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# Studies with a Spontaneous Murine Tumour—I. Indetectibility of Host Immune Resistance with Tumour-activated Elaboration of T Cell Stimulatory Lymphokines *in Vitro**

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**Abstract**—*A spontaneous tumour SP/N-1 which arose in a BALB/c female mouse and histologically consistent with breast origin was studied* in vivo *and* in vitro *to assess host responses to the tumour. Examination by means of the established methods of tumour cell challenge and cell transfer assays following 'immunization' of the isogeneic host against the tumour failed to provide clear evidence of host recognition and reactivity. Cell transfers conducted using critical numbers of tumour cells in which tumour to spleen cell ratios of up to 1 : 1000 were employed did not show any sign of antitumour activity within the pretreated host spleen cells. Nor was there any evidence of host resistance when presensitized mice were challenged with graded doses of tumour cells ranging from $10^4$ cells, with no take of tumours, to $5 \times 10^4$ cells, with total acceptance of the tumour by the challenged mice. Despite failures to demonstrate clear immune recognition of tumour by the above criteria, normal spleen cells when incubated with the tumour cells* in vitro *elaborated factors with stimulatory and possibly suppressive properties. At least one of these factors was shown to stimulate T cells as evidenced by stimulation of DNA synthesis in thymocytes. This could be shown readily in the presence of a submitogenic concentration of Con A.*

## INTRODUCTION

MOST investigations on tumour immunology are based on animal neoplasms induced experimentally by such means as chemical carcinogens, viruses or ultra-violet irradiation. A common feature of many of these tumours is their readily demonstrable immunogenicity within an isogeneic or autochthanous host. Very often a degree of concomitant immunity develops in the tumour bearer [1]. In certain situations augmentation of the antitumour activity of the tumour bearer resulted in regression of well-developed tumours [2, 3]. Despite their usefulness in investigations in tumour immunology, these tumours do not share several important characteristics of the naturally arising tumours. Hewitt *et al.* [4] failed to show evidence of immune reactivity in some 27 spontaneous tumour systems studied in the mouse and concluded that the only appropriate models for human cancer are the spontaneous tumours. Arguments concerning the nature and immunogenicities of spontaneous tumours have been discussed by Woodruff [5] and Woodruff *et al.* [6].

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There is clearly a need for greater interest in naturally arising tumours whatever their unknown etiology. Most of these tumours are either weakly immunogenic or immunogenicity cannot be demonstrated in them because of the insensitivity of the existing methods. We report here observations on a spontaneous tumour in the isogeneic BALB/c strain in which no clear evidence of immune reactivity could be found when examined by the established *in vivo* techniques, although host lymphoid cells can in fact recognize the tumour and respond to it by the elaboration and release of 'lymphokine factors' *in vitro*.

## MATERIALS AND METHODS

Techniques used have been described previously [1] and only departures from these and new techniques are described below.

### *Animals*

Inbred BALB/c female mice 3–4 months of age were used in all experiments. They were maintained as an inbred colony in the University's Animal House and originated from a nucleus obtained from the Medical Research Council Laboratory Animal Centre in Surrey. Food and water were supplied *ad libitum*.

*Tumours*

Tumour SP/N-1 arose spontaneously in a BALB/c female mouse anterolaterally in the shoulder region, while MC677 was induced in a BALB/c female by the injection of 3-methylcholanthrene (3-MC) in olive oil into the thigh musculature. The use of the spontaneous tumour was confined to its first 5 transplant generations, while the MC677 was within its 10th to 15th transplant generations.

*Media*

Eagle's Minimum Essential Medium (MEM) was used for preparation of cell suspensions and contained 100 IU of penicillin and 100 $\mu$g of streptomycin/ml. Cell culture medium consisted of RPMI 1640 (Gibco) containing 10% heat-inactivated foetal calf serum and other additives according to the formula used by Rosenberg *et al.* [7], and was called 'complete medium'.

*Preparation of cell suspensions*

Tumour cells were prepared as previously described. Spleen cells were separated by teasing out the tissues into MEM using 19G syringe needles. The residual pieces in MEM were gently sucked into a 1-ml syringe and released, and this was repeated three or four times to liberate easily recoverable cells. The cell suspension was filtered through 200 mesh stainless steel gauze, spun for 7 min at 250 **g** and red blood cells were lysed by means of hypotonic MEM and then washed twice again before use in cell transfer assays or in the preparation of cell cultures.

