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Autolymphocyte Therapy—I. *In Vivo* Tumour-specific Adoptive Cellular Therapy of Murine Melanoma and Carcinoma Using *Ex Vivo* Activated Memory T-Lymphocytes

J.E. Gold and M.E. Osband

Autolymphocyte therapy (ALT) is tumour-specific adoptive cellular therapy of neoplastic disease based upon non-specific *ex vivo* activation of autologous peripheral blood lymphocytes (PBL), using the supernatant derived from a previously prepared one-way mixed lymphocyte culture (MLC). To determine the requirement for tumour antigen during the activation process, splenocytes from C57BL/6J healthy syngeneic mice (HSM) and tumour-bearing mice (TBM) were activated *ex vivo* using a MLC-supernatant (MLCS). *Ex vivo* activation was performed both in the presence (HSM splenocytes) and absence (TBM splenocytes) of a 3M KCl syngeneic tumour-antigen (STA) extract prepared from Lewis lung (3LL) carcinoma, B16 melanoma, or normal lung. Immunophenotyping of splenocytes pre- and post-activation by MLCS plus STA or MLCS only revealed expansion of activated CD44$^+$ (memory) T-cells. *Ex vivo* tumour-specific cytotoxicity was demonstrated using MLCS-activated (TBM) or MLCS + STA-activated (HSM) splenocytes against 3LL or B16 target cells. CD44$^+$ T-cells (ALT-cells) were then infused into syngeneic 3LL and B16 TBM. Significant antitumour activity was detected in 3LL and B16 TBM receiving cells from normal mice that were activated with MLCS in the presence of 3LL or B16 STA, respectively, and in 3LL and B16 TBM receiving splenocytes from 3LL-TBM and B16-TBM, respectively, activated by MLCS alone. Infusions of 3LL-derived or B16-derived ALT-cells into HSM provided specific immunity on tumour challenge. No antitumour activity was seen in 3LL and B16 TBM receiving fresh TBM splenocytes, ALT-cells derived from HSM which were activated *ex vivo* using MLCS without antigen, normal lung tissue as antigen, or using MLCS-activated splenocytes without STA derived from reciprocal TBM. *Ex vivo* depletion of CD44$^+$ cells or Thy-1.2$^+$ T-cells abrogated all antitumour activity in TBM and in HSM challenged with tumour. Depletion of NK-1.1$^+$ natural killer (NK)-cells had no effect on antitumour efficacy. These data suggest that tumour-specific adoptive cellular therapy is possible using *ex vivo* activated HSM splenocytes with STA, *or* TBM splenocytes activated *ex vivo* without STA, and that these antitumour effects are dependent on CD44$^+$ memory T-cells.

Key words: autolymphocyte therapy, memory T-cell, tumour-specific immunotherapy
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INTRODUCTION

ADOPTIVE CELLULAR therapy of neoplastic disease is the use of antitumour immunoreactive cells to control or eradicate the neoplasm. Clinical trials of adoptive cellular therapy of cancer in humans have focused on peripheral blood lymphocytes (PBL) activated *ex vivo* by interleukin-2 (IL-2) [1]. Incubation of PBL with high concentrations of IL-2 results in the generation of lymphokine-activated killer (LAK) cells [2] which *ex vivo*, are able to lyse a variety of fresh tumour targets [3, 4]. LAK-cells combined with exogenous IL-2 *in vivo* have demonstrated some antitumour efficacy in patients with metastatic renal cell carcinoma (RCC) and melanoma [5–7]. LAK activity appears to be mediated by subsets of natural killer (NK) cells, which explains the apparent lack of antitumour specificity since killing of tumour targets by LAK-cells is non-specific and non-MHC-restricted [4, 8]. In addition, most of the clinical benefit seen with the combined use of LAK/IL-2 is attributed to IL-2, although there appears to be marginal clinical advantage of adding LAK cells to the IL-2.

In an effort to generate cells with more specific antitumour activity, some investigators have used tumour biopsy specimens and expanded the immune cells within the specimen. These

Correspondence to J.E. Gold at The Mount Sinai Medical Center, 920 Park Avenue, New York, NY 10028, U.S.A.
J.E. Gold is at the Divisions of Haematology and Neoplastic Diseases, Department of Medicine, The Mount Sinai Hospital and Mount Sinai School of Medicine of the City University of New York, NY; M.E. Osband is at the Division of Paediatric Haematology-Oncology, Department of Paediatrics, Boston City Hospital and Boston University School of Medicine, Boston, MA; Odyssey Therapeutics, Cambridge, MA; and Cellcor, Inc., Newton, MA, U.S.A.
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tumour-infiltrating lymphocytes (TIL) consist predominantly of a T-cell infiltrate [9, 10], and tend to be functionally impaired when isolated from the tumour biopsy specimens [9, 11]. However, when incubated *ex vivo* with IL-2, TIL proliferate and can then be infused into TBH as adoptively transferred, antitumour T-cells [12]. Initial experiments indicated that TIL/IL-2, in combination with cyclophosphamide (CY), were 50–100 fold more potent on a per cell basis than LAK-cells in mediating antitumour effects in murine models [13]. Trials of adoptively transferred TIL and IL-2 with and without CY have been performed in humans with metastatic tumours. The responses seen in melanoma have been generally superior to previous LAK/IL-2 data, but not other solid tumours [14, 15]. In addition, the putative tumour specificity of TIL was apparent *ex vivo* only in TIL isolated from melanoma specimens, while TIL from RCC and other tumour specimens had broader tumour lytic capabilities [12, 16, 17]. Shu and Chang have also attempted to improve upon antitumour specificity of adoptive cellular therapy using *in vitro* sensitised (IVS) T-cells as a variation of TIL therapy. In IVS therapy, tumour-draining lymph node (TDLN) T-cells are immunised *ex vivo* against autologous irradiated tumour cells in conjunction with IL-2 [18, 19]. Preliminary results of these adoptively transferred IVS T-cells in animal models with poorly immunogenic sarcomas have been encouraging in terms of both the tumour specificity and therapeutic efficacy of IVS [20]. Limiting features of both LAK and TIL therapies include the requirement for labour-intensive generation of cells, as well as the severe toxicities due to the dependence of these cells on constant exogenous IL-2 supplementation [21, 22]. The need for tumour tissue in TIL and IVS therapies also makes the potential for widespread use of these therapies somewhat problematic since tumour tissue is not available in many human cancers.

