which increased linearly with doubling increments of attacker:target ratios (Fig. 4). This killing was not due to a non-specific activation of spleen cells resulting from culture in the presence of FCS. The culture of spleen cells alone

Table 3. Incidence (%) of small lymphocyte subsets in blood and tumour

Host	Tissue	Thy-1$^+$	S-Ig$^+$	Null	Lyt$^+$	Ly-1$^+$	Ly 23$^+$	Ly 123$^+$
W-164	Blood	56	30	14	53	19	32	1
10d	Tumour	39	41	20	33	14	14	5
MPC-11	Blood	55	32	18	50	17	24	9
15d	Tumour	36	40	22	35	10	15	10

Table 4. Test for killer cell generation* *in vivo* in MPC-11 transplanted mice

Tumour age (days)		Spleen Attacker:target ratio 100:1	50:1	25:1	Left inguinal nodes Attacker:target ratio 100:1	50:1	25:1
0		7±3	6±1	4±2	—	—	—
3		12±2	7±2	5±2	6±2	6±2	4±1
	αThy-1+C′	11±2	7±2	4±1			
7		7±2	6±2	4±2	5±2	3±2	3±1
10		5±2	4±1	2±1	4±2	3±2	2±1
14		10±2	9±2	6±2	6±1	4±2	4±1
	αThy-1+C′	11±2	8±2	5±2			
21		6±2	5±1	4±2	—	—	—

* Mean percentage specific lysis of MPC-11 cells (± SE), derived from quadruplicate wells of lymphoid cells pooled from four mice at each interval.

in the absence of tumour cells caused a detectable but only a very minor rise in the lytic ability compared with fresh spleen cells, as previously reported by Burton, Chism & Warner (1977). Treatment of killer cells with anti-Thy-1+C′ or anti-Ly-2+C′ or anti-Ly−1+C′ caused a substantial (50%–60%) reduction in the lytic ability, the extent of the reduction being highest with the former. The abrogation of lytic ability must have been due to removal of appropriate phenotype bearing subsets from the attacker population rather than toxic effects of sera or complement, since a prior treatment of attacker cells with complement alone or an irrelevant antiserum (anti-Ia^k) plus complement did not affect their lytic ability (Fig. 4). These results indicate that the killer cells generated *in vitro* are Thy-1 +ve cells, which also include Ly-1 and 2 bearing cells. Since a combination of anti-Ly-1 and anti-Ly-2 sera caused only a minor increment (not significant at $P=0{\cdot}05$) in the abrogation of lytic function compared with that seen with each antiserum alone (Fig. 1), it appears that the T_c phenotype in the current experiment is Ly-12. However, a minor subset of Ly-1 or Ly-2 only cells amongst the effector population cannot be excluded. Residual lytic activity remaining after a single anti-Thy-1 treatment (Fig. 4) was primarily considered to be the result of incomplete T-cell removal with the present protocol, since a second treatment with anti-Thy-1 was found to remove most of the lytic ability (data not shown).

Suppressor mechanism in vivo *(adoptive transfer experiments)*. Pilot experiments revealed that an inoculum of $1–2\times10^7$ cells taken from the thymus as well as the spleen of tumour-bearing mice exhibited the ability of enhancing tumour growth when adopti-