*Preparation of lymphokine-enriched cell culture filtrates*

Spleen cells prepared as above were resuspended in complete medium and mixed with tumour cells so that the mixture contained $5 \times 10^5$ tumour cells plus $5 \times 10^6$ spleen cells/ml of complete medium. The cell cultures were incubated in sterile plastic Universals or in 30-ml culture flasks at 37°C for 18–20 hr. The supernatants were recovered by spinning at 250 **g** for 10 min, followed by a second spin at 1000 **g** for 10 min before 0.45 $\mu$m millipore filtration and storage until use at −80°C.

*Assessment of T cell stimulatory activity*

Thymocytes from normal donor mice were separated by teasing out into MEM and the liberated cells were washed twice in MEM before suspending in complete medium or in dilutions of supernatants derived from tumour plus spleen cell co-cultures (TSCS) or from spleen cell cultures in the absence of tumour cells (SCS). Thymocytes were cultured in U-bottomed wells of a microtitre tissue culture plate using $5 \times 10^5$ cells per well in 0.25 ml of the culturing medium. For each dilution of the culture medium, 3 replicates of thymocyte cultures were set up. The cell cultures were incubated at 37°C in 5% $CO_2$ air for a total period of 48 hr. Five hours prior to harvesting 0.5 $\mu$Ci of tritiated thymidine ([$^3$H]-TdR) (5 Ci/mM, Radiochemical Centre, Amersham, U.K.) was added to each well and the cells were harvested in a Mini Mash cell harvester (Dynatech Laboratories Ltd., U.K.) on to glass fibre filter paper (Flow Laboratories, U.K.) and washed with distilled water. The filters were dried at 37°C for 24 hr or more and filter discs were placed in mini-scintillation vials to which 2 ml of scintillation fluid was added. Tritiated thymidine incorporation was determined in a liquid scintillation counter (LKB Wallac, 1216, Rack Beta, LKB Instruments Ltd., U.K.). The activity was expressed as the mean counts/min minus the background counts/min of thymocyte cultures incubated with complete medium in presence of 0.2 $\mu$g/ml Con A.

*Statistical evaluation of data*

Most data are expressed as their arithmetic means ±S.D. or S.E.M. Means are compared by Student's *t*-test and levels of significance are given as *P*-values. Proportions of animals with tumours were compared between groups and the significance was tested by means of Fisher's Exact Test for $2 \times 2$ tables.

## RESULTS

In vivo *tests for antitumour activity by cell transfer assays*

*Experiment* 1. Trocar pieces of tumour measuring approximately $2 \times 2$ mm were implanted s.c. into a group of 10 normal mice, and 10 days later, when the tumours reached 3–6 mm in diameter, they were excised. A further 10 days later the excision site was carefully examined for regrowths of tumour, and spleens were obtained from tumour-free animals. Spleen cells were prepared as described and tumour cells were obtained by trypsinization of the tumour. The cells were mixed together at a ratio of 500:1 and were inoculated into a group of 8 normal mice, each receiving $5 \times 10^7$ spleen cells plus $10^5$ tumour

cells s.c. Controls consisted of a group of 8 normal mice inoculated with normal spleen cells plus tumour cells identically. Observation was made over a period of 4 months following cell transfers.

Table 1 shows the proportion of animals that developed tumours in each group and also their mean diameters. There was no significant difference in either the proportions of mice developing tumours or in the mean diameters of the tumours between the test and control groups at any stage during tumour development.

*Experiment* 2. A second cell transfer assay was conducted using spleen cells obtained from tumour-excised mice as previously, but the assay was made more sensitive by halving the number of tumour cells per mouse and increasing the ratio of spleen to tumour cells to 1000:1. Controls consisted of a group of mice inoculated with normal spleen cells plus tumour cells, identically. There were 7 animals in each group.

Results of this experiment shown in Table 1 indicated tumour enhancement rather than resistance by presensitized spleen cells.

*Tumour cell challenge assays*

*Experiment* 1. Experiments were performed to see whether mice whose first transplant of tumour had been surgically excised were resistant to a challenge implant of the tumour. A group of 30 mice were s.c. implanted with approximately 2×2 mm size pieces of the tumour by means of a trocar. When the tumours were 3–8 mm in diameter 10 days later they were excised. All tumour-excised mice were left for possible development of antitumour activity for a further 11 days and were challenged s.c. with graded doses of the tumour into the opposite flank. Group 1, which consisted of 10 mice, were given $10^5$ tumour cells. Groups 2–4, each with 6 mice, were given $5\times10^4$, $2\times10^4$ or $10^4$ tumour cells respectively in 0.1 ml of MEM s.c. Four equal groups of normal mice were identically challenged with tumour cells as controls.

