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Patho- and immunobiology of malignant mesothelioma: characterisation of tumour infiltrating leucocytes and cytokine production in a murine model

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Abstract. Malignant mesothelioma (MM) is an aggressive, uniformly fatal serosal tumour, usually associated with asbestos exposure, for which there currently is no effective treatment. In order to gain insight into the mechanism(s) whereby MM might escape immune surveillance, a murine model for MM was used (a) to characterise the tumour-infiltrating lymphocytes (TIL) and macrophages (TIM) phenotypically, (b) to examine systemic immune recognition of MM, and (c) to examine the possible influence of tumour-derived cytokines on systemic and local pathobiological manifestations of MM. A profound down-regulation of lymphocyte surface markers, known to be involved in T cell activation, was found in TIL. Likewise, although TIM were present in large numbers, their expression of MHC class II antigen and integrins was weak or absent, suggestive of altered functional activity. Significant amounts of cytokines, in particular transforming growth factor β, interleukin-6 (IL-6), IL-1 and tumour necrosis factor were produced during the course of MM tumour development – directly by the MM cells and/or indirectly in response to tumour growth. These factors may contribute both to derangement of antitumour effector mechanisms and to the clinical and pathological manifestations of the disease.

Key words. Malignant mesothelioma – TIL – CD3 – TGFβ – Interleukin-6

Introduction

Malignant mesothelioma (MM) is an aggressive, uniformly fatal serosal tumour, usually associated with asbestos exposure. MM is unresponsive to conventional therapy, including surgery, chemo- and radiotherapy [36], and immunotherapy, attempted on a small-scale trial basis, has so far shown modest results [12]. A prerequisite for development of more effective treatment-protocols will be a better understanding of the basic immunobiology of MM.

Several reports have considered mononuclear cell (MNC) infiltration in tumours to be a sign of good prognosis [45], while others have affirmed the contrary (reviewed in [23]). A frequent observation has been that the infiltrating MNC are rarely in close contact with tumour cells, but rather tend to congregate in fibrous tracts separating strands or islands of tumour cells, thus, casting doubts on their direct functional involvement in antitumour activities [23, 32]. Likewise, the role of tumour-infiltrating macrophages (TIM) remains controversial, with both pro- and antitumour activities being described in various cancers [19]. Studies in a number of animal tumour-models have also clearly indicated that there need not be a direct correlation between the immune response or the effector cells in the periphery and in the solid tumour mass [21]. A consensus view of the abundant literature on tumour-infiltrating lymphocytes (TIL) and TIM in various cancers, both in humans and in experimental animal models, suggests that no conclusions should be drawn on a comparative basis, and that each tumour type will require individual characterisation.

Only a few selective descriptions of TIL in MM have been reported [22]. Likewise, there is still a relative paucity of knowledge of other aspects of mesothelioma immunobiology, including cytokine or growth factor requirements and production, expression of cell-surface markers on tumour cells and the immune responses elicited.

In order to overcome the logistic and ethical constraints inherent in using material from human patients for such studies, we have established a murine model of MM [13], the relevance of which is based on the fact that it closely

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parallels the human disease in a number of important aspects including (a) the inducing agent (asbestos), (b) a long latency but rapid late clinical tumour development, (c) clinical behaviour, (d) growth factor biology, and (e) certain preliminary immunobiological features (MHC expression, natural killer cell insensitivity, lymphokine-activated killer cell sensitivity and partial responsiveness to interferon α therapy) [5, 12, 18, 32].

The aims of the present studies were to further the immunobiological characterisation of MM in the murine model and specifically (a) to characterise the tumour-infiltrating leucocytes phenotypically, (b) to examine cytokine production during tumour development as a possible contributor to the pathobiological manifestations of MM, and (c) to examine systemic immune recognition of MM. Our results demonstrate that, despite immune recognition of the tumours, the malignant cells may escape effector mechanisms by adversely affecting lymphocytes and macrophages in the local tumour microenvironment, as well as systemically. This effect may be, at least in part, due to transforming growth factor β (TGFβ) production by the tumour cells.

Materials and methods

Animals. BALB/c (H-2^d) and CBA/CAH (H-2^k) mice (specific-pathogen-free, female, 6–8 weeks old) were obtained from the Animal Resources Centre (Murdoch, Western Australia) and maintained under standard conditions in the University Department of Medicine animal holding area. All animal experimental procedures were approved by the UWA Animal Welfare Committee, and followed the NH&MRC Recommendations for Experiments in Animals.

Antibodies. The following monoclonal antibodies (mAb) to murine antigens were used for analysis of surface antigen expression, T cell stimulation, and/or as blocking Ab in cytokine bioassays: RM2.1, rat anti-CD2 [49], 145-2C11, hamster anti-CD3ε [29], KT3.2, rat anti-CD3, GK1.5, rat anti-CD4, 53.6.72, rat anti-CD8, I21.1.1, rat anti-CD18, the β chain of LFA-1, M1/70, rat anti-CD11b, F4/80, rat anti-macrophage (all hybridomas from the American Type Culture Collection, ATCC, Rockville, Maryland), 53.7.3, rat anti-CD5 (Boehringer Mannheim, Castle Hill, NSW, Australia), Lyt2, rat anti-CD8 (Becton-Dickinson, Mountain View, Calif.), B3B4, rat anti-CD23 [39], 7D4, rat anti-CD25 (European Type Culture Collection), M5/114, rat anti-Ia (Boehringer Mannheim, catalogue no. 1199293), 11B11, rat anti-IL-4 (ATCC), S4B6, rat anti-(interleukin-2) (IL-2), and XMG1.2, rat anti-(interferon γ) [11], 6B4, rat anti-IL-6 [44], SXC1 and SXC4, rat anti-IL-10 [35]. The MHC-class-I and -II-specific mAb have been described previously [13]. Where no commercial supplier is mentioned the antibodies were purified from hybridomacultures propagated in serum-free medium by ammonium sulphate precipitation followed by extensive dialysis. Secondary reagents comprised biotinylated rabbit anti-(rat Ig) and streptavidin/horseradish peroxidase from Dako a/s (Glostrup, Denmark), and fluorescein-isothiocyanate(FITC)-conjugated goat anti-(rat Ig) and anti-(mouse Ig) from Tago Inc. (Burlingame, Calif.).