Autolymphocyte therapy (ALT) is adoptive cellular therapy of neoplastic disease based upon the infusion of autolymphocytes (ALT-cells) that are activated *ex vivo* from autologous PBL by the supernatant derived from a previously prepared one-way mixed lymphocyte culture (MLC). We demonstrated that activation of autologous PBL from human TBH results in the expansion of the memory T-cell subset [23–25]. ALT consists of monthly infusions of these memory T-cells combined with daily oral cimetidine (to reduce tumour-associated suppressor cell activity), and has been shown to significantly prolong survival, induce durable tumour responses, and is accompanied by only minimal toxicity in patients with metastatic RCC [24–26]. We previously reported ALT as successful antigen-specific adoptive cellular therapy using lymphocytes from tumour-bearing patients and mice which were immunised *ex vivo* using an autologous 3M KCl tumour extract as tumour antigen, and MLC supernatant (MLCS) as a stimulator [24]. However, like TIL and IVS therapy, many patients do not have tumour tissue available for use with ALT. Antigen-specific secondary responses can be recalled *ex vivo* by non-specific activation without the use of the specific antigen [27]. In a preliminary model, ALT was able to provide tumour-specific activity following non-specific *ex vivo* activation [28]. Therefore, in this report, we tested, in an animal model, whether adoptive cellular therapy using ALT-cells derived from lymphocytes activated *ex vivo* in the presence or absence of tumour tissue (antigen) with MLCS was tumour-specific, dependent on the presence of memory T-cells for antitumour effects, and whether adoptive transfer of these ALT-cells could impart tumour-specific immunity in healthy mice challenged with different tumours.

MATERIALS AND METHODS

Animals and tumour cell lines

C57BL/6J and DBA/2 mice, 6–8 weeks old, were obtained from Jackson Laboratories (Bar Harbor, Maine, U.S.A.), kept in a pathogen-free environment, and fed *ad libitum*. Murine B16 melanoma and 3LL carcinoma were obtained from the Division of Cancer Treatment Tumor Repository (Frederick, Maryland, U.S.A.).

Preparation of syngeneic tumour antigen (STA)

5×10^6 B16 melanoma or 3LL (Lewis Lung) carcinoma cells were implanted in the footpads of C57BL/6J mice and metastatic tumours were allowed to develop in the lungs. At various times following implantation, tumour-bearing mice were sacrificed, the lungs were aseptically harvested, and syngeneic tumour antigen was prepared by a 3M KCl extraction as previously described [29]. Normal lung tissue was obtained from healthy C57BL/6J mice and similarly treated to prepare normal lung antigen.

Preparation of MLCS

The source of the *ex vivo* stimulation for both the specific and non-specific portions of these experiments was the supernatant from a one-way MLC in which the responding cells were splenocytes from C57BL/6J healthy syngeneic mice, and the stimulator cells were splenocytes derived from healthy allogeneic DBA/2 mice. Spleens from these animals were removed aseptically. Single cell suspensions were prepared mechanically by pressing with the blunt end of a 10-ml plastic syringe plunger in HBSS (GIBCO, Grand Island, New York, U.S.A.). The cell suspension was filtered through a layer of no. 100 nylon mesh (Nitex; TETKO Inc., Elmsford, New York, U.S.A.), centrifuged, and erythrocytes were lysed by re-suspension of the cell pellet in ammonium chloride–potassium lysing buffer (8.29 g NH_4Cl, 1.0 g $KHCO_3$ and 0.0372 g EDTA/l, pH 7.4) for 2 min at room temperature. The cells were washed twice and suspended in complete medium (CM) which was composed of RPMI 1640 with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamicin, 0.5 mg/ml Fungizone (all from GIBCO), and 5×10^{-5} M 2-ME (Sigma). The stimulator cells were subjected to 3000 cGy of gamma irradiation and mixed at a 1:1 ratio with the responding cells. Following incubation for 48 h at 37°C in a moist-air incubator containing 5% CO_2, the cultures were harvested and centrifuged. The supernatant from the culture (MLCS) was sterilised by filtration, aliquoted, and then stored at −70°C until use.

Preparation of ALT-cells

Spleens from C57BL/6J healthy syngeneic mice and tumour-bearing mice were removed aseptically and splenocytes harvested as described above. Splenocytes from tumour-bearing mice were harvested 7–10 days after tumour implantation, and these were able to lyse B16 or 3LL cells *ex vivo* following stimulation with MLCS (see below). The splenocytes were depleted of suppressor T-cells prior to activation by exposing them to 50 cGy of γ-irradiation. The suppressor cell-depleted splenocytes were then resuspended at 10^6 cells/ml in RPMI 1640 containing 0.5% syngeneic and 9.5% fetal calf sera, 25% MLCS, in the presence or absence of 0.1 mg/ml of the 3M KCl extract of the various tissue antigens (3LL, B16 or normal lung) to be used. The ALT-cells were cultured for 7 days at 37°C in a moist-

air incubator containing 5% CO_2. This consistently resulted in a 10–20 fold expansion in cell numbers. At the end of 7 days, the ALT-cells were washed extensively and resuspended in HBSS at 1–2 × 10^7 cells/ml.

Lymphocyte immunophenotype

Fresh splenocytes and ALT-cells (approximately 5 × 10^5 cells) were stained with conjugated anti-mouse monoclonal antibody (MAb) washed in PBS and fixed in 2% paraformaldehyde/PBS. The MAbs used for staining were anti-Ly-4 ($CD4^+$ T-cells), anti-Ly-2 ($CD8^+$ T-cells), anti-Thy 1.2 (all peripheral T-cells), anti-Ly-43/IL-2Ra (IL-2 receptor, CD25), and anti-pgp-1 ($CD44^+$ memory T-cells) (PharMingen, San Diego, California, U.S.A.). The MAbs used in a direct immunofluorescence technique were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Control MAbs used were murine IgG_1-FITC and IgG_2-PE. The surface antigens were analysed by single and/or dual two colour flow cytometric study on a FACS 440 cytofluorometer (Becton-Dickinson, Mountain View, California, U.S.A.).

Depletion of $CD44^+$, Thy-1.2^+, or NK-1.1^+ Cells from the ALT-cell population

B16-derived or 3LL-derived ALT-cell populations were generated by antigen-specific or non-specific *ex vivo* activation as described above, incubated for 30 min at 4°C with either PE-labeled anti CD44 (pgp-1), anti Thy-1.2, or anti NK-1.1, washed, and sorted on a FACStar plus cell sorter (Becton-Dickenson) to obtain a highly purified cell population. The purity of the sorted antibody-sorted cell populations exceeded 98%. CD44-depleted, Thy-1.2-depleted, or NK-1.1-depleted ALT-cells were then used as effector cells in both the tumour-bearing mice and in the healthy syngeneic mice treated with adoptively transferred ALT-cells prior to tumour challenge. NK-1.1-depleted ALT-cells were also used in determination of tumour-specific *ex vivo* cytotoxicity.