Results of this experiment are presented in Table 2. It can be seen that $10^5$ tumour cells grew in 10/10 mice in both control and test groups. Group 2 given $5\times10^4$ cells also developed tumours in all 6 animals of the test

*Table* 1. *Effect of 'presensitized' spleen cells on growth of tumours following mixed cell transfers*

| Group | Cell inoculum | No. of mice in group | | Days 10 | 17 | 30 | 40 |
|---|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | | |
| Test | $10^5$ SP/N-1 cells plus $5\times10^7$ 'sensitized' spleen cells | 8 | Proportion with tumours | *6/8 | 8/8 | 8/8 | 8/8 |
| | | | Mean dia. (mm) | 2.6 | 1.8 | 8 | 10 |
| Controls | $10^5$ SP/N-1 cells plus $5\times10^7$ normal spleen cells | 8 | Proportion with tumours | 6/8 | 8/8 | 8/8 | 8/8 |
| | | | Mean dia. (mm) | 1.4 | 0.8 | 7 | 9.9 |

| | | | | Days 10 | 14 | 18 | 25 | 34 | 45 | 56 |
|---|---|---|---|---|---|---|---|---|---|---|
| Experiment 2 | | | | | | | | | | |
| Test | $5\times10^4$ SP/N-1 cells plus $5\times10^7$ 'sensitized' spleen cells | 7 | Proportion with tumours | 4/7 | 4/7 | 4/7 | 6/7 | 5/7 | 5/7 | 5/7 |
| | | | Mean dia. (mm) | | | | | 8.2 ±4 | 12.5 ±1.5 | 14.2 ±1 |
| Controls | $5\times10^4$ SP/N-1 cells plus $5\times10^7$ normal spleen cells | 7 | Proportion with tumours | 1/7 | 4/7 | 5/7 | 6/7 | 6/7 | 5/7 | 5/7 |
| | | | Mean dia. (mm) | | | | | 5.5 ±3 | 8.2 ±3.6 | 7.3 ±5 |

*No. with tumour. / total No. in group

Mean dia. = mean diameter of developed tumours in group ± S.D. In Experiment 2 the mean diameter of controls was significantly greater than in test group at 45 days ($P<0.05$) and at 56 days ($P<0.05$). In Experiment 1, 10-day-old trocar implants of tumour SP/N-1 were excised 10 days prior to obtaining 'sensitized' spleens for cell transfers. In Experiment 2 similarly implanted tumours were excised 7–20 days prior to obtaining spleens.

*Table 2. Effect of 'presensitization' on the ability of host to reject challenge inocula of tumour cells*

| | Group | Treatment | No. in group | Challenge cell inoculum | Days 12 | 20 | 30 | 40 | 50 |
|---|---|---|---|---|---|---|---|---|---|
| | Experiment 1 | | | | | | | | |
| Test | 1 | *'Presensitized' | 10 | $10^5$ | +8/10 | 10/10 | 10/10 | 10/10 | — |
| | 2 | to the tumour | 6 | $5 \times 10^4$ | 1/6 | 4/6 | 4/6 | 4/6 | 6/6 |
| | 3 | | 6 | $2 \times 10^4$ | 0/6 | 0/6 | 2/6 | 2/6 | 2/6 |
| | 4 | | 6 | $10^4$ | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Controls | 1 | Normal | 10 | $10^5$ | 6/10 | 10/10 | 10/10 | 10/10 | — |
| | 2 | Controls | 6 | $5 \times 10^4$ | 1/6 | 5/6 | 5/6 | 5/6 | 6/6 |
| | 3 | | 6 | $2 \times 10^4$ | 0/6 | 0/6 | 1/6 | 1/6 | 1/6 |
| | 4 | | 6 | $10^4$ | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Test | Experiment 2 | ‡Presensitized' to the tumour | 10 | $5 \times 10^4$ | 9/10 | 10/10 | 10/10 | — | — |
| Controls | | Normal | 10 | $5 \times 10^4$ | 9/10 | 10/10 | 10/10 | — | — |

*Tumour implanted by trocar at day 0 and excised at day 10 when tumours were 3–8 mm in diameter. Challenge inoculation was done 11 days later.

†No. with tumours. / total No. in group

‡Tumours were implanted by trocar on day 0 and they were excised after 14 days. The mice were given $10^6$ mitomycin-treated tumour cells on three occasions at 10–27 day intervals and challenged with tumour cells 13 days after the last 'immunization'.

group as well as in control mice. Group 3 had tumours in 2/6 of the test group animals and 1/6 of the controls and Group 4 did not develop tumours in both groups.