Reagents. Recombinant cytokines for use as standards in cytokine bioassays comprised recombinant murine interferon γ (rmIFNγ; Genzyme), recombinant human IL-β1 (rhIL-β1), rhIL-6 and recombinant human tumour necrosis factor α (rhTNFα) (Boehringer-Mannheim), and rhTGFβ1 (Genzyme). Phytohemagglutinin was purchased from Wellcome (Dartford, Kent, UK), concanavalin A from Pharmacia (Uppsala, Sweden). Lipopolysaccharide, diaminobenzidine, methyl green, hydrogen peroxide, DNase and hyaluronidase were purchased from Sigma (Castle Hill, N.S.W.), and OCT compound was from Bayer. Collagenase was obtained from Boehringer Mannheim, and Ficoll/Hypaque and Percoll were purchased from Pharmacia (North Ryde, N.S.W.). Heparin was purchased from David Bull Labs. (Melbourne, Victoria, Australia), and micro-blood tubes (Microtainer) were from Becton-Dickinson.

Tumour cell lines. Murine mesothelioma cell lines were established from malignant cells in peritoneal effusions arising 7–14 months after injection of sterile asbestos fibres into the peritoneal cavity of BALB/c and CBA/CAH female mice [13]. Their basic characterisation, including cytology, ultrastructure, tumourigenicity and MHC antigen profile, has been previously described in detail [13]. Of the 16 original cell lines, 4 were used in this study: AB1 and AB22 (BALB/c-derived), and AC29 and AC31 (CBA-derived). The cell lines were maintained in Dulbecco's minimal essential medium (Gibco, Glen Waverley, Vic.) supplemented with 20 mM HEPES, 50 μ M 2-mercaptoethanol, 10 μU/l penicillin (Glaxo Australia, Boronia, N.S.W.), 50 mg/l gentamicin (David Bull Labs), and 5% fetal bovine serum (FBS; Gibco). For animal inoculations, cells from fewer than 20 in vitro passages were used in all cases except for line AB1 (passage levels 71–85).

Tumour challenge protocol and pathology studies. Single-cell suspensions in pyrogen-free saline (Astra) were inoculated at indicated cell doses, either s.c. or i.p. into syngeneic mice. Mice were observed daily, and killed when s.c. tumours reached a diameter of 1 cm or, in the case of i.p. inoculated mice, their clinical status had deteriorated significantly (see Results for further detail). Anaesthetized animals were bled by cardiac puncture immediately before euthanasia, and the serum was stored at –85° C until tested. A post-mortem examination was conducted on all animals and tissues collected for further processing: (a) cell procurement for in vitro studies, (b) tumour embedding in OCT for cryosectioning (stored at –85° C), (c) fixation of selected tissues in 4% buffered formaldehyde for histopathological examination. Routine preparation of tissues for histology was performed by the Department of Pathology, UWA.

Lymphocyte procurement and in vitro functional assays. Single-cell suspensions were made from lymph nodes and spleens by teasing, followed by several sequences of washing in ice-cold phosphate-buffered saline (PBS) with 5% FBS. Spleen cells were subsequently spun over Ficoll/Hypaque to remove erythrocytes and dead cells. Tumour-infiltrating leucocytes were procured by digesting excised tumours for 3–4 h at room temperature in a mixture of collagenase (2 mg/ml), hyaluronidase (5 U/ml) and DNase (0.2 mg/ml) in RPMI medium/5% FBS, followed by filtration through sterile gauze and centrifugation on a discontinuous Percoll gradient (adapted from [4]). TIL were enriched in the 65%/75% interphase and in the pellet to a purity above 65% and 95% respectively, and these cell fractions were either pooled or used separately.

Proliferative responses to and cytokine induction by mitogens were assessed by incubating triplicate cultures of 1×10^5 MNC/well in 150 μl RPMI medium/5% FBS in 96-well round-bottomed microculture plates (Nunc, Roskilde, Denmark) without or with various doses of phytohaemagglutinin or concanavalin A. After 48 h incubation at 37° C, 5% CO_2 in air, 100 μl culture medium was removed from each well and stored at –85° C. The cultures were replenished with 50 μl fresh medium and incubated for a further 24 h. During the last 8 h cultures were pulsed with 1 μCi [^{3}H]dT (Amersham, Castle Hill, N.S.W.) and frozen/thawed once; the DNA was harvested onto glass filter-paper using a PHD cell harvester (Cambridge Tech. Inc., Watertown, Mass., USA), and thymidine incorporation measured by liquid scintillation. In the case of TIL, cells were stimulated with either phytohaemagglutinin or plate-bound mAb 145–2C11, in the presence of IL-2 and irradiated (30 Gy), syngeneic or allogeneic, spleen feeder cells.

Procurement of peritoneal macrophages and induction of cytokine/production. The peritoneal cavities of normal or tumour-bearing mice were washed out with ice-cold Mg^{2+}/Ca^{2+}-free PBS containing 50 U/ml heparin. In the case of mice with i.p. tumours red blood cell contam-

Table 1. Clinical and post-mortem features of mice with abdominal mesothelioma (MM)

MM tumour cell line	Clinical presentation	Post-mortem features
AB22	Cachexia (but not anorexia until moribund); diarrhoea; complete depletion of body fat stores. Subcutaneous haemorrhages easily induced	300 µl–1.0 ml blood-tinted ascites. Usually a single large (>1.5 cm diameter) well-vascularized tumour mass centrally in mesenterium, often with evidence of haemorrhage (haematoma). Minimal connective-tissue formation. Necrosis variable. Grossly apparent liver damage, in some some cases frank necrosis. Thymus atrophy (often >90% reduction)
AC29	Progressive (over 24–96 h) loss of vigour/activity/grooming ending in frank depression and a moribund state. Minimal to moderate loss of body weight. Progressive abdominal distension	3–6.5 ml blood-tinted ascites. Nodular tumour growth throughout mesenterium with particularly prominent growth around pancreas and the mesenteric lymph nodes. Considerable connective-tissue component and low-moderate vascularization. Widespread necrosis, independent of tumour size. Occasional intestinal obstruction and penetration by tumour mass. "Fleshy" plaque-like tumour growth on diaphragm. Focal to widespread coalescing areas of liver necrosis. Thymus and spleen atrophy (50%–90%)
AC31	Per-acute to acute development of symptoms described for AC29	As for AC29

The latency period from tumour cell inoculation to first signs of disease was strictly dependent on both the tumour cell line used and the challenge dose. However, when clinical signs appeared the course was independent of the initial challenge dose

ination was removed by Ficoll separation. Following several washes in ice-cold medium the cells were seeded in flat-bottomed 96-well tissue-culture plates (Nunc) at a cell density adjusted to approximately 2×10^5 macrophages/well, and incubated overnight. Non-adherent cells were removed with several changes of cold PBS and the adherent cells (above 98% pure as assessed by non-specific esterase staining [4]) reincubated in 150 µl/well RPMI medium/5% FBS without or with the following stimuli: 100 U/ml rmIFNγ, 10 µg/ml lipopolysaccharide or both. Following a 48-h incubation the culture supernatants were collected and stored at –85° C until tested for cytokine activities. The final cell density per well was determined by counting non-specific-esterase-positive cells, and the cytokine data were adjusted accordingly to allow comparisons to be made between experimental days.