Measurement of ex vivo *tumour-specific cytotoxicity of ALT-cells*

Splenocytes from tumour-bearing mice were harvested 7–10 days after tumour implantation. Detection of lytic activity was performed using a 4-h and 18-h chromium 51 (^{51}Cr) release assay. Various concentrations of MLCS-activated (splenocytes from tumour-bearing mice) or MLCS + STA-activated (splenocytes from healthy syngeneic mice) 3LL-derived or B16-derived ALT-cells, NK-1.1-depleted 3LL-derived or B16-derived ALT-cells, and fresh splenocytes from tumour-bearing mice were added to U-bottom 96-well plates to achieve effector target ratios (E : T) of 1 : 1, 20 : 1 and 40 : 1. Prior to cytotoxicity assays, B16 or 3LL target cells were labelled with 250 μCi of $Na^{51}CrO_4$ (New England Nuclear, Boston, Massachusetts, U.S.A.) washed three times, and then suspended in CM at a concentration of 10^5 viable cells/ml. Thereafter, target cells (10^4/100 μl) were added to 96-well plates. The plates were incubated for 4 or 18 h at 37°C in 5% CO_2, and the culture supernatants were harvested with the Skatron harvesting system; the amount of released ^{51}Cr was determined in a Beckman Gamma 4000 counter. Maximum ^{51}Cr release was produced by incubation of the targets with 0.1 N HCl. Spontaneous release was measured in target cells to which medium alone was added. The percent specific lysis was calculated as:

$$\text{percent specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100\% .$$

All determinations were made in triplicate and the data reported as the mean.

Adoptive transfer studies of ALT-cells in tumour-bearing mice

An illustration of the ALT models in TBM is shown in Figure 1. B16 melanoma or 3LL carcinoma cells were implanted in the footpads of C57BL/6J mice on day 1. Metastatic tumour was allowed to develop in the lungs of these animals with the appearance of multiple, macroscopic, grossly visible lesions by days 5–10. ALT was administered as 1–2 × 10^7 ALT-cells in 1.0 ml of HBSS, infused intravenously into tumour-bearing mice on day 5, in order to simulate adoptive cellular therapy of advanced metastatic disease in humans. The ALT-cells were prepared in two ways. In one set of experiments, ALT-cells were generated from syngeneic C57BL/6J splenocytes derived from normal hosts that underwent primary *ex vivo* immunisation with MLCS in the presence of a 3M KCl syngeneic tissue extract derived from either B16 melanoma, 3LL carcinoma, or normal syngeneic lung tissue (control group). In the second set of experiments, ALT-cells were generated from splenocytes of C57BL/6J mice in which B16 melanoma or 3LL carcinoma cells had been implanted. In these experiments, no 3M KCl syngeneic tissue extract was used in the *ex vivo* activating cultures. Splenocytes from C57BL/6J healthy syngeneic mice were used as controls for both sets of experiments. In addition, tumour-bearing mice in both sets of experiments also received 3LL-derived or B16-derived ALT-cells that underwent subsequent CD44, Thy-1.2, or NK-1.1 depletion. Three measurements were made in tumour-bearing mice to evaluate the therapeutic efficacy of the various cell populations; sequential size of the primary tumour as measured by footpad thickness, number of lung metastases on day 21, and median survival. Each group receiving antigen-derived or non-antigen-derived ALT with different ALT-cells consisted of 10–15 animals. All mice were used for evaluation of the primary tumour by serial measurement of footpad thickness over the first 15 days. To count pulmonary metastases, five mice in each group were sacrificed on day 21 and 15% India ink was instilled into the trachea of 3LL-tumour-bearing mice. Lungs were harvested and those containing 3LL were bleached with Feteke's solution. 3LL tumours appeared as discrete white nodules against a black background and were readily visible. B16 tumours appeared as black nodules against lung tissue. If metastases were greater than 250, those were counted as 250. The remaining mice in each group were used for determination of survival.

Adoptive transfer studies of ALT-cells in healthy syngeneic mice

Healthy syngeneic mice were given 50 mg/kg intraperitoneal (i.p.) CY (Mead Johnson Co., Evansville, Indiana, U.S.A.) and within 48–72 h, received B16-derived or 3LL-derived ALT-cells via tail vein injection. ALT-cells were prepared as above using splenocytes from either B16- or 3LL-tumour-bearing mice and activated *ex vivo* with MLCS alone, or with splenocytes derived from normal hosts that underwent primary *ex vivo* immunisation with MLC-supernatant in the presence of a 3M KCl syngeneic tissue extract derived from either B16 melanoma or 3LL carcinoma. Healthy syngeneic mice also received 3LL-derived or B16-derived ALT-cells that had been depleted of

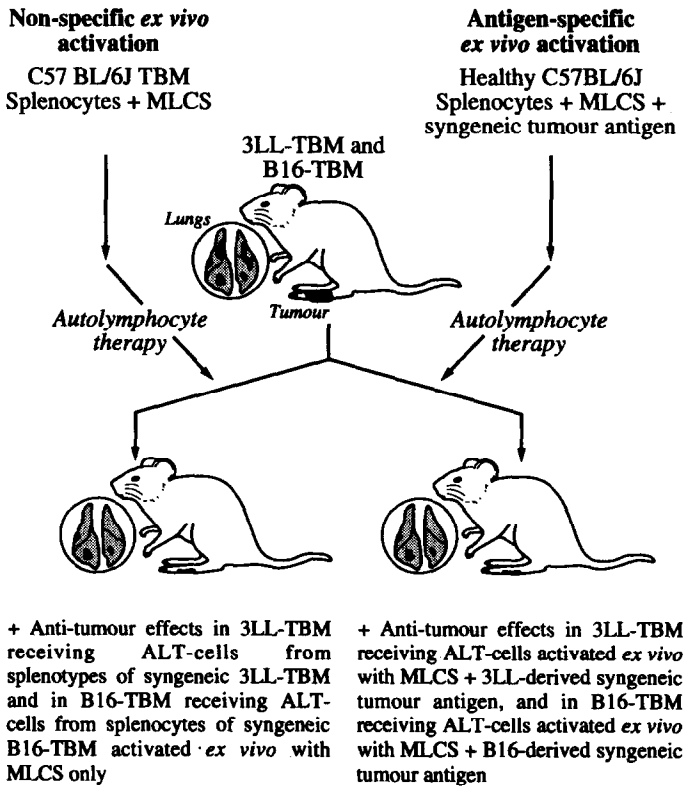

Figure 1. ALT of tumour-bearing mice with 3LL carcinoma and B16 melanoma. As shown, 3LL- and B16-tumour-bearing mice received ALT derived from either antigen-specific activation of syngeneic splenocytes from healthy C57BL/6J mice with syngeneic 3LL or B16 tumour antigen and MLC-supernatant (MLCS), or non-antigen-specific activation with splenocytes from syngeneic 3LL- or B16-tumour-bearing mice and MLCS only. As illustrated here and described in the text, 3LL- or B16-tumour-bearing mice demonstrated antitumour effects when receiving *ex vivo* activated splenocytes from 3LL- or B16-tumour-bearing mice, respectively, using MLCS only or splenocytes from healthy C57BL/6J mice activated *ex vivo* using MLCS and 3LL or B16 tumour antigen extract, respectively.