*Experiment* 2. A second tumour cell challenge experiment was conducted using $5 \times 10^4$ tumour cells, which appeared to be a critical inoculum of tumour cells. A group of 10 mice, implanted with tumour as for Experiment 1, had their tumours excised 14 days later. The mice were repeatedly given s.c. inoculations of $10^6$ mitomycin-treated SP/N-1 cells on three occasions 10–27 days apart, and were tumour-challenged 13 days after the last inoculation. A group of normal controls were identically challenged with the tumour simultaneously. Results presented in Table 2 for Experiment 2 failed to provide any indication of tumour rejection or delayed tumour formation in the pretreated animals. Tumours developed in all 10 animals of both groups.

### In vitro *studies on tumour recognition by spleen cells and secretion of lymphokines*

Culture supernatants of normal spleen cells incubated with tumour cells (TSCS) or without tumour cells (SCS) as described were tested for T cell (thymocyte) stimulatory activities in the presence of a submitogenic concentration of Con A (0.2 $\mu$g/ml). In addition to the SP/N-1 tumour a demonstrably immunogenic MC-induced tumour MC677 was also utilized for the preparation of TSCS. All TSCS as well as SCS preparations used in each experiment were made simultaneously using identical spleen cells and under identical conditions. Their T cell stimulatory activities were also tested simultaneously under the same conditions.

Table 3 shows the incorporation of [$^3$H]-TdR by thymocytes cultured in doubling dilutions of TSCS and SCS preparations made in complete medium in two experiments. It can be seen that in Experiments 1 and 2, TSCS stimulated DNA synthesis in thymocytes significantly more than SCS preparations. Thus, in Experiment 1 the significance levels at various dilutions for SP/N-1 SUPS were: 1/2, $P < 0.01$; 1/4, $P < 0.0$; 1/8, $P < 0.001$, and in Experiment 2: 1/2, $P < 0.001$; 1/4, $P < 0.05$. Similarly, highly significant stimulation of DNA synthesis was also observed in MC677 SUPS preparations at dilutions ranging from 1/2 to 1/16.

## DISCUSSION

The SP/N-1 tumour studied showed no clear evidence of immunogenicity when examined by the cell transfer and tumour cell challenge assay techniques. In cell transfers (Table 1) conducted using $10^5$ tumour cells plus $5 \times 10^7$ 'sensitized' spleen cells, no significant difference in tumour development could be detected between groups. The second cell transfer experiment was made highly sensitive by the selection of a critical number of tumour cells ($5 \times 10^4$) and increasing the ratio of spleen to tumour cells to 1000:1. Despite improvement of sensitivity of the assay system, no anti-

*Table 3. Stimulation of DNA synthesis in thymocytes by the supernatants of mixed tumour–spleen cell cultures (TSCS) in the presence of a submitogenic concentration* (0.2 μg/ml) *of concanavalin A* (counts/min ± *S.D.*)

| | Group | Derivation of supernatant: tumour-activating spleen cells in culture | Dilution of supernatant 1/2 | 1/4 | 1/8 | 1/16 |
|---|---|---|---|---|---|---|
| Experiment 1 | 1 | SP/N-1 | 1916 ± 196 | 2235 ± 547 | 1263 ± 164 | 126 ± 415 |
| | 2 | MC-677 | 1443 ± 205 | 2281 ± 168 | 2016 ± 204 | 1559 ± 749 |
| | 3 | Control (spleen cells incubated without tumour—SCS) | 1217 ± 127 | 70 ± 268 | −191 ± 54 | −366 ± 131 |
| Experiment 2 | 1 | SP/N-1 | 4048 ± 390 | 3851 ± 1925 | 1225 ± 988 | 1403 ± 1052 |
| | 2 | MC-677 | 3042 ± 588 | 4449 ± 1075 | 825 ± 143 | 680 ± 411 |
| | 3 | Control (spleen cells incubated without tumour—SCS) | 570 ± 241 | 157 ± 289 | 96 ± 100 | 130 ± 55 |