Cytofluorometric analysis. Binding of murine sera to surface components on tumour cells was assessed by flow cytometry according to a previously described protocol [13]. TIL, spleen and lymph node cells were analysed for cell-surface antigens with the above-listed rat mAb and secondary FITC conjugate according to a previously described protocol [17]. Cells were stored overnight at 4° C in the dark before analysis on a FACScan flow cytometer (Becton Dickinson).

Immunocytochemistry. Serial cryosections (10 µm) collected on poly-L-lysine-coated, precleaned slides were fixed in 20% acetone in PBS/0.5% bovine serum albumin [3] following removal of OCT, and immunolabelled in a triple-layer protocol using mAb and biotin/streptavidin- conjugates listed above. All incubations were at room temperature: 1 h with primary Ab, and 0.5 h with each of the conjugates, with 3 5-min washes in TRIS-buffered saline in between. Incubation with diaminobenzidine/H_2O_2 substrate was for 5–10 min at room temperature, followed by counterstaining with methyl green and mounting in DPX. Usually at least two sections from each tumour were labelled for each CD marker, and tumours were randomised with respect both to the labelling procedure and to the final assessment. For each section five representative 40× objective fields were selected and *all* positively labelled cells counted. Areas with extensive necrosis were excluded as were areas compromised by artefactual (processing-related) distortion of tissue structure. All mAb were initially titrated on normal lymphoid tissues and any changes in labelling intensity of TIL and TIM were judged in relation to that of cells in spleen or thymus.

Cytokine bioassays. Stored MM, MNC and macrophage culture supernatants, sera and cell-free ascitic fluids were assayed for cytokine bioactivities by previously published methods, using the recombinant protein standards listed above, or, in the case of IL-3/granulocyte/macrophage-colony-sstimulating factor(GM-CSF), conditioned medium from constitutively producing cytokines producing cells (WEHI-3B) [26]. Briefly, IL-1 was assayed using D10.G4.1 cells [25] in a cofactor-dependent stimulation assay. IL-6 was assayed as described [17] using the factor-dependent B9 cell line. This cell line is reportedly also responsive to IL-11 [9]; however, blocking studies using the mAb 6B4 or goat anti-mIL-6 (British Biotech. Prod. Ltd.) showed that the activities detected in our samples were indeed due to IL-6 (data not shown). GM-CSF/IL-3 activity and IFNγ were detected using FD5/12 and WEHI-279 cell lines respectively, and protocols described by Kelso [26]. TNFα was assayed using L929 cells as described [20], and TGFβ was detected by the CCL64 growth-inhibition assay [18]. The sensitivity of the various bioassays was, in our hands, as follows: IL-1, 0.5 pg/ml or 0.025 U/ml; IL-3/GM-CSF, 0.1 U/ml; IL-6, 0.03 U/ml; IFNγ, 0.02 U/ml; TNFα and TGFβ, 50 pg/ml.

Delayed-type hypersensitivity (DTH) reactivity. To assess whether a hypersensitivity recall reaction to MM in previously exposed animals could be elicited, 1×10^5 MM cells in 25 µl pyrogen-free saline were inoculated into the right hind footpad of either naive animals or mice previously inoculated i.p. or s.c. with 1×10^6 irradiated (150 Gy) but viable MM cells. The left footpad received saline only. The thickness of the footpads was measured, using calipers, before and every 6–12 h over a 96-h period following injections.

Analysis for TGFβ mRNA by polymerase chain reaction (PCR) and Southern blotting. A detailed description of primers and PCR conditions has been given elsewhere [18]. Briefly, total RNA extracted from cryosectioned tumours or tumour cells grown in vitro was reverse-transcribed into cDNA using random primers and avian myeloblastosis virus reverse transcriptase, and amplified by PCR using TGFβ1- and TGFβ2-specific primer pairs. Amplification was limited to 27 cycles to remain within the logarithmic phase and co-amplified β-actin was used in each tube as an internal control. The resulting products were separated on a 2% agarose gel and transferred to nylon membranes. The membranes were hybridized with biotinylated TGFβ1- and -β2-specific probes and the binding visualized using streptavidin-peroxidase-coupled luminol-based enhanced chemiluminescence [18].

Results

Clinical and pathological features of subcutaneous and intraperitoneal murine MM

Animals inoculated subcutaneously with 1×10^6 MM cells developed palpable tumour growth within 6–21 days, and for most tumour cell lines all inoculated mice developed palpable tumours within a few days of each other; the exception was line AB1, where only 80% of animals develop

