Eur J Cancer Clin Oncol, Vol. 18, No. 11, pp. 1063–1068, 1982.
Printed in Great Britain.

0277-5379/82/111063-06$03.00/0

Studies with a Spontaneous Murine Tumour—II. Transformation of Non-inhibitory Lymph Node Cells (LNC) of Tumour Bearers into Tumour Inhibitor Cells *in Vitro**

KURUPPU D. CHANDRADASA and JANET BLEARS
Department of Immunology, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Abstract—*Syngeneic BALB/c mice repeatedly pre-exposed to mitomycin-c-treated cells of a 'spontaneous' tumour SP/N-1 failed to develop resistance against the same tumour when tested by cell transfer assays. No difference could be observed between tumour-bearer, tumour-pretreated or normal donor LNC, or spleen cells by this assay system. When tumour-bearer LNC were cultured* in vitro *for* 45 hr *in the absence of tumour cells, these LNC generated a relatively strong ability to suppress tumour outgrowth* in vivo *following cell transfers. The tumour suppression appears to be due to tumour cell destruction since observation over four months or more in some experiments failed to reveal the presence of tumours at the site of inoculation. These findings clearly demonstrate that naturally arising tumours so often considered as devoid of, or weak in, tumour-associated transplantation antigens (TATA) may not necessarily be so. Despite the fact that the syngeneic host may be capable of recognizing TATA as foreign, the immune response initiated may not progress to completion in at least a proportion of these tumour-host systems. The defect appears to reside in the failure of differentiation of the pre-effector cells into the mature effector form. We interpret this as being due to a strong activation of certain inhibitors of essential lymphokine mediators such as interleukin 2, needed for differentiation and expansion of effector cell clones. It remains to be seen whether the mechanism of non-responsiveness or weakness of the response against many other members of this category of tumours—the 'spontaneous' or natural tumours—can be explained on the same basis as that of the SP/N-1 system studied here.*

INTRODUCTION

IN A previous communication [1] it was shown that SP/N-1 would normally have been considered as a non-immunogenic tumour, judging by the well-accepted criteria of host resistance detectable by the tumour immunization/challenge technique as well as by the cell transfer assay. The inability to demonstrate tumour-associated transplantation antigens (TATA) in many of the 'spontaneously' arising tumours has been reported by several groups of investigators [2–5]. Where a degree of immune resistance has been detected, i.e. in tumours other than those of viral aetiology, the level of tumour cell rejection that could be induced in syngeneic hosts over that of the normal threshold by immunization against the tumour has been minimal [3, 6, 7]. The naturally arising tumours are therefore, as a category, markedly different from the chemically, virally or u.v. irradiation-induced tumours, where the norm is immunogenicity, which is often strong. The reason for the weakness of immunogenicity of the spontaneous tumours has been considered by some to be due to selection pressure against antigenic cell clones [8] during the long tumour progression [9, 10] period of the naturally arising tumours. The mechanism of non-immunogenicity or weakness of immunogenicity of the natural tumours has not been clarified. However, in the laboratory-induced tumours, immunosuppression mediated by several mechanisms has been de-

Accepted 15 June 1982.
*Supported by the North-West Cancer Research Fund.

monstrated. Thus suppressor cells have been known to cause failure of tumour rejection or to enhance tumours [11–15]. Another mechanism demonstrated both in animal and human tumour systems has been the blocking factor-mediated prevention of target cell destruction [16–19].

We report here the development of strong effector cell activity and suppression of outgrowth of a 'non-immunogenic spontaneous' tumour when non-inhibitory lymphoid cells of tumour bearers have been incubated *in vitro* for a short period of approximately two days prior to testing for their inhibitory activity against the tumour cells.

MATERIALS AND METHODS

These have been described in previous communications [1, 11] and are only briefly dealt with here.

Animals

Inbred BALB/c female mice aged 3–4 months were used in all experiments.