Whole thymocytes ($5 \times 10^5$) were incubated in dilutions of TSCS or SCS and DNA synthesis assessed by the incorporation of [$^3$H]-TdR. Activity incorporated is expressed as mean counts per minute − background ± standard deviation (counts/min ± S.D.). Each mean counts/min is derived from 3 replicate cultures. Following differences are significant: Experiment 1, Group 1 ∝ Group 3, dilution 1/2, $P < 0.01$, dilution 1/4, $P < 0.01$, dilution 1/8, $P < 0.001$; Group 2 ∝ Group 3, dilution 1/4, $P < 0.001$, dilution 1/8, $P < 0.001$, dilution 1/16, $P < 0.001$. Experiment 2, Group 1 ∝ Group 3, dilution 1/2, $P < 0.001$, dilution 1/4, $P < 0.05$; Group 2 ∝ Group 3, dilution 1/2, $P < 0.001$, dilution 1/4, $P < 0.01$, dilution 1/8, $P < 0.02$.

tumour effect could be observed. Lack of reactivity to tumour was also observed in the tumour cell challenge assays (Table 2), which consistently failed to demonstrate any significant differences between test and control groups. A vague indication that in this system presensitized spleen cells may enhance the tumour came from cell transfer Experiment 2. It was observed that the larger mean diameters of the test group tumours compared with those of the controls reached the 5% level of significance ($P < 0.05$) after 45 days and 56 days, but no significant difference could be found after 35 days. There is thus good evidence that this tumour did not arouse an immune response in the syngeneic mice which contributed to immunological rejection or inhibition of tumour cells. However, an interesting feature of this tumour system is the ability of the syngeneic normal animals to reject as many as $2 \times 10^4$ tumour cells when inoculated s.c. This is in contrast to the inability of non-immune mice to reject $10^4$ cells of a moderately immunogenic MC-induced tumour MC677 [8]. It is possible that SP/N-1 is more susceptible to inhibition by such para-immune mechanisms as the activity of natural killer cells [9], macrophages [10, 11] or interferon [12] than to truly immune antitumour activity.

Evidence that this tumour is not totally lacking in tumour-associated antigens (TAA) recognizable by the syngeneic host came from studies on lymphokines present in co-cultures of tumour cells plus normal spleen cells. As shown in Table 3, factors present in SCS preparations were weak in ability to stimulate [$^3$H]-TdR incorporation by thymocytes in the presence of 0.2 μg/ml of Con A under these experimental conditions. However, TSCS preparations from the two tumour systems studied showed a highly significant ($P < 0.001$) level of T cell-stimulating activity when compared with that of SCS preparations. In the SP/N-1 system this difference was strikingly shown in the various dilutions of the TSCS preparations examined ranging from 1/2 to 1/8 with a very high level of significance ($P < 0.001$). However, the observed secretion of T cell stimulatory factors in response to tumour bore no direct relationship to the development of antitumour activity within the host. This is seen clearly in our studies with tumour MC677, which, in contrast to SP/N-1, has the capacity to immunize the syngeneic host to reject 50 to 100-fold more tumour cells than the cell dose that would regularly form tumours in normal mice (6/6 vs 0/6). The TSCS preparations we have used in our studies were crude supernatants which invariably contained a host of factors—some stimulatory, others suppressive. These preparations most likely contained, amongst other molecules, lymphocyte-activating factor or interleukin-1, which is considered to be a macrophage product [13, 14], and possibly also interleukin-2, known to be a product of T cells [15–17]. These factors have been shown to be mediators of cytotoxic T cell differentiation and clonal expansion in the immune response [18]. Clearly, further studies are needed to clarify the nature of the activities present in

our TSCS preparations, and we are at present investigating the origin of these molecules, their detection and their biological roles in tumour systems. These findings will be reported in subsequent communications.

Several possibilities exist as to why lymphoid cells with antitumour activity could not be detected in the SP/N-1 system despite T cell lymphokine responses comparable with that of the immunogenic tumour MC677. It can be considered that the SP/N-1 system causes a stronger activation of the suppressor T cell system which would abrogate the differentiation of effector cells with antitumour activity. It is also possible that cytotoxic T cell differentiation and proliferation are inhibited *in vivo* by the presence of inhibitors of certain lymphokines which may be absent or inactive *in vitro*. Although the mechanism(s) of interaction are not clear at present, the observations reported here suggest that lymphokine factors may be of importance in the pathway of tumour 'inhibitory' T cell as well as NK cell generation or its abrogation.

Secondly, if our observation on tumour enhancement reflects a true *in vivo* situation in view of the significance ($P < 0.05$) of the finding, it may lend further support to Prehn's hypothesis of immunostimulation of tumour growth [19–22; Chandradasa, unpublished observation] and raise the interesting possibility that certain lymphokines or lymphokine-like factors may also promote the proliferation of tumour cells.

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