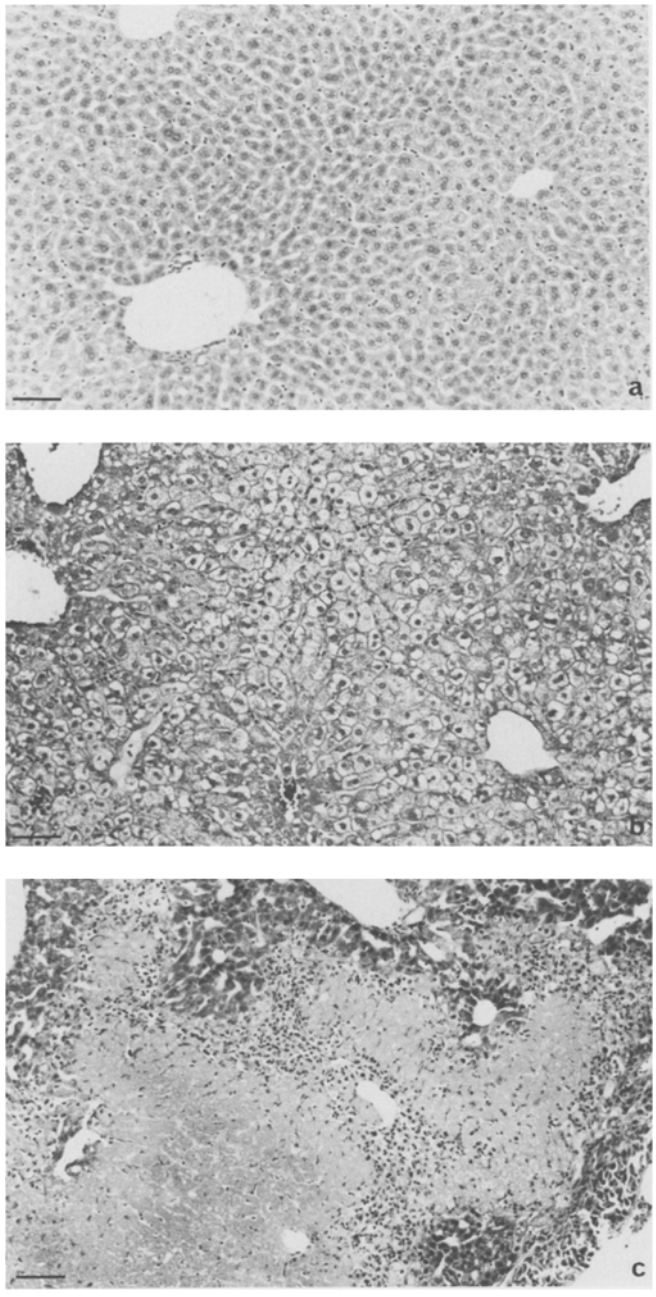

Fig. 1 a–c. Microphotographs of liver pathology in murine malignant mesothelioma (i.p. growth). **a** Normal murine liver; **b** liver changes in AB22-tumour-bearing mice consisting in vacuolation and swelling of hepatocytes; **c** liver changes in AC29/AC31-bearing mice consisting in focal coagulation necrosis, here accompanied by infiltration of mononuclear leucocytes. *Bars* = 108 μm

tumours [13]. Clinically the mice remained apparently normal, and only in the case of AB22- and AC31-inoculated mice were minor histopathological changes found in internal organs, restricted to slight vacuolation and swelling of hepatocytes and minimal infiltration of MNC and polymorphonuclear cells (PMN) in liver triads; thymus and other lymphoid organs appeared histologically normal (data not shown).

In contrast, mice receiving i.p. inoculations of MM cells developed clinical symptoms reflecting systemic as well as local effects of tumour growth. The onset of symptoms was typically sudden and led to death within 12–96 h. In the case of AC29 and AC31 tumour challenges, mice presented with progressive abdominal swelling, rough coat, dehydration and inactivity progressing to a moribund state and death. AB22-inoculated mice presented with cachexia, usually leading to a 25% loss of body weight in 3–4 days, diarrhoea and rough coat, but only relatively late in the tumour development did they become anorexic, dehydrated and listless (Table 1).

Gross and histopathological findings for i.p. tumours are summarised in Table 1. Histologically, all four tumours presented as mixed-type MM [32]. Necrotic foci were characterized by lack of an inflammatory response in the surrounding intact tissue, but frequently a massive PMN infiltration within the boundaries of the necrotic foci. Characteristically, tracts of necrotic tissue would occur 15–20 cell layers under the surface of AC29 and AC31 tumours, or in apparently well-vascularized parts of AB22 tumours. Haemorrhage and haematoma formation were also prominent features of AB22 tumours.

Tumour infiltration/seeding in other organs was rare, except that in most cases tumour growth had disrupted the gross structure of the pancreas, but interfered minimally with the histological appearance of the glandular acini or pancreatic islands. The most prominent extratumour changes were found in the thymus and liver. The former was usually completely depleted of cortical thymocytes, and the medulla, although structurally distinguishable, was noticeably depleted of lymphocytes. In AB22-inoculated mice the liver changes consisted of significant vacuolation and swelling of hepatocytes, in milder cases in a zonal distribution around the central vein, but in many cases uniformly throughout the organ (Fig. 1b). In AC29- and AC31-inoculated mice focal acute coagulation necrosis was widespread in the liver, in most cases without an accompanying inflammatory response although, in a few more protracted cases, MNC infiltration surrounding the necrotic areas featured prominently (Fig. 1c). Degenerative changes in other organs were usually only seen when tumour growth directly obstructed the organ, such as the ureters or intestines. Lymph nodes contained no or only few germinal centres as did the spleen.

In situ phenotyping of TIL and TIM

Examination of serial sections of tumours immunolabelled for a range of CD markers revealed a number of notable features. The predominant leucocyte was the macrophage, which in both subcutaneous and i.p. AC29 tumours constituted up to 40%–50% of all nucleated cells. Of these only a subpopulation expressed detectable MHC class II and CD11b/CD18 antigens (Fig. 2). This positive subpopulation tended to locate near small vessels or in connective tissue tracts, whereas F4/80$^+$ cells were found throughout the tumour (Fig. 3a). No or exceedingly few B cells were found and the CD4:CD8 ratio appeared to vary between tumour lines (Fig. 2). However, the most remarkable finding was that the CD3 expression was apparently down-regulated, as suggested by two features: (a) the total number of CD5$^+$, or