Tumour

SP/N-1 arose anterolaterally in the shoulder region of a female BALB/c mouse and was histologically consistent with breast origin. The tumour was passaged by trocar transplantation of solid pieces of the tumour subcutaneously s.c. in female BALB/c mice. In present experiments the tumour was used within 10 transplant generations from its origin.

Media

Eagle's Minimum Essential Medium (MEM) and RPMI 1640 were used. These have been described previously [1].

Preparation of single-cell suspensions

The methods used for preparation of tumour cells [11] and spleen cells [1] have been described. In the preparation of LNC, axillary brachial and inguinal lymph nodes were taken. They were washed ×5 in MEM and the cells were liberated into MEM in a Petri dish using 19G syringe needles. The cells were removed and residual pieces were gently sucked with MEM into a 1-ml syringe without the needle and released. This was repeated 4–5 times to obtain the easily recoverable cells. The cells were filtered through a 200 mesh stainless steel gauze and washed ×2 in MEM prior to their use for cell transfers or for LNC culture.

Mitomycin-c (MMC) treatment of tumour cells

Tumour cells were concentrated to approximately 25×10^6 cells/ml and MMC was added at a concentration of 60 μg/ml. The cell suspension was incubated for 1 hr at 37°C in a Bijou or Universal with intermittent gentle shaking. At the end of incubation the cells were washed ×3 in MEM.

Tumour pre-treatment of mice/'immunization'

The MMC-treated tumour cells were suspended in MEM at a concentration of 5×10^6/ml and 0.1 ml of cells were inoculated s.c. into the shaved flank of groups of mice using a 1-ml syringe. Tumour cells were inoculated on 3 occasions 10–14 days apart and lymph nodes were taken for assays 9/10 days later.

LNC culture

LNC prepared as described were over 70% viable. The cells were suspended in RPMI 1640 (Gibco) medium containing 10% heat-inactivated foetal calf serum and other additives according to the formula of Rosenberg *et al.* [20]. The suspended cells were incubated in 200-ml tissue culture flasks at a cell concentration of 5×10^5/ml. Cell cultures were harvested after 45 hr incubation and were washed ×1 in MEM prior to use in cell transfer assays.

Cell transfer assays

Tumour SP/N-1 cells were derived from a trypsin-disaggregated tumour as described previously [11]. The cells were washed ×2 in MEM and were mixed together with LNC or spleen cell preparations. The volume was adjusted to give 5×10^5 tumour cells plus the desired number of LNC or spleen cells in 1 ml of medium. The well-mixed cells were inoculated s.c. into the shaved and 70% alcohol-sterilized flanks of normal mice so that each received 0.1 ml containing 5×10^4 tumour cells together with the lymphoid cells. Tumour formation was detected by palpation at the site of inoculation after 7–10 days and at regular intervals thereafter.

RESULTS

Antitumour activity of tumour-pre-treated or normal mouse LNC

Experiment 1. A group of 10 BALB/c mice were repeatedly inoculated with 5×10^5 MMC-treated SP/N-1 cells s.c. in 0.1 ml of MEM at 10–14 day intervals. Ten days following the third inoculation, their axillary brachial and inguinal lymph nodes as well as spleens were removed. Control lymph nodes and spleens were obtained from a group of normal mice. Single-cell suspensions of spleen cells and LNC were prepared as described and were mixed with tumour cells at ratios of 50:1 or 100:1 for LNC and 500:1 for spleen cells. The

well-mixed cell suspensions were inoculated into groups of 10 normal recipients s.c. Tumour formation at the site of inoculation was monitored by palpation, starting 10 days later. As shown in Table 1, tumours appeared in 9/10 and 10/10 recipients of tumour cells plus spleen cells from tumour-pre-treated or normal donors respectively. Similarly, 10/10 mice in each of the groups inoculated with tumour cells together with 100 times as many LNC also developed tumours and no significant difference was noted in groups which received tumour cells mixed with 50 times as many LNC.