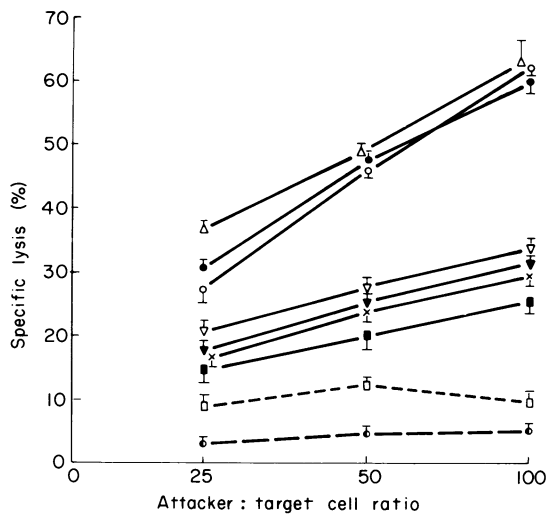

Figure 4. Cytotoxicity of BALB/c lymphoid cells against MPC-11 tumour target cells under different conditions. In the case of induced spleen cells anti-tumour cytotoxicity was induced by 5 days of co-cultivation with irradiated MPC-11 cells. Induced spleen cells: (●) untreated; (○) C′ alone; (△) anti-Ia^k+C′; (■) anti-Thy-1+C′; (▽) anti-Ly-1+C′; (▼) anti-Ly-2+C′; (x) anti-Ly-1+anti-Ly-2+C′. Fresh spleen cells (◐) as well as spleen cells cultured in the absence of MPC-11 cells (□) are also included as controls. To identify the effector cell phenotype, attacker cells were subjected to antibody+C′ mediated killing immediately before the assay. Each point represents mean result of four samples ±SE.

vely transferred i.v. into fresh hosts at the time of tumour transplantation. The tumour-enhancing ability of the thymocytes was detectable as early as day 7 of tumour growth in the donor animal, but the effects of splenocytes were reproducible only when taken from donors bearing tumours of more than 2 weeks duration (data not shown). For these reasons, more detailed studies were confined to later stages of tumour growth. Table 5 shows the results with spleen cells, and Fig. 5 the results with thymus cells taken from MPC-11 bearing mice at 21 days of tumour growth. In both cases, the recipients at the time of adoptive transfer were presensitized 3 weeks earlier with mitomycin C treated MPC-11 cells. This procedure evidently did not produce effective immunity to MPC-11 tumour transplantation. Nevertheless, tumour growth was enhanced by an adoptive transfer of spleen cells (Table 5) as well as thymus cells (Fig. 5), when these cells were obtained from mice bearing the same tumour. Tumour growth enhancement on both these occasions was revealed by a 3-day shortening in the lag period for the appearance of palpable tumours. Once palpable, the tumour growth rate *per se* did not appear to be different. The enhancement was completely abrogated by pretreatment of lymphoid cells with anti-Thy-1+C′ (Table 5) or anti-Ly-2+C′ (Fig. 5), but not with anti-Ly-1+C′ (Fig. 5). Thus, the lymphoid cell subset having the tumour growth enhancing ability was Thy-1$^+$, Ly-1$^-$, 2$^+$.

Table 5. Effects of adoptive transfer of spleen cells on MPC-11 tumour growth (mean diameter in mm ± SE)

Day	Normal spleen cells (2×10^7)	MPC-11 spleen cells (2×10^7)	
		Untreated	α-Thy-1+C′ treated
5	0	0	0
6	0	2·0±0·8	0
9	3·7±1·0	8·0±2·0	3·7±2·0
13	12·0±1·6	16·0±2·0	13·0±2·0
19	18·8±1·8	23·0±2·0	19·0±1·8

Table 6 presents the data on W-164 tumour growth in unprimed BALC/c mice after adoptive transfer of 2×10^7 thymocytes taken from BALB/c mice bearing 21-day-old tumours, as compared with that noted after no cell transfer or transfer of anti-Lyt+C′ treated thymocytes from tumour-bearing mice. In this case,