$CD44^+$ cells, Thy-1.2^+ T-cells, or NK-1.1^+ NK-cells. Each group receiving some form of ALT-cells consisted of eight to ten mice. Animals were then challenged with 10^5 B16 or 3LL tumour cells intravenously (i.v.) or by footpad implantation in order to test systemic and local immunity, respectively. Some mice were killed 3 weeks after i.v.; tumour injection and pulmonary metastases were evaluated as above. Mice that had received footpad tumour challenges were evaluated by serial measurement of footpad thickness. The remaining mice in each group were observed for 100 days for evidence of tumour recurrence.

Statistical analysis

The Student *t*-test and generalised Wilcoxon test were used to determine differences in the immunophenotypes of splenocytes and ALT-cells, the size of the primary footpad tumours, number of lung metastases, and difference in survival time of each group. A P value below 0.05 was considered significant.

RESULTS

Immunophenotype of fresh splenocytes and ALT-cells

The mean phenotypes of the ALT-cells from 20 tumour-bearing mice following activation by MLCS alone and splenocytes from healthy syngeneic mice immunised *ex vivo* with MLCS and STA are shown in Table 1. No significant difference in ALT-cell phenotype was noted between both groups. These data demonstrate that *ex vivo* activation of splenocytes from tumour-bearing mice by MLCS without STA or splenocytes from healthy syngeneic mice immunised *ex vivo* with MLCS and STA results in the generation of activated T-cells with a memory phenotype.

Ex vivo *tumour-specific cytotoxicity of ALT-cells*

ALT-cells were generated using splenocytes from normal syngeneic mice activated *ex vivo* using the MLCS, in the presence or absence of STA extract derived from B16 tumour, 3LL tumour, or normal lung tissue. ALT-cells were also generated without STA from the splenocytes of tumour-bearing mice with either B16 melanoma or 3LL carcinoma by activation with MLCS only. NK-1.1-depleted ALT-cells were derived from

Table 1. Mean percentage of positive-staining splenocytes and ALT-cells†

Antigen	Fresh splenocytes	ALT-cells
Thy-1.2^+	61	93
$CD4^+$	35	55
$CD8^+$	18	31
Thy-1.2^+ $CD44^+$	23	62*
$CD4^+$ $CD44^+$	11	33*
$CD8^+$ $CD44^+$	8	20*
$CD25^+$ Thy-1.2^+	2	17*

* $P < 0.005$ in comparison with unstimulated cells.

† ALT-cells derived from splenocytes of 3LL and B16 TBM activated *ex vivo* by MLCS or from splenocytes of healthy syngeneic mice immunised *ex vivo* using MLCS and STA.

3LL- or B16-tumour-bearing mice activated *ex vivo* with MLCS and then depleted of NK-cells. As seen in Table 2, 3LL-derived ALT-cells using MLCS-activated splenocytes from 3LL-tumour-bearing mice or MLCS + 3LL-STA-activated splenocytes from healthy syngeneic mice demonstrated significant cytotoxicity over fresh splenocytes from 3LL-tumour-bearing mice against 3LL tumour targets but not B16 tumour targets. Similar but reciprocal findings were seen with B16-derived ALT-cells with significant cytotoxicity observed against B16 but not 3LL tumour targets. NK-1.1-depleted ALT-cells also demonstrated the same *ex vivo* tumour-specificity, although there was some impairment noted of the 4- but not 18-h lysis. This demonstrates that *ex vivo* tumour-specific cytotoxicity is possible using ALT-cells derived from healthy syngeneic mice or tumour-bearing mice in the presence or absence of STA, respectively, and that this effect is mediated principally by *ex vivo* activated T-cells.

Activity of ALT-cells generated in the presence of tumour antigen in tumour-bearing mice

ALT-cells were generated using splenocytes from normal syngeneic mice activated *ex vivo* using the MLCS, in the presence or absence of STA extract derived from B16 tumour, 3LL tumour, or normal lung tissue. Tumour-bearing mice also received antigen-derived ALT-cells that had been depleted of $CD44^+$ cells, Thy-1.2^+ T-cells, or NK-1.1^+ NK-cells. Each group receiving antigen-derived ALT-cells consisted of 10–15 mice. As seen in Tables 3 and 4, 3LL-tumour-bearing mice that received ALT with cells derived from splenocytes of healthy syngeneic mice and activated *ex vivo* with STA derived from 3LL-tumour-bearing mice, had significantly greater antitumour activity (decreased primary tumour size, decreased mean number of lung metastases at day 21, and prolonged median survival) when compared with the mice who received the other antigen-specific ALT preparations (B16 melanoma or normal lung

Table 2. Ex vivo *tumour-specific cytotoxicity of ALT-cells*

Tumour target	Source of ALT-cells	Effector/target ratio (E:T)	4-h Mean % lysis	18-h Mean % lysis
3LL	Fresh 3LL-TBM splenocytes	1:1	0.7	0.9
		20:1	1.1	1.9
		40:1	2.1	3.6
3LL	**MLCS + STA-activated 3LL-HSM splenocytes**	**1:1**	**4.6***	**10.5***
		20:1	**9.2***	**21.2***
		40:1	**16.5***	**49.7***
3LL	MLCS + STA-activated B16-HSM splenocytes	1:1	1.0	0.9
		20:1	1.7	2.1
		40:1	4.1	3.4
3LL	MLCS-activated 3LL-TBM splenocytes	1:1	5.2*	**11.3***
		20:1	11.8*	**18.8***
		40:1	25.4*	**60.7***
3LL	MLCS-activated B16-TBM splenocytes	1:1	0.5	1.0
		20.1	0.9	1.8
		40:1	2.2	2.7
3LL	MLCS-activated 3LL-TBM NK-1.1-depleted	1:1	2.8	**9.2***
		20:1	5.5	**22.5***
		40:1	9.2	**40.0***
B16	Fresh B16-TBM splenocytes	1:1	1.1	1.4
		20:1	1.4	2.2
		40:1	3.3	3.9
B16	**MLCS + STA-activated B16-HSM splenocytes**	**1:1**	**5.8***	**9.8***
		20:1	**14.4***	**21.7***
		40:1	**22.1***	**39.2***
B16	MLCS + STA-activated 3LL-HSM splenocytes	1:1	0.4	0.7
		20:1	1.5	1.4
		40:1	3.1	3.9
B16	**MLCS-activated B16-TBM splenocytes**	**1:1**	**6.1***	**12.7***
		20:1	**13.7***	**20.4***
		40:1	**30.8***	**54.1***
B16	MLCS-activated B16-TBM NK-1.1-depleted	1:1	3.1	**8.9***
		20:1	6.7	**19.6***
		40:1	11.4	**45.5***

* $P < 0.001$ in comparison with control mice.