Antitumour activity of pre-cultured vs fresh LNC

Experiment 2. In experiment 2 the antitumour activity of LNC derived freshly from 30-day tumour bearers with 8 to 10-mm diameter tumours, as well as that of tumour-pre-treated and normal donors, were compared with the antitumour activity of similar tumour-bearer or repeatedly tumour-pre-treated donor LNC which were pre-cultured *in vitro* for 45 hr. Tumour cells were mixed with LNC at a ratio of 1:50 and the mixtures were inoculated s.c. into groups of 5 normal recipients. The results of this experiment are shown in Table 2. Fresh LNC from none of the

Table 1. Lack of tumour inhibition by LNC and spleen cells of mice repeatedly pre-exposed to mitomycin-c-treated tumour cells (sensitized LNC or spleen cells)

Group	Composition of cell mixture	Ratio of lymphoid cells to tumour cells	Proportion of mice developing tumours
1	sensitized LNC + tumour cells	50:1	10/10*
	normal LNC + tumour cells	50:1	8/10
2	sensitized LNC + tumour cells	100:1	10/10
	normal LNC + tumour cells	100:1	10/10
3	sensitized spleen cells + tumour cells	500:1	10/10
	normal spleen cells + tumour cells	500:1	9/10

Sensitized lymphoid cells were obtained from a group of mice that were inoculated s.c. with 5×10^5 mitomycin-*c*-treated SP/N-1 cells on three occasions at 10 to 14-day intervals. The nodes and spleens were taken 10 days after the last inoculation. All cell transfers contained 5×10^4 tumour cells together with appropriate number of lymphoid cells in MEM, and all inoculations were made s.c. in 0.1 ml of medium.

*Indicates the number of mice with tumours/number of mice in group after an observation period of 6 weeks.

Table 2. Comparison of tumour inhibitory activity of LNC obtained from tumour-bearers or tumour-pre-treated mice with or without culture and from normal mice

LNC donor group	LNC cultured/fresh	Ratio of LNC to tumour cells in inoculum	Proportion with tumours on days: 12	15	20	30	40
Tumour bearers	Fresh	50:1	5/5	5/5	5/5	5/5	5/5
	Cultured	50:1	0/5	0/5	0/5	0/5	0/5
Tumour-pre-sensitized	Fresh	50:1	5/5	5/5	5/5	5/5	5/5
	Cultured	50:1	4/5	4/5	4/5	4/5	4/5
Normal	Fresh	50:1	5/5	5/5	5/5	5/5	5/5

Tumour-pre-sensitized donors were inoculated with MMC-treated tumour cells on three occasions 10–14 days apart. LNC were taken 9 days after the third inoculation and cultured for 45 hr at 37°C prior to cell transfer assay. Tumour-bearer LNC donors had tumours of 30-day duration with mean tumour diameters of 8–10 mm. All cell transfers consisted of 5×10^4 tumour cells in mixture with 2.5×10^6 LNC which were inoculated s.c. in 0.1 ml of MEM.

three groups had any protection against tumour outgrowth and tumours developed in 5/5 mice in each of the inoculated groups of mice. LNC from tumour-pre-treated mice which were pre-cultured *in vitro* for 45 hr prior to mixing with tumour cells failed to prevent tumour development in 4/5 recipients. In contrast, tumour-bearer LNC pre-cultured for 45 hr developed inhibitory activity against the tumour cells and conferred total protection against the tumour cells present in the inoculum, leaving 5/5 recipient mice free of tumours.

Antitumour activity of tumour-bearer LNC, fresh vs pre-cultured

Experiment 3A. Experiment 2 was repeated using LNC derived from tumour bearers of two months duration which had tumours of 1–2 cm mean diameter. Tumour inhibitory activity of freshly derived tumour-bearer LNC was compared with tumour-bearer LNC pre-cultured for 45 hr exactly as in experiment 2. There were 10 mice in each of the tumour cells plus LNC recipient groups. The results are presented in Table 3, experiment 3A. Pre-cultured LNC protected 8/10 of the inoculated mice against tumour development, whilst only 1/10 in the group which received freshly derived tumour-bearer LNC remained free of tumour. After 4 months of observation no change in the proportions freed of tumour was noted.