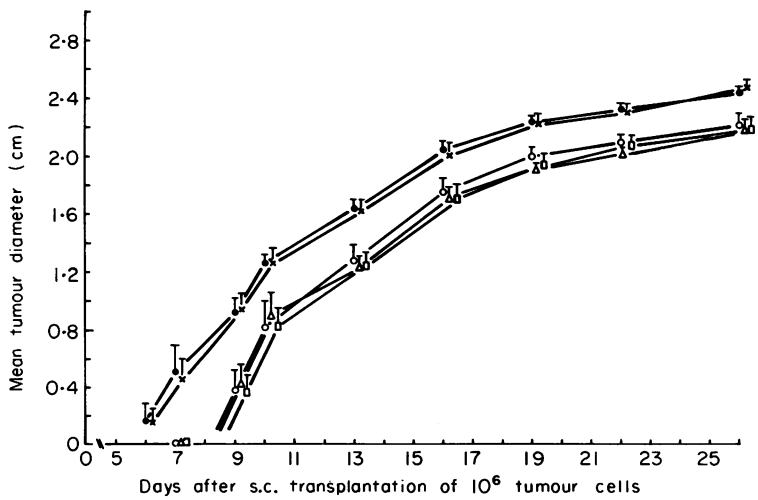

Figure 5. Effects of adoptive intravenous transfer of thymocytes taken from mice bearing 21-day-old MPC-11 tumours on the growth of fresh MPC-11 transplants into BALB/c mice which had been inoculated with mitomycin C treated MPC-11 cells 3 weeks before the assay. This presensitization failed to produce a lasting immunity. Transfer of no thymocytes or thymocytes from unrelated (WEHI-164) tumour-bearing BALB/c mice served as controls. To identify the Lyt phenotype of the tumour growth enhancing cells, thymocytes were treated with appropriate antibody +C′ immediately before the transfer. Data represent means from six animals ±SE. (○) Tumour cells alone; (●) 2×10^7 MPC-11 host thymocytes i.v.; (△) 2×10^7 anti-Ly-2+C′ treated MPC-11 host thymocytes i.v.; (×) 2×10^7 anti-Ly-1+C′ treated MPC-11 host thymocytes i.v.; (□) 2×10^7 W-164 host thymocyte i.v.

Table 6. Effects of adoptive transfer of thymocytes on W-164 tumour growth (mean diameter in mm ± SE)

	Adoptive transfer of				
			W-164 thymocytes (2×10^7)		
Day	No thymocytes	Normal thymocytes (2×10^7)	Untreated	αLy-1+C′ treated	αLy-2+C′ treated
5	0	0	2·5±0·8	2·6±0·7	0
7	0	0	5·0±1·5	4·8±1·6	0
10	3·4±1·0	3·5±1·0	8·5±1·3	8·2±1·2	3·5±1·1
12	4·5±1·5	4·3±1·6	10·0±1·4	9·8±1·3	4·4±1·5
15	6·0±1·8	7·1±1·6	11·4±1·5	11·2±1·5	6·6±1·7
19	8·8±2·1	8·5±1·8	16·3±2·2	15·8±2·1	9·0±2·0
21	13·0±2·4	13·5±1·5	18·5±1·9	18·2±1·8	13·3±2·3
24	14·0±2·0	14·0±2·1	22·7±1·8	22·2±1·7	14·1±2·3
26	16·0±2·3	17·0±2·5	23·1±1·8	23·0±1·8	16·6±2·4

thymocytes from tumour-bearing mice caused a reduction in the lag period (by approximately 5 days) for tumour appearance, and also some acceleration of growth rate after the tumours appeared. Normal thymocytes had no effect, and the effect was completely abrogated by pretreatment of inocula with anti-Ly-2+C′ but not anti-Ly-1+C′, indicating the involvement of an Ly-1$^-$, 2$^+$ thymocyte subset.

Similar experiments were also carried out in CBA mice transplanted with W-1 fibrosarcoma. In one study, unprimed CBA mice were employed (Fig. 6). In this case, adoptive transfer of thymocytes from 21 day tumour-bearing mice did not produce any appreciable difference in the latency period for tumour appearance. On the other hand, once tumours were palpable, they appeared to grow at a faster rate than seen in those receiving no thymocytes. The differences in tumour sizes became significant (at $P = 0{\cdot}05$) from day 17 onwards. Thymocytes taken from normal or unrelated syngenic tumour (W-54) bearing mice had no effect. The effect in the experimental group was abrogated by a prior treatment of thymocytes with