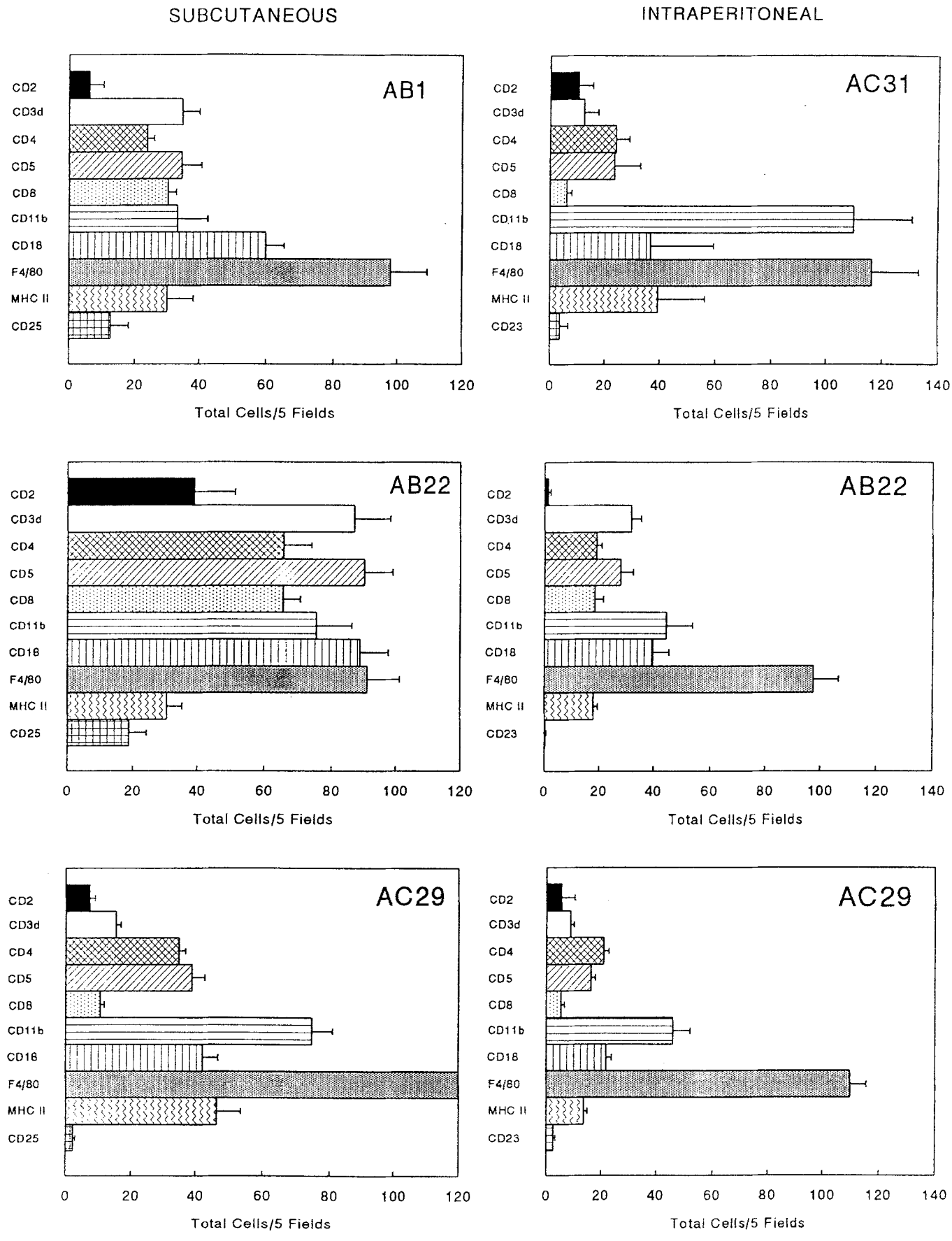

Fig. 2. In situ phenotyping of tumour-infiltrating lymphocytes (TIL)/tumour-infiltrating macrophages (TIM) in subcutaneously and intraperitoneally growing malignant mesothelioma (MM) tumours in mice. The data shown represent mean numbers ± SEM of 15–36 animals per tumour type and inoculation route

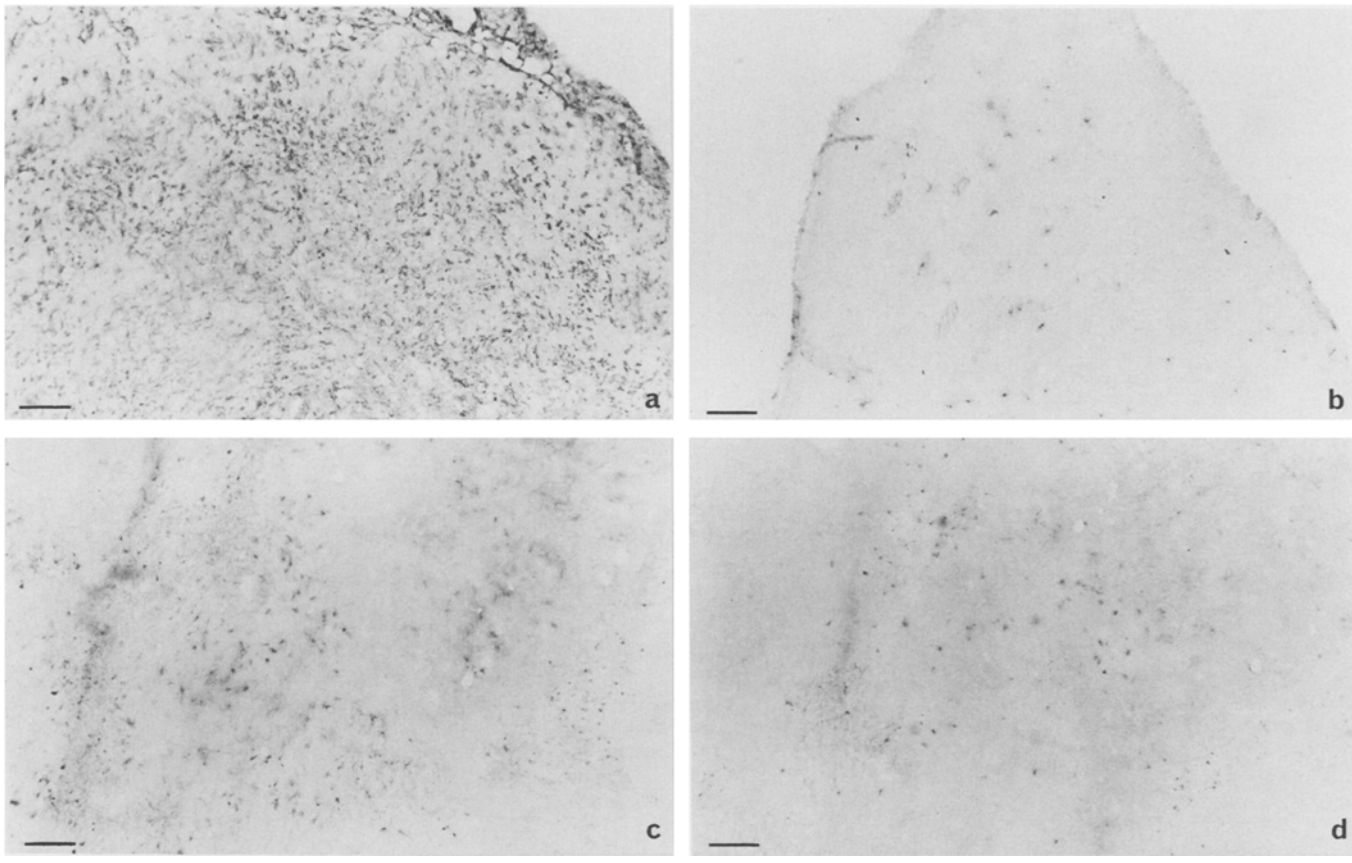

Fig. 3 a–d. Photomicrographs of immunocytochemically labelled cells: **a** F4/80+ cells (i.e., macrophages), **b** CD3+ T cells, **c** CD4+ T cells, and **d** CD8+ T cells in murine AC29 MM tumours. *Bars* = 272 μm