Experiment 3B. The experiment was repeated for the third time using LNC from tumour bearers of 50-day duration with tumours of 1–2 cm mean diameter. The results shown in Table 3, experiment 3B confirmed those of the previous two experiments. When inoculated in mixture with tumour cells, cultured LNC protected all 5 recipients whilst tumours developed in all 5 mice inoculated with fresh tumour-bearer LNC and tumour cells. No change was noted during an observation period of over 60 days.

DISCUSSION

We have presented in this communication very clear evidence that tumour-bearer LNC derived from syngeneic hosts of what might normally have been considered a non-immunogenic 'spontaneous' tumour developed marked tumour inhibitory activity when these cells were pre-cultured *in vitro* for approximately 2 days. Evidence provided in an earlier report [1] showed that mice pre-exposed to the tumour SP/N-1 by excision and eradication of a previous s.c. implant of tumour with or without further treatment using 'MMC-inactivated' tumour cells failed to develop elevated resistance against SP/N-1. This was observed by tumour cell challenge as well as by cell transfer assays in which 1000-fold excess of spleen cells as a critical number of tumour cells were utilized. Since repeated inoculation with small numbers of MMC-treated tumour cells was considered to be an effective method of immunization against tumours in general, as has been found by other workers [21, 22], this method was adopted in the present series of experiments, in which both spleen cells as well as LNC were tested for antitumour activity. Despite the use of 500-fold excess of spleen cells or 100-fold excess of LNC as tumour cells, in cell transfer assays no inhibitory effect could be observed in pre-sensitized donor lymphoid cells, nor was there any tumour inhibitory effect in normal mouse LNC (Table 1). The cell transfer assay system has been considered to be even more sensitive than the immunization/challenge technique for the detection of antitumour activity, attributed to generation of suppressor cells *in vivo*. Since tumour cell challenge technique has been observed to be superior to the membrane immunofluorescence technique in the detection of TATA in spontaneously arising rat tumours [6], the assay utilized here can be considered to be a sensitive technique for detection of tumour inhibitory lymphoid cells. From the above criteria one might conclude that non-inducibility of immune resistance against SP/N-1 could be due

Table 3. Comparison of tumour inhibitory activity of tumour-bearer LNC with and without pre-culture for 45 hr in vitro

	LNC donor group	Cultured/fresh	Ratio of LNC to tumour cells	Proportion with tumours on days: 20	30	40	60	80	120
Experiment 3A	Tumour bearer	Cultured	50:1	2/10	2/10	2/10		2/10	2/10
		Fresh	50:1	7/10	9/10	9/10		9/10	9/10
Experiment 3B	Tumour-bearer	Cultured	50:1				0/5		
		Fresh	50:1				5/5		

Tumour bearers had tumours of 50 to 60-day duration, with tumours of 1–2 cm mean diameter at the time nodes were taken for 45 hr. LNC cultures and fresh LNC were prepared on the day of cell transfers. The cell transfer inoculum consisted of 5×10^4 tumour cells plus 2.5×10^6 LNC inoculated s.c. in 0.1 ml MEM.

either to absence or weakness of TATA or to a genetic lack of responsiveness of the host to the tumour-associated antigens which may nevertheless have potential to act as tumour rejection antigens. This latter situation could arise if the tumour host fails to elaborate one or more of the mediators of effector cell differentiation, but the same effect could be obtained if the tumour activated individual generates inhibitor(s) of such a mediator(s) *in vivo*.