MLCS, mixed lymphocyte culture supernatant; STA, syngeneic tumour antigen; HSM, healthy syngeneic mice; TBM, tumour-bearing mice; NK-1.1-depleted, ALT-cells derived from TBM splenocytes and then NK-1.1-depleted.

Table 3. Adoptive cellular therapy using antigen-specific activation of ALT-cells in 3LL and B16 tumour-bearing mice

TBM	Source of splenocytes	Source of antigen	Mean number lung metastases at day 21 (S.E.M.)	Median survival (days)
3LL-TBM	C57BL/6J HSM	None	250 (0)	27
3LL-TBM	**C57BL/6J HSM**	**3LL**	**92 (10)***	**54***
3LL-TBM	C57BL/6J HSM	B16	228 (18)	30
3LL-TBM	C57BL/6J HSM	Normal lung	250 (0)	31
3LL-TBM	C57BL/6J HSM	3LL/ CD44-depleted	243 (7)	30
3LL-TBM	**C57BL/6J HSM**	**3LL/ NK-1.1-depleted**	**98 (16)***	**50***
3LL-TBM	C57BL/6J HSM	3LL/ Thy-1.2-depleted	250 (0)	27
B16-TBM	C57BL/6J HSM	None	250 (0)	29
B16-TBM	C57BL/6J HSM	3LL	202 (15)	33
B16-TBM	**C57BL/6J HSM**	**B16**	**76 (11)***	**59***
B16-TBM	C57BL/6J HSM	Normal lung	231 (18)	31
B16-TBM	C57BL/6J HSM	B16/ CD44-depleted	250 (0)	28
B16-TBM	**C57BL/6J HSM**	**B16/ NK-1.1-depleted**	**105 (10)***	**51***
B16-TBM	C57BL/6J HSM	B16/ Thy-1.2-depleted	250 (0)	26

* $P < 0.001$ in comparison with control mice.
HSM, healthy syngeneic mice; TBM, tumour-bearing mice.

tissue), the control group, CD44-depleted, or Thy-1.2-depleted ALT-cells. 3LL-Tumour-bearing mice that received 3LL-derived NK-1.1-depleted ALT-cells also demonstrated significant antitumour effects (Tables 3 and 4). Similar but reciprocal results were obtained with B16-tumour-bearing mice that received ALT with cells derived from splenocytes of healthy syngeneic mice and activated *ex vivo* with STA derived from B16. This demonstrates that antigen-specific adoptive cellular therapy is possible using antigen-specific *ex vivo* activated splenocytes and that the antitumour effects are dependent on $CD44^+$ (memory) T-cells.

Activity of ALT-cells generated without tumour antigen in tumour-bearing mice

ALT-cells were generated without STA from the splenocytes of healthy syngeneic mice, 3LL- or B16-tumour-bearing mice by activation with MLCS only. Tumour-bearing mice also received MLCS-derived CD44-depleted ALT-cells. Each group of animals receiving MLCS-activated ALT-cells consisted of 10–15 mice. As seen in Tables 4 and 5, 3LL-tumour-bearing mice that received ALT using cells prepared from the splenocytes of other 3LL-tumour-bearing mice demonstrated significantly greater antitumour activity (decreased primary tumour size, decreased mean number of lung metastases on day 21 and prolonged median survival) than those that received splenocytes derived from B16-tumour-bearing mice, healthy syngeneic mice or CD44-depleted ALT-cells. Similar but reciprocal results were demonstrated with B16-tumour-bearing mice that received ALT-cells prepared from the splenocytes of other B16-tumour-bearing mice (Tables 4 and 5). B16- and 3LL-tumour-bearing mice that received B16-derived or 3LL-derived ALT-cells, respectively, that were NK-1.1-depleted also demonstrated significant antitumour effects (Tables 4 and 5). This demonstrates that tumour-specific adoptive cellular therapy is possible and dependent on $CD44^+$ (memory) T-cells generated by non-specific *ex vivo* activation of splenocytes from tumour-bearing hosts.

Table 4. Adoptive cellular therapy and primary tumour burden in 3LL and B16 tumour-bearing mice

TBM	Source of ALT-cells	Day 5 Mean FT	Day 8 Mean FT	Day 10 Mean FT	Day 12 Mean FT	Day 14 Mean FT
3LL-TBM	None	3.0	3.2	5.1	5.3	6.2
3LL-TBM	**3LL-STA**	**2.5**	**2.7**	**2.4***	**2.3***	**2.3***
3LL-TBM	B16-STA	3.2	3.7	4.7	5.5	6.4
3LL-TBM	**3LL-TBM Splenocytes**	**3.0**	**3.0**	**2.5***	**2.5***	**2.4***
3LL-TBM	B16-TBM Splenocytes	3.1	4.2	5.1	5.9	6.4
3LL-TBM	3LL-derived †CD44-dep	2.6	4.1	4.7	5.9	6.6
3LL-TBM	3LL-derived ‡CD44-dep	2.9	3.6	4.9	6.0	6.3
3LL-TBM	3LL-TBM Thy 1.2-dep	3.0	4.0	5.0	5.8	6.0
3LL-TBM	**3LL-TBM NK-1.1-dep**	**2.7**	**2.7**	**2.4***	**2.4***	**2.4***
B16-TBM	None	2.9	3.8	5.0	5.6	5.9
B16-TBM	**B16-STA**	**3.1**	**3.3**	**2.7***	**2.7***	**2.6***
B16-TBM	3LL-STA	3.0	4.1	4.9	5.8	6.0
B16-TBM	**B16-TBM Splenocytes**	**3.0**	**3.1**	**2.7***	**2.6***	**2.5***
B16-TBM	3LL-TBM Splenocytes	2.8	4.2	4.7	5.7	6.2
B16-TBM	B16-derived †CD44-dep	2.5	4.3	5.0	5.5	6.1
B16-TBM	B16-derived ‡CD44-dep	2.9	4.3	5.4	6.0	6.4
B16-TBM	B16-TBM Thy-1.2-dep	3.1	3.9	5.0	6.2	6.5
B16-TBM	**B16-TBM NK-1.1-dep**	**2.5**	**2.6**	**2.4***	**2.2***	**2.2***

* $P < 0.01$ in comparison with control mice.