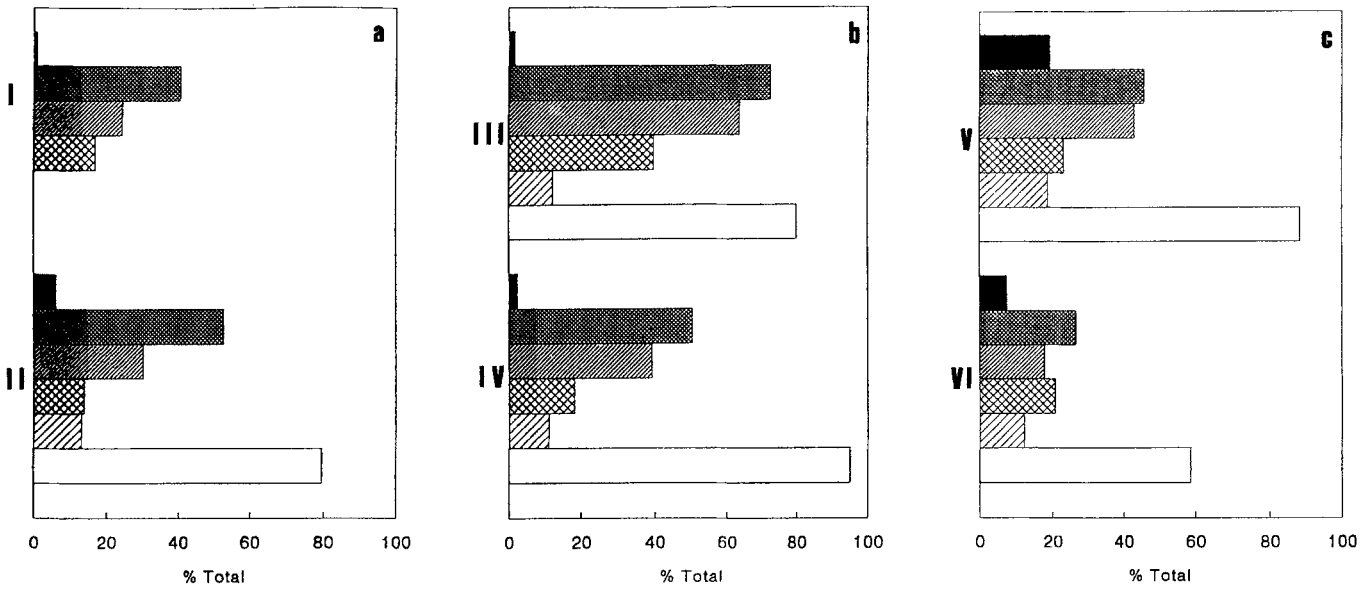

Fig. 4 a–c. Phenotypic profile of TIL, isolated from subcutaneous (**a**) AC29 and (**c**) AB22 mesotheliomas, compared with spleen and lymph node T cells (**b, c**), as determined by flow cytometry. TIL in (*I*) Percoll pellet and (*II*) in 65%/75% interphase. Spleen (*III*) and lymph node (*IV*) cells from AC29-bearing mice. *V* Spleen cells from AB22-bearing mice. *IV* TIL in pellet plus 65%/75%-interphase. ■ CD2, ▩ CD3, ▨ CD5, ▩ CD4, ▨ CD8, □ CD18