It was therefore interesting that, despite inability to detect immunogenicity in this tumour system, we were able to obtain strong tumour inhibitory activity when LNC derived from tumour bearers with tumours of up to 2 cm diameter and of long duration were cultured *in vitro* for a short period of 45 hr. Such pre-cultured LNC regularly inhibited an inoculum of 5×10^4 tumour cells when mixed at a ratio of 50:1 and inoculated s.c. into normal recipients, quite in contrast to the inability of tumour-bearer or normal LNC to bring about suppression of tumours. The results obtained in three separate experiments (Tables 2, 3) show an almost all-or-nothing fashion of activities of these LNC populations under our experimental conditions. Interestingly, pre-cultured LNC from tumour-pre-treated donors also failed to inhibit tumours and, as shown in Table 2, 4/5 mice in this group developed tumours, compared with 0/5 in the pre-cultured tumour-bearer LNC recipients.

We concluded from our findings that indetectability of TATA in the SP/N-1 system following tumour pre-treatment could not have been due to absence of TATA, but to a total, or almost total, 'suppression' of the immune response *in vivo*, presumably by the suppressor T cells and/or their products. The suppression cannot be due to blocking factors acting at the effector or target cell level as has been shown to be operative in certain tumour systems [16–19], since attempts to immunize the syngeneic host were met with failure, as reported previously [1] as well as shown in this investigation (Table 1).

On the other hand, the generation of a relatively strong effector activity *in vitro* after a short period of *in vitro* culture in the absence of added tumour antigen indicates that the tumour-activated effector cell precursors already existed in the LNC population of the tumour bearers. We therefore hypothesize that the effector cell transformation event occurred *in vitro* and that this was a terminal step in the chain of events leading to the differentiation of the effector cells. The failure to develop effector cells *in vivo* may have been due to the generation of a powerful inhibitor of essential lymphokine factors such as interleukin 2 (IL 2), which is known to be a product of T cells important in the differentiation and clonal expansion of the effector population in allogeneic systems [23–25]. This rapidity of generation of effectors *in vitro* from LNC of tumour bearers can be explained on the basis that pre-effector cells existed in an activated form in the LNC population in contact with tumour antigen. When incubated *in vitro*, elaboration of IL 2 and its release by helper T cells into the medium, where the inhibitor is too dilute or because its source of origin is inactive, enabled pre-effectors to transform into the effector form.

The above conclusion is supported by our observations reported in a previous communication [1], where we have shown that culture supernatants derived from co-cultures of SP/N-1 cells and spleen cells contained T cell-stimulating factors as well as suppressors. We have also observed similar activities in culture supernatants of tumour-bearer spleen cells and node cells and in short-term cultures of tumour cells containing tumour-infiltrated lymphoid cells in other tumour systems (Chandradasa, unpublished observations).

REFERENCES

1. CHANDRADASA KD, BLEARS J. Studies with a spontaneous murine tumour—I. Indetectability of host immune resistance with tumour-activated elaboration of T cell stimulatory lymphokines *in vitro*. *Eur J Cancer Clin Oncol* 1982, **18**, 853–859.
2. PREHN RT, MAIN JM. Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* 1957, **18**, 769–778.
3. RIGGINS RS, PILCH YH. Immunity to spontaneous and methylcholanthrene-induced tumours in inbred mice. *Cancer Res* 1964, **24**, 1994–1996.
4. BALDWIN RW. Tumour-specific immunity against spontaneous rat tumours. *Int J Cancer* 1966, **1**, 257–264.
5. HEWITT HB, BLAKE ER, WALDER AS. A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumours of spontaneous origin. *Br J Cancer* 1976, **33**, 241–259.
6. BALDWIN RW, EMBLETON MJ. Detection and isolation of tumour specific antigen associated with spontaneously arising rat mammary carcinoma. *Int J Cancer* 1970, **6**, 373–382.