FT, footpad thickness (mm); TBM, tumour-bearing mice; HSM, healthy syngeneic mice; 3LL-STA, ALT-cells derived from HSM splenocytes activated with MLCS and STA derived from 3LL tumours; B16-STA, ALT-cells derived from HSM splenocytes activated with MLCS and STA derived from B16 tumours; † CD44-dep, ALT-cells derived from splenocytes of HSM immunised *ex vivo* with MLCS and STA and then CD44-depleted; ‡CD44-dep, ALT-cells derived from TBM splenocytes immunised *ex vivo* with MLCS only and then CD44-depleted; Thy-1.2-dep, ALT-cells derived from TBM splenocytes and then Thy-1.2-depleted; NK-1.1-dep, ALT-cells derived from TBM splenocytes and then NK-1.1-depleted.

Activity of ALT-cells in healthy syngeneic mice

Healthy syngeneic mice given CY followed by ALT-cells were challenged 2–4 weeks later with tumour cells i.v. or by footpad implantation of tumour cells. Healthy syngeneic mice given 3LL-derived ALT-cells using splenocytes from 3LL-tumour-bearing mice activated *ex vivo* with MLCS alone, or with splenocytes derived from normal hosts that underwent primary *ex vivo* immunisation with MLC-supernatant in the presence of an STA derived from 3LL carcinoma, were protected against both systemic (i.v.) and local (footpad) 3LL tumour challenge but not B16 tumour challenge (Tables 6 and 7). Similar but reciprocal results with B16-derived ALT-cells were observed (Tables 6 and 7). Adoptive transfer of fresh splenocytes from 3LL- or B16-tumour-bearing mice, CD44-depleted, or Thy-1.2-depleted ALT-cells did not protect against tumour challenge, but NK-1.1-depleted ALT-cells were able to protect against local and systemic challenge from their respective tumour cells (Tables 6 and 7). All mice given 3LL- or B16-derived ALT-cells remained free of 3LL or B16, respectively, at day 100 after tumour challenge. These data demonstrate that tumour-specific

Table 5. Adoptive cellular therapy using non-specific activation of ALT-cells in 3LL- and B16-tumour-bearing mice

TBM	Source of splenocytes	Source of antigen	Mean number of lung metastases at day 21 (S.E.M.)	Median survival (days)
3LL-TBM	**3LL-TBM**	**None**	**85 (10)***	**61***
3LL-TBM	B16-TBM	None	195	(28) 29
3LL-TBM	C57BL/6J HSM	None	250 (0)	33
3LL-TBM	3LL-TBM CD44-depleted	None	239 (9)	27
3LL-TBM	**3LL-TBM NK-1.1-depleted**	**None**	**101 (17)***	**53***
3LL-TBM	3LL-TBM Thy-1.2-depleted	None	250 (0)	24
B16-TBM	3LL-TBM	None	250 (0)	30
B16-TBM	**B16-TBM**	**None**	**92 (15)***	**58***
B16-TBM	C57BL/6J HSM	None	218 (20)	35
B16-TBM	B16-TBM CD44-depleted	None	245 (5)	28
B16-TBM	**B16-TBM NK-1.1-depleted**	**None**	**92 (14)***	**52***
B16-TBM	B16-TBM Thy-1.2-depleted	None	250 (0)	26

* $P < 0.001$ in comparison with control mice.
HSM, healthy syngeneic mice; TBM, tumour-bearing mice.

immunity can be adoptively transferred by and is dependent on ALT-cells consisting of $CD44^+$ memory T-cells.

DISCUSSION

Adoptive cellular therapy has become a recognised modality of cancer treatment. Various lymphocyte populations have been used for this purpose, including PBL activated by interleukin-2 (LAK cells), those expanded from tumour tissue (TIL), TDLN T-cells (IVS T-cells), and memory T-cells activated in a non-specific manner (ALT-cells). We previously reported that out-patient treatment of patients with ALT significantly prolongs survival, induces durable tumour responses, and is accompanied by minor toxicity [24, 26].

The underlying assumption for all tumour immunotherapy is that malignant tissue is antigenic and capable of inducing a clinically effective immune response. Nevertheless, few of the methods used for inducing immunoreactive cells for adoptive cellular therapy use tumour tissue or antigen, and previous reports have demonstrated methods to generate antitumour effectors *ex vivo* in the absence of tumour tissue. In murine models, another method of generating antitumour effectors was allo-sensitisation of lymphocytes from tumour-bearing mice in a one-way MLC [30–32]. Zarling and co-workers demonstrated that tumour-bearing mice were capable of generating antitumour effectors when stimulated with multiple allogeneic donor lymphocytes [33]. As a further extension of these observations, Hurrell and Zarling noted that the pool-priming MLCS was also capable of generating antitumour effects from syngeneic PBL [34]. This is analogous to the situation in this report, where the MLCS was capable of generating antitumour activity in both experiments. Tuttle and associates were able to generate tumour-specific effectors using non-antigen specific activation of TDLN T-cells activated *ex vivo* by bryostatin 1 and ionomycin [35].

This report describes *ex vivo* tumour-specific cytotoxicity and *in vivo* tumour-specific efficacy of adoptive cellular therapy using *ex vivo* activated splenocytes from tumour-bearing mice with advanced metastatic disease. *Ex vivo* incubation of 3LL or B16 tumour cell targets with 3LL- or B16-derived ALT-cells, respectively, demonstrated tumour-specific lysis of target cells by ALT-cells generated from splenocytes from tumour-bearing mice using MLCS only, or healthy syngeneic mice splenocytes using MLCS + STA (Table 2). In addition, NK-cell-depleted ALT-cells also followed this same pattern of *ex vivo* tumour-specific-lysis, although with somewhat less lysis noted at the 4-h but not the 18-h assay. We have also shown that *ex vivo* incubation of tumour targets with ALT-cells derived from supernatant-activated splenocytes from tumour-bearing mice results in the generation of high levels of IFN-γ only with 3LL tumours incubated with 3LL-derived ALT-cells or B16 tumour targets incubated with B16-derived ALT-cells [43]. This implies tumour-specific recognition and lysis as demonstrated by us [40] and other investigators [41, 42], which is dependent on *ex vivo* activated T-cells.