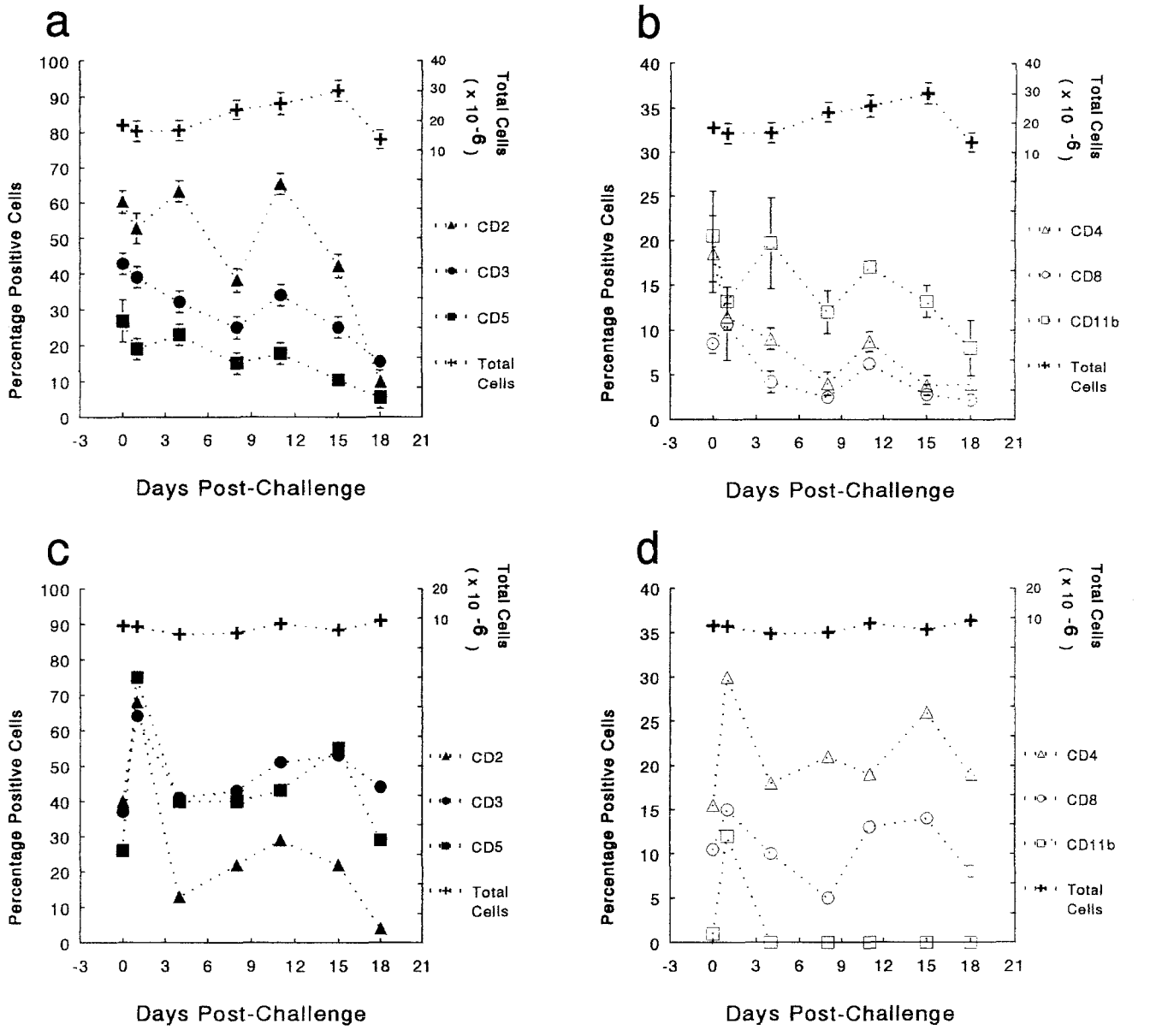

Fig. 5 a–d. Phenotypic profile of spleen **(a, b)** and lymph node **(c, d)** cells isolated from mice during intraperitoneal AC29 MM tumour development, as determined by flow cytometry. Tumour growth became apparent, upon post-mortem examination, on days 8–11, and the first clinical signs were noted on day 13 after inoculation. On day 18 the remaining mice were all in a poor condition, although not yet moribund. The data represent means ± SEM of 4–6 animals for each assay time

CD4+ plus CD8+, cells exceeded the number of CD3+ cells, and (b) a gradual decrease in labelling intensity of CD3+ cells occurred both over time during tumour development (not shown) and within larger tumours from the periphery to the centre of the tumours. This phenomenon was particularly apparent in AB1, AC29 and AC31 tumours (Fig. 3b), and less apparent in AB22 tumours, which also contained relatively more CD3+ cells (Fig. 2). By mRNA analysis it was found that CD3ε and CD5 mRNA expression remained relatively constant, whereas CD3δ mRNA levels decreased progressively during tumour development [24].

Leucocyte procurement and in vitro activities during tumour development

Repeated attempts were made to induce in vitro growth of purified TIL from AB1, AB22, AC29 and other MM tumours not described here. Examples of the phenotypic profile of TIL preparations more than 80% pure, from subcutaneous AB22 and AC29, tumours are shown in Fig. 4. However, despite a relative enrichment for CD2+/CD3+ cells, as compared to the in situ frequency (Figs. 2, 3), they appeared unable to proliferate in response to phytohaemagglutinin, solid-phase anti-CD3ε mAb, IL-2 or autologous tumour cells (all in the presence of syngeneic spleen feeder cells). Only when stimulated with allogeneic spleen cells plus IL-2 plus LAK cell-conditioned medium

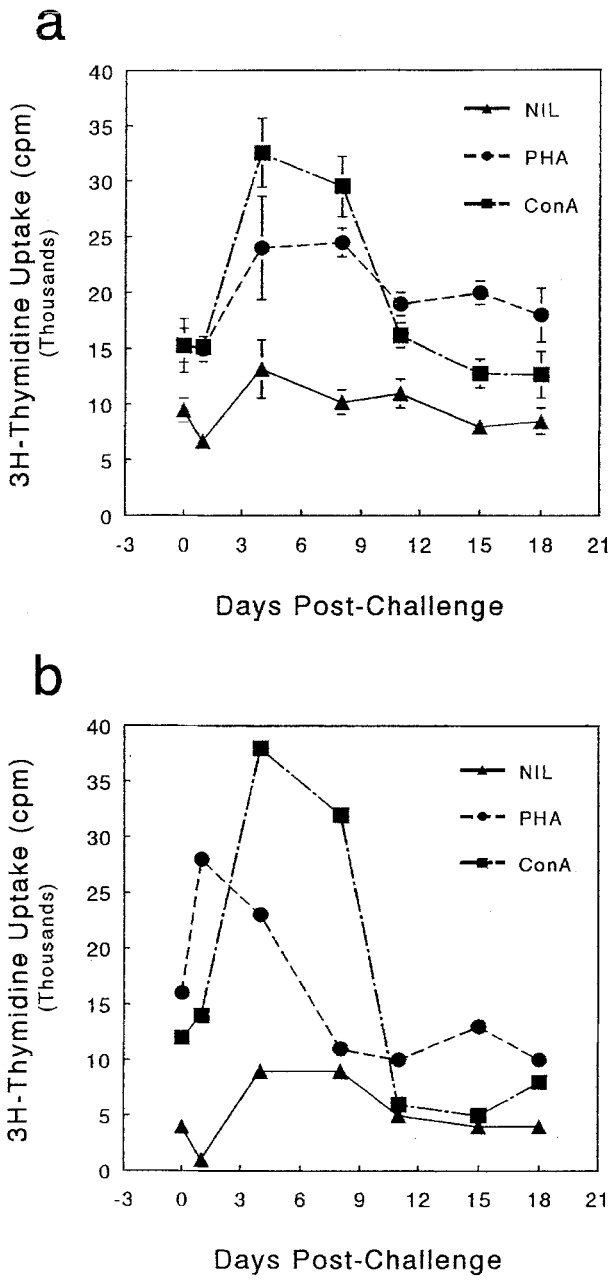

Fig. 6a, b. Proliferative response to mitogens of (**a**) spleen and (**b**) lymph node cells retrieved from animals with intraperitoneally developing AC29 MM tumours (same animals as in Fig. 5). The data are representative for the responses seen to a range of mitogen concentrations and, in the case of spleen cells, are mean radioactivities (cpm) ± SEM of 4–6 animals at each asssay time, and, in the case of lymph node cells, radioactivities (cpm) of pooled cells from 4–6 mice

did a small fraction (less than 1:35 000) of cells proliferate and they could, following limiting-dilution cell cloning, be maintained for 5–8 weeks under those culture conditions. However, these clones, whether of the CD4 or CD8 phenotype, lacked tumour-antigen specificity as assessed in cytotoxicity, proliferation and cytokine-production assays (data not shown).