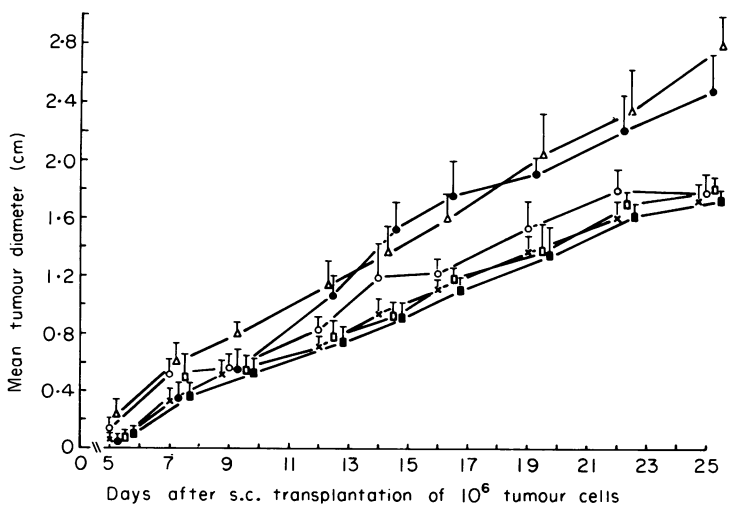

Figure 6. Effects of adoptive intravenous transfer of thymocytes taken from mice bearing 21 day old W-1 transplants into normal CBA mice. Transfer of no thymocytes or thymocytes from unrelated (W-54) tumour-bearing CBA mice served as controls. (○) Tumour cells alone; (□) 10^7 CBA thymocytes i.v.; (△) 10^7 W-1 host thymocytes i.v.; (●) 10^7 anti-Ly-1+C′ treated W-1 host thymocytes i.v.; (×) 10^7 anti-Ly-2+C′ treated W-1 host thymocytes i.v.; (■) 10^7 W-54 host thymocytes i.v.

anti-Ly-2+C′ but no anti-Ly-1+C′, indicating again that the effect was owing to Ly-1$^-$, 2$^+$ cells.

Tumour growth enhancing effects of 2×10^7 thymocytes taken from W-1 tumour-bearing mice were found to be more easily identifiable when mice surgically immunized against W-1 tumour were employed in the assay (Table 7). In this case, tumours

Table 7. Effects of adoptive transfer of thymocytes on W-1 fibrosarcoma growth in W-1 immune CBA mice (mean diameter in mm ± SE)

		Adoptive transfer of	
		W-1 host (21d) thymocytes 2×10^7	
Day	No thymocytes	Untreated	αLy-2 C′ treated
5	1·2±0·7	1·3±0·8	1·3±0·8
7	3·9±1·0	6·1±1·6	4·1±1·1
10	5·5±1·2	8·0±2·0	5·9±1·2
13	7·5±1·5	11·8±1·9	8·1±1·3
15	8·0±1·7	14·3±1·8	8·8±1·3
17	5·0±1·6	14±1·8	5·5±1·5
19	1·8±1·6	11·9±1·9	2·8±1·6
21	0	6·5±1·9	2·0±1·0

eventually regressed in all mice, but the growth promoting effect was significant (at $P = 0{\cdot}05$) from day 15 onwards. Again, the effect was abrogated by pretreatment of thymocytes with anti-Ly-2$^+$ C′.

DISCUSSION

The first part of the present study characterized small lymphocyte subsets in tumour-bearing mice at the morphological level using a radioautographic technique on the basis of several surface markers: s-Ig, Thy-1 and Lyt (Ly-1, 2 and 3) antigens. Subcutaneous transplantation of two completely different tumours in syngeneic BALB/c mice was found to cause similar patterns of changes in the host lymphocyte subsets. Of these two were most noteworthy: there was an increase in the levels of null small lymphocytes and also of the Ly-23$^+$ subset of T small lymphocytes in the host blood, spleen and thymus as well as at the tumour site. The absolute number of these subsets increased on all occasions excepting the null cell rise in the thymus, which reflected a relative change. The magnitude and the kinetics of increase for the two cell classes were somewhat different depending on the site. For example, the magnitude of rise in the null cell number was highest in the spleen, while that for the Ly-23$^+$ cells was large both in the thymus and the spleen. Furthermore, the rise in the level of Ly-23$^+$ cells occurred earliest in the thymus.