In vivo, mice with advanced metastatic (5 day) 3LL carcinoma or B16 melanoma demonstrated benefit if the ALT-cells they received were either: (1) obtained from syngeneic 3LL- or B16-

Table 6. Adoptive transfer of ALT-cells provides systemic tumour-specific immunity in healthy syngeneic mice

ALT-cells*	Mean number of lung metastases (S.E.M.) at day 21	
	3LL tumour challenge	B16 tumour challenge
3LL-derived ALT-cells†	**0 (0)**	**250 (0)**
B16-derived ALT-cells†	**250 (0)**	**0 (0)**
CD44-depleted ALT-cells (3LL)†	242 (9)	ND
CD44-depleted ALT-cells (B16)†	ND	245 (5)
Thy-1.2-depleted ALT-cells (3LL)†	250 (0)	ND
Thy-1.2-depleted ALT-cells (B16)†	ND	247 (4)
NK-1.1-depleted ALT-cells (3LL)†	**0 (0)**	**250 (0)**
NK-1.1-depleted ALT-cells (B16)†	**238 (10)**	**0 (0)**
Fresh 3LL-TBM splenocytes	224 (21)	250 (0)
Fresh B16-TBM splenocytes	240 (8)	231 (15)
3LL-derived ALT-cells‡	**0 (0)**	**220 (22)**
B16-derived ALT-cells‡	**250 (0)**	**0 (0)**
CD44-depleted ALT-cells (3LL)‡	250 (0)	ND
CD44-depleted ALT-cells (B16)‡	ND	238 (10)
Thy-1.2-depleted ALT-cells (3LL)‡	250 (0)	ND
Thy-1.2-depleted ALT-cells (B16)‡	ND	234 (12)
NK-1.1-depleted ALT-cells (3LL)‡	**0 (0)**	**225 (18)**
NK-1.1-depleted ALT-cells (B16)‡	**250 (0)**	**0 (0)**

* HSM received CY 50 mg/kg 48–72 h before ALT-cell infusion.
† ALT-cells prepared using HSM splenocytes, MLCS, and STA.
‡ ALT-cells prepared using TBM splenocytes and MLCS.
ND = not done.
TBM, tumour-bearing mice; HSM, healthy syngeneic mice.

tumour-bearing mice, respectively, and activated with MLCS alone; or (2) obtained from normal mice, and activated by a combination of MLCS and tissue extract from 3LL or B16 tumour, respectively (Figure 1, Tables 3–5). No antitumour activity was seen with ALT-cells that were: (1) obtained from reciprocal tumour-bearing mice and activated with MLCS alone; (2) obtained from normal mice, and activated by a combination of MLCS and extract derived from either reciprocal tumours, or normal lung tissue (Tables 3–5); or (3) depleted of $CD44^+$ cells or Thy-1.2^+ T-cells. Depletion of NK-1.1^+ NK-cells had no discernible effect on the antitumour activity of ALT-cells. These data demonstrate that the observed antitumour activity in tumour-bearing mice is dependent on $CD44^+$ (memory) T-cells.

Adoptive transfer of 3LL- or B16-derived ALT-cells was also capable of providing both systemic and local tumour-specific immunity in healthy syngeneic mice. These demonstrated local and systemic tumour-specific immunity if the ALT-cells they received were either: (1) obtained from syngeneic 3LL or B16 TBM, respectively, and activated with MLCS alone, or, (2) obtained from normal mice, and activated by a combination of MLCS and tissue extract from 3LL or B16 tumour, respectively. No specific antitumour immunity was seen with fresh splenocytes from tumour-bearing mice or ALT-cells that were: (1) obtained from reciprocal tumour-bearing mice and activated with MLCS alone; (2) obtained from normal mice, and activated by a combination of MLCS and extract derived from a reciprocal tumour; or (3) depleted of $CD44^+$ cells or Thy-1.2^+ T-cells. Administration of NK-1.1-depleted ALT-cells to healthy syngeneic mice was also effective in imparting local and systemic tumour-specific immunity. Therefore, adoptive transfer of tumour-specific immunity to healthy syngeneic mice is dependent on $CD44^+$ T-cells.

These data suggest that lymphocytes can be activated *ex vivo* and can possess tumour-specific activity that is dependent on $CD44^+$ T-cells even in the absence of tumour antigen. Alternatively, lymphocytes can be activated *ex vivo* in the presence of antigen, thereby conferring tumour-specific activity that is also dependent on $CD44^+$ T-cells. The former scenario is the clinically relevant setting, and is the model for patients with metastatic RCC treated with ALT. The clinical results from 36 patients with metastatic RCC treated with ALT using cells activated with MLCS have been previously reported [24, 39]. The activation cultures for these patients also contained a 3M KCl extract of autologous tumour tissue.

The data reported here suggest that the cells activated in this approach are $CD44^+$ (memory) T-cells, consistent with other reports of memory T-cell activation *ex vivo* without the requirement for antigen to be present in the culture system [23]. This prompted a multi-site, randomised clinical trial of ALT in patients with metastatic RCC [24, 25]. Again it was found that ALT prolonged survival, induced durable tumour responses in some patients, and was accompanied by only minimal toxicity. In addition to MLCS, a combination of anti-CD3 MAb and a previously prepared mixture of autologous cytokines, similar to the MLCS, termed T3CS, was used. This was based on previous studies that demonstrated T3CS is capable of expanding the memory T-cell subset in human cancer patients [23, 40], and that in tumour-bearing mice, expansion with T3CS also resulted in long-term tumour-specific immunity mediated by donor memory T-cells in healthy congenic mice [36]. Some patients in the multi-site randomised ALT study had their cells activated in the absence of autologous tumour antigen. No difference in clinical outcome was noted with or without the use of tumour antigen or in those whose PBL were activated *ex vivo* with

Table 7. Adoptive transfer of ALT-cells provides local tumour-specific immunity in healthy syngeneic mice