7. MORTON DL. Successful immunization against a spontaneous mammary tumour on C3H/HEN mice. *Proc Am Assoc Cancer Res* 1962, **3**, 346.
8. KLEIN G, KLEIN E. Immune surveillance against virus-induced tumours and non-rejectability of spontaneous tumours: contrasting consequences of host versus tumour evolution. *Proc Natl Acad Sci USA* 1977, **74**, 2121–2125.
9. FOULDS L. Mammary tumours in hybrid mice. Growth and progression of spontaneous tumours. *Br J Cancer* 1949, **3**, 345–375.
10. FOULDS L. *Neoplastic Development.* Academic Press, London, 1969.
11. CHANDRADASA KD. The stimulation and specific suppression of concomitant immunity in two syngeneic tumour-host systems. *Int J Cancer* 1973, **11**, 648–662.
12. FUJIMOTO S, GREENE MI, SEHON AH. Regulation of the immune response to tumour antigens. I. Immunosuppressor cells in tumour bearing hosts. *J Immunol* 1976, **116**, 791–799.
13. FUJIMOTO S, GREENE MI, SEHON AH. Regulation of the immune response to tumour antigens. II. The nature of the immunosuppressor cells in tumour bearing hosts. *J Immunol* 1976, **116**, 800–806.
14. TREAVES AJ, CARNAUD C, TRAININ N, FELDMAN M, COHEN R. Enhancing T lymphocytes from tumour bearing mice suppress host resistance to a syngeneic tumour. *Eur J Immunol* 1974, **4**, 722–727.
15. UMIEL T, TRAININ N. Immunological enhancement of tumour growth by syngeneic thymus derived lymphocytes. *Transplantation* 1974, **18**, 244–250.
16. HELLSTRÖM I, HELLSTRÖM KE. Studies on cellular immunity and its serum-mediated inhibition in Moloney virus-induced mouse sarcomas. *Int J Cancer* 1969, **4**, 587–600.
17. HELLSTRÖM I, HELLSTRÖM KE. Some aspects of immune defence against cancer. *Cancer* 1971, **28**, 1269–1271.
18. SJÖGREN HO, HELLSTRÖM I, BANSAL SC, HELLSTRÖM KE. Suggestive evidence that the "blocking antibodies" of tumour bearing individuals may be antigen-antibody complexes. *Proc Natl Acad Sci USA* 1971, **68**, 1372–1375.
19. BALDWIN RW, PRICE MR, ROBINS RA. Blocking of lymphocyte-mediated cytotoxicity for rat hepatoma cells by tumour-specific antigen-antibody complexes. *Nature New Biol* 1972, **238**, 185–186.
20. ROSENBERG SA, SCHWARS S, SPIESS PJ, BROWN JM. *In vitro* growth of murine T cells III. Method for separation of T cell growth factor (TCGF) from concanavalin A and biological activity of the resulting TCGF. *J Immunol Methods* 1980, **33**, 337–350.
21. WRATHMELL AB, ALEXANDER P. Immunogenicity of a rat leukaemia of spontaneous origin (SAL). *Br J Cancer* 1976, **33**, 181–186.
22. WOODRUFF MFA, WHITEHEAD VL, SPEEDY G. Studies with a spontaneous mouse tumour. I. Growth in normal mice and response to *Corynebacterium parvum*. *Br J Cancer* 1978, **37**, 345–355.
23. TALMAGE DW, WOOLNOUGH JA, HEMMINGSEN H, LOPEZ L, LAFFERTY KJ. Activation of cytotoxic T cells by non-stimulating tumour cells and spleen cell factors. *Proc Natl Acad Sci USA* 1977, **74**, 4610–4614.
24. WAGNER H, HARDT C, HEEG K *et al.* T–T cell interactions during cytotoxic T lymphocyte (CTL) responses: T cell derived helper factor (Interleukin 2) as a probe to analyse CTL responsiveness and thymic maturation of CTL progenitors. In: MÖLLER G, ed. *Immunological Reviews.* Copenhagen, Munksgaard, 1980, Vol. 51, 215–255.
25. SANDERSON R, VESOLE D, JAKWAY J, TALMAGE D. A lymphocyte growth factor made by human lymphoid cell line. In: MÖLLER G, ed. *Immunological Reviews.* Copenhagen, Munksgaard, 1980, Vol. 51, 176–191.