Current findings of an increase in the host null lymphocyte levels are in agreement with our previous reports (Garnis & Lala, 1978; Lala & Kaizer, 1977) in other host-tumour systems, suggesting that this may be a universal phenomenon in tumour-transplanted mice. We have also reported this phenomenon shortly before the clinical onset of spontaneous mammary tumours in C3H mice which may be harbouring subclinical tumours of non-palpable size (Santer *et al.*, 1980). Furthermore, a null cell rise is also seen in pregnant mice, more notably during allogeneic pregnancy, the temporal patterns of the rise in various lymphoid organs suggesting a response originating in the bone marrow (Chatterjee-Hasrouni *et al.*, 1980). A further characterization of the splenic null lymphocytes in tumour-transplanted mice (Lala *et al.*, 1979a) revealed a minor incidence of cells bearing C′3 or Fc receptors or cells capable of forming B lymphocyte colonies, but a major incidence (~40%) of Ia antigen-bearing cells. They also include natural killer cells both in active and inactive forms (Lala, Libenson & Santer, submitted). Thus, the null cell rise in these situations may reflect an early, non-specific response to a variety of antigens, e.g. embryonic antigens (during syngenic or allogenic pregnancy), alloantigens (explaining the enhanced response during allogenic pregnancy) and tumour antigens, of a class of young postmitotic marrow derived lymphocytes having heterogeneous functional potentials. Evidently further work is needed to identify fully their significance.

The second part of our study analysed functional T-cell subsets and their surface Lyt phenotypes in syngeneic tumour-bearing mice with two objectives: (i) to understand the implication of these findings for the tumour-host relationship, and (ii) to relate these findings to the changes in the host T-cell subsets identified on the basis of Lyt surface markers alone.

Our failure to detect appreciable levels of T_c *in vivo* is consistent with other reports (Takei, Levy & Kilburn, 1976; Wang, Berczi & Sehon, 1980) showing that their appearance *in vivo* is a transient event seen only with small-sized tumour inocula. This was not attributable to the lack of antigenicity of MPC-11 cells, as sufficient T_c was generated against this tumour *in vitro* with unprimed spleen cells. Generation of tumour-specific T_c has been accomplished for the MPC-11

(Burton & Warner, 1977; Giorgi & Warner, 1981) and many other syngeneic murine tumours by a variety of techniques (Wagner & Rollinghoff, 1973; Rollinghoff & Warner, 1973; Takei *et al.*, 1976; Shiku *et al.*, 1976; Mills & Paetkau, 1980). Some of the T_c have also been successfully maintained as long term culture lines (Giorgi & Warner, 1981). In view of the above evidence, failure of the host to contain the growth of otherwise immunogenic tumours would imply suppressor mechanism(s) which can either suppress the generation of T_c *in vivo* or inactivate them once they are generated. The present study has identified in the spleen and the thymus of tumour-bearing mice a T-cell subset which, on adoptive transfer into unprimed mice, can specifically enhance the growth of three syngeneic tumours: MPC-11 plasmacytoma and W-164 fibrosarcoma in BALB/c mice and W-1 fibrosarcoma in CBA mice transplanted shortly after the lymphoid cell transfer. This enhancement was seen either as an earlier appearance of tumours, or as an acceleration of tumour growth, or both. Similar findings have also been reported for a sarcoma line 1509A (Fujimoto, Green & Sehon, 1976a, b; Greene, Fujimoto & Sehon, 1977) and also other tumours employing either *in vivo* assays (Treves, Carnaud, Trainin & Feldman, 1974; Wang *et al.*, 1980) or *in vitro* assays (Takei *et al.*, 1976; Takei, Levy & Kilburn, 1977) of suppressor function. The precise kinetics of the appearance of the suppressor cell lineage in host lymphoid organs varies somewhat depending on the tumour-host system, tumour inoculum size, and relative sensitivity of the assay systems employed to detect suppression.