Antitumour effectors	Tumour challenge	Day 5 Mean FT	Day 8 Mean FT	Day 10 Mean FT	Day 12 Mean FT	Day 15 Mean FT
3LL-derived ALT-cells†	**3LL/B16**	**2.4/2.6**	**2.2/3.5**	**2.0/4.7**	**2.0*/5.6**	**2.0*/6.1**
B15-derived ALT-cells†	**3LL/B16**	**2.5/2.4**	**4.0/2.2**	**5.1/2.2**	**5.3/2.1***	**6.0/2.0***
3LL/CD44d ALT-cells†	3LL	2.7	3.8	4.5	5.5	5.8
B16/CD44d ALT-cells†	B16	2.5	4.0	4.8	5.1	5.9
B16/Thy1.2d ALT-cells†	B16	2.6	3.7	4.6	5.8	6.1
3LL/Thy1.2d ALT-cells†	3LL	2.8	4.0	4.8	5.7	6.4
3LL/NK1.1d ALT-cells†	**3LL/B16**	**2.5/2.6**	**2.3/4.0**	**2.3/5.0**	**2.2*/5.9**	**2.2*/6.3**
B16/NK1.1d ALT-cells†	**3LL/B16**	**2.6/2.6**	**3.5/2.6**	**4.9/2.5**	**5.7/2.3***	**6.0/2.2***
3LL-TBM splenocytes	3LL	2.6	3.9	4.4	4.9	5.5
B16-TBM splenocytes	B16	2.8	3.7	4.6	5.8	6.5
3LL-derived ALT-cells‡	**3LL/B16**	**2.5/2.5**	**2.4/4.1**	**2.2/4.9**	**2.2*/5.2**	**2.1*/6.0**
B16-derived ALT-cells‡	**3LL/B16**	**2.7/2.3**	**3.8/2.3**	**4.2/2.3**	**5.2/2.2***	**5.7/2.2***
3LL/CD44d ALT-cells‡	3LL	2.6	3.3	3.9	4.7	5.5
B16/CD44d ALT-cells‡	B16	2.5	4.0	5.0	5.7	5.9
B16/Thy1.2d ALT-cells‡	B16	2.8	4.3	4.9	5.7	6.3
3LL/Thy1.2d ALT-cells‡	3LL	2.6	4.0	5.0	5.9	6.4
3LL/NK1.1d ALT-cells‡	**3LL/B16**	**2.4/2.7**	**2.4/4.3**	**2.4/5.1**	**2.2*/6.0**	**2.2*/6.3**
B16/NK1.1d ALT-cells‡	**3LL/B16**	**2.9/2.4**	**3.9/2.5**	**5.1/2.5**	**5.8/2.4***	**6.1/2.3***

* $P < 0.01$ in comparison with control mice.
CD44d, CD44-depleted ALT-cells; Thy-1.2d, Thy-1.2-depleted ALT-Cells; NK-1.1-d, NK-depleted; TBM, tumour-bearing mice.
† ALT-cells prepared using HSM splenocytes, MLCS and STA.
‡ ALT-cells prepared using TBM splenocytes and MLCS.

MLCS or T3CS. Analysis of the cytokines in these supernatants revealed high levels of IL-1, granulocyte macrophage-colony stimulating factor (GM-CSF), interferon (IFN-γ), IL-6, IL-8, and tumour-necrosis factor (TNF), but low levels of IL-2 [37, and in preparation]. This is consistent with the non-IL-2-dependent mechanism of ALT.

We have demonstrated here that MLCS expands the $CD44^+$ T-cell subset in murine tumour-bearing hosts. Therefore, non-specific activation of lymphocytes from tumour-bearing hosts using allogenic lymphocytes, MLCS, or T3CS is capable of generating specific antitumour effectors. The selective memory T-cell subset expansion in tumour-bearing mice and human cancer patients suggests that these memory T-cells may be provoking a specific antitumour recall response in these patients via non-specific stimulation. In addition, studies performed on *ex vivo* lysis of human autologous tumour targets by ALT-cells

have shown that depletion of the memory T-cell population markedly impairs tumour lysis [40]. Whether these memory T-cells are directly cytotoxic to tumour cells or exert their effects indirectly via induction of cytotoxicity is not clear. Dye and North have shown that memory T-cells are indeed capable of direct cytotoxicity, but on a delayed rather than immediate basis [38]. Further studies using *ex vivo* activation of memory T-cells are ongoing in order to optimise ALT and the generation of specific antitumour effectors using non-specific *ex vivo* activation.

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Prognostic Significance of pS2 mRNA in Breast Cancer

S.J. Wysocki, B.J. Iacopetta and D.M. Ingram

The oestrogen-inducible pS2 protein has previously been associated with good prognosis for breast cancer patients. In 1987–1988 a series of 145 primary breast cancers were examined for pS2 mRNA using northern blots. On recent examination of mortality data, we were unable to find any association between tumour pS2 positivity and patient survival. One patient in 6 died within 5 years of surgery, regardless of pS2 status. In the oestrogen receptor positive/progesterone receptor positive tumour subgroup of patients, we found no evidence of increased survival for pS2-positive tumours. These results do not support use of pS2 as an indicator of increased survival in an average breast cancer patient population.
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INTRODUCTION

THE IDENTIFICATION of a gene coding for an oestrogen-inducible protein termed pS2 in breast cancers led to anticipation that expression of the pS2 gene would help in the identification of patients likely to respond to hormone therapy [1]. Preliminary investigations supporting this experimental approach have been published [2–4], although more recent data are less supportive of pS2 as a predictor of endocrine response [5]. A large retrospective study demonstrated that the measurement of pS2 protein in breast cancers provided independent prognostic information about patient outcome [6]. In accordance with this finding, high pS2 gene expression was shown to be an indicator of good prognosis for breast cancer patients [7]. However, the value of pS2 estimation in breast cancers has been challenged by several groups [8–10] and is now somewhat controversial. The latter studies all used immunohistochemistry, whereas investigators supporting pS2 estimation used an immunoradiometric assay of pS2 protein and northern blot analysis of pS2 mRNA. The question still remains as to whether differences in methodology are responsible for inconsistent results regarding the usefulness of pS2 quantitation in breast cancers. In the present study, we have correlated pS2 mRNA in breast cancers with patient survival at 5 years after surgery.

Correspondence to S.J. Wysocki.
The authors are at the Departments of Surgery, University of Western Australia, Fremantle Hospital, Fremantle, 6160 and Queen Elizabeth II Medical Centre, Nedlands 6009, Western Australia, Australia.
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PATIENTS AND METHODS

A total of 150 primary breast cancers were collected from Western Australian women who underwent surgical excision of these tumours during the period 1987–1988. 5 of the patients died within 5 years from causes other than disseminated breast cancer and were censored. The average age of the remaining 145 breast cancer patients was 56 years (range 27–88) with 41% of the patients less than 50 years of age. Tumours were frozen in liquid nitrogen prior to quantitation of pS2 mRNA using standard northern blot methodology [11]. A RNA ladder (Gibco BRL, U.S.A.) was used in sizing of bands on gels. Intensities of pS2 mRNA bands were visually assessed and assigned a signal strength on a graded scale from very weak to very strong, taking into account the relative signal for the ubiquitous 36B4 mRNA from the same specimen [12]. Tumours displaying strong and very strong mRNA signals were judged to be positive for pS2 and those with weaker signals were classed as negative. Oestrogen receptor (ER) and progesterone receptor (PR) measurements were carried out using an enzyme immunoassay kit (Abbott Laboratories, Chicago, Illinois, U.S.A.) and expressed in fmol/mg protein. Tumours were ER+ if 4 fmol/mg protein or greater were detected, and PR+ if 10 fmol/mg protein or greater were detected. Patients dying from breast cancer within 5 years of surgery were identified from a Death Register