In the present study, the phenotype of the anti-MPC-11 T_c generated *in vitro* was found to be Ly-12. Clonal T_c lines generated against this tumour by Giorgi & Warner (1981) in long term culture have been phenotypes as Ly-23 on most occasions and Ly-123 on one occasion. Our data together with these results cannot distinguish between two possibilities: (i) that both T_c phenotypes may be generated from a Ly-123 precursor population, or (ii) that a repeated antigenic stimulation, employed by Giorgi and Warner, may cause a reduction or loss of Ly-1 antigens. T_c phenotypes generated against other murine syngenic tumours have also been reported as either Ly-12 (Shiku *et al.*, 1976; Shimizu & Shen, 1979), or Ly-23 (Stutman, Shen & Boyse, 1977; Maier, Levy & Kilburn, 1980). One report (Mills & Paetkau, 1980) detected Ly-1, but did not test for Ly-2. Tumour-inhibiting immune lymphocytes detectable with a Winn assay have been found to have Ly-1 phenotype (Perry & Greene, 1981; Nelson, Nelson, McKenzie & Blanden, 1981), but such cells may represent either antigen-specific delayed-type hypersensitivity (DTH) effector T cells which can recruit cytotoxic macrophages, or antigen-specific T helper cells which can recruit T_c in the test animal.

The phenotype of tumour growth promoting T cells in the present study was found to be Ly-2 (most likely Ly-23, as these two phenotypes go together) in all three syngenic tumour-host systems employed. These cells may theoretically represent T suppressor (T_s) cells, or suppressor-precursor cells, or suppressor-inducer cells. A chain of T suppressor cells of various orders has been postulated in the immune regulatory network (Benacerraf, 1981), in which the third order (T_s3) presumably expresses Ly-2 phenotype. In view of the paucity of data in the literature on the Ly phenotype of tumour-specific T_s, it is difficult to compare our findings with other tumour systems. In one case where the T_s was generated *in vivo* by injecting soluble membrane extracts of P815 mastocytoma cells in DBA/2 mice and detected by an *in vitro* assay, the phenotype has been reported as Ly-1^+, 2^- (Maier *et al.*, 1980). In other syngenic tumour systems, tumour-specific T_s has been reported to express I-J antigenic marker (Greene, Perry & Benacerraf, 1979; Yamauchi, Fujimoto & Tada, 1979) which becomes an integral component of the suppressor factor released by these cells. Levy, Maier & Kilburn (1979) observed that the tumour-specific suppressor cells were Ia^+, but were not restricted with respect to H-2K or D phenotypes on the responder cells.

We do not know what proportion of the Ly-23 subset of T cells represents the specific tumour-enhancing suppressor lineage in the functional assay. Nevertheless, the striking similarity in their temporal patterns of enrichment in the thymus and spleen during tumour development may suggest that studies on surface markers alone may have some predictive or prognostic value in tumour-bearing hosts. If these reflect a functional parallel, then one would predict that the balance of the cellular immune responses at the systemic as well as at the local (tumour) level tips heavily in favour of the tumour during the tumour development *in vivo*. One possible reason for this phenomenon, postulated by Yamauchi *et al.* (1979), is a differential activation of T_c and T_s because of the differential nature of the antigenic determinants recognized by the two cell lineages. Using sarcoma S1509A

in A/J mice, they noted that T_s recognized determinants present in a soluble membrane extract, but T_c could only be activated by the determinants associated with self antigens present on the surface of live tumour cells. Thus, an early activation of T_s may be due to a release of soluble tumour antigens.

A human parallel of our findings in mice is the report by two different laboratories (Cobleigh *et al.*, 1980; Kaszubowski, Husby, Tung & Williams, 1980) of an increase in the incidence of circulating T cells bearing Fc receptors for IgG (Tγ, which are known to include the suppressor cell category) in patients bearing a variety of advanced or disseminated malignant conditions. Simultaneous *in vitro* tumour-specific functional assays of T cells in such patients may be valuable.

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