Whereas s.c. growth of the AB1 and AB22 MM cell lines caused an increase in total spleen cell numbers (two- to sevenfold), s.c. growth of AC29 and AC31, as well as

Table 2. Phenotypic profile of peritoneal cells from mice with intraperitoneal AC29 MM tumour growth

Time after tumour challenge (days)	Total cells retrieved ($\times 10^{-6}$)	Percentage of cells positive for:				
		NSE	CD3	CD4	CD8	MHC class II[a]
control (0)	1.49 ±0.19	73.1 ±5.6	5.0 ±0.3	3.0 ±0.1	1.6 ±0.3	58.8 ±0.6
1	1.85 ±0.18	71.3 ±2.9	3.3 ±0.3	3.6 ±0.6	<1	49.6 ±0.6
4	2.05 ±0.33	75.0 ±2.7	6.4 ±1.8	5.0 ±2.2	1.3 ±0.3	ND
8	2.39 ±0.26	76.2 ±0.6	7.9 ±1.5	4.5 ±0.3	1.5 ±0.3	44.7 ±4.1
11	2.85 ±0.64	80.2 ±2.2	6.7 ±1.8	4.6 ±0.7	<1	27.8 ±3.1
15	8.20 ±0.67	71.2 ±6.1	8.6 ±1.4	6.9 ±1.0	2.4 ±1.2	23.6 ±4.8
>21	4.40[c] ±1.15	76.7[b] ±3.3	19.1 ±1.2	11.7 ±2.6	4.7 ±0.8	27.5 ±3.1

Other cells in the preparations that did not immunolabel included polymorphonuclear neutrophils (PMN), mast cells, basophils and tumour cells. NSE, non-specific esterase

[a] All MHC-class-II-positive cells were judged, by morphological criteria, to be macrophages

[b] Determined by immunolabelling for CD11b, which may occur on both macrophages and PMN

[c] At this point in tumour development, poor viability of peritoneal leucocytes was a consistent finding

i.p. growth of AB22, AC29 and AC31 MM tumours caused a notable decrease (15%–85%). Intraperitoneal growth of AB22 also caused a progressive loss of peripheral lymph node cells, whereas only minor variations were seen in animals with AC29 tumours (Fig. 5, and data not shown). Phenotypic analysis of spleen cells from animals with i.p. AC29 tumours, sampled over an 18-day postinoculation period, demonstrated a progressive down-regulation of all lymphocyte markers and/or a loss of marker-positive spleen cells. In contrast, increases or no change occurred in the lymph node cells during the same period, except for an early disappearance of $CD11b^+$ cells and a generalised loss of surface-marker expression on day 18, when the animals were in a clinically poor condition (Fig. 5). These changes were accompanied by changes in the mitogen responsiveness (Fig. 6), characterised by an initial increase followed by a return to control levels or a depressed response. The changes were most marked in lymph node cells and with respect to the concanavalin A response (Fig. 6). A similar response pattern was seen when the mitogen-induced IL-3/GM-CSF, IFNγ and IL-6 production were assessed (data not shown). In additional experiments it was repeatedly found that lymphocytes isolated from peripheral lymphoid organs of animals terminated *in extremis* were devoid of mitogen responsiveness, proliferation or cytokine production, or that the mitogen optimum had shifted (data not shown). No MM-tumour-cell-specific response (proliferative or cytotoxic) was detected in spleen or lymph node cells freshly isolated from tumour-bearing or tumour-immunised mice (data not shown).

Table 3. Cytokine production by peritoneal macrophages from mice with abdominal AC29 MM-growth

Time after tumour inoculation (days)	IL-6 (U/ml) in response to:				IL-1 (U/ml) in response to:			
	Nil	LPS	IFNγ	LPS+ IFNγ	Nil	LPS	IFNγ	LPS+ IFNγ
0	5.8 ±2.5	75.9 ±30	22.5 ±10	239.2 ±6.6	31.3 ±1.5	322 ±1.3	14.9 ±3.5	350.4 ±24
1	51.4 ±20	117 ±5.5	59.9 ±8.4	188 ±18	35.2 ±5.2	53.9 ±4.3	26.2 ±8.9	124.2 ±44
4	60.2 ±36	164 ±69	18.9 ±6.5	359.7 ±184	80.4 ±18	561 ±22	17.2 ±2.5	199.9 ±76
8	34.4 ±12	113 ±37	16.1 ±3.0	438.1 ±95	27.6 ±8.8	397 ±73	22.1 ±7.8	244.8 ±23
11	33.3 ±23	156 ±86	74.2 ±51	1494 ±531	166 ±83	302 ±99	35.0 ±15	422.3 ±124
15	501 ±268	1573 ±802	341 ±161	2032 ±814	90.1 ±32	448 ±251	72.7 ±41	290.4 ±75.5

Bioactivity released into the culture medium during 48 h incubation of 2×10^5 macrophages without stimulus or with lipopolysaccharide (LPS; 10 ng/ml), recombinant murine interferon γ (IFNγ; 100 U/ml) or their combination. Results are means ± SEM of 3–5 animals per assay time

In animals with i.p. growth of AC29 MM tumours the number of peritoneal macrophages and lymphocytes increased significantly (macrophages up to 3.6-fold, lymphocytes up to 14-fold). Notably, however, MHC class II antigen expression by peritoneal macrophages decreased during tumour development, whereas the CD4:CD8 ratio remained in the range of 2:1 to 4:1, regardless of the increase in total peritoneal lymphocyte numbers (Table 2). Similar findings were made in animals with AB22 and AC31 MM tumours (data not shown). Peritoneal macrophages, procured from mice during the development of intraperitoneal AC29 tumours, were assessed functionally with respect to cytokine production. As shown in Table 3, spontaneous and lipopolysaccharide-inducible IL-6 release increased during tumour development, culminating in release of large amounts on day 15 after challenge, when also the first clinical signs of MM were apparent (not shown). Macrophages from the last sampling time, day 18 after inoculation when the animals were *in extremis*, adhered poorly and could therefore not be assayed. Some less consistent increases over time of IL-1 production were also observed (Table 3). No or only marginal levels of TNFα were detected in the macrophage culture fluids at any time after tumour challenge (not shown).

Immunorecognition of MM

Since it was not possible to demonstrate MM-specific T cell proliferation or cytotoxicity in vitro, in vivo evidence of tumour-specific immunorecognition was sought instead. Two approaches were taken: (a) using flow cytometry, sera from tumour-immunised [31] and/or tumour-cell-challenged mice were examined for MM-specific antibodies. A

Table 4. Features of representative murine malignant mesothelioma models

Feature	Tumour cell line			
	AB	AB22	AC29	AC31
Strain derivation	BALB/c	BALB/c	CBA	CBA
MHC class I expression	H-2d (100%)	H-2d (100%)	H-2k (95%)	H-2k (97%)
MHC class II expression: Constitutive inducible	–/–	–/–	–/±	–/–
Tumorigenicity (%)				
Subcutaneous	80	100	100	100
Intraperitoneal	Variable	100	100	100
Protection after immunisation	++	–	–	–
Enhanced tumorigenesis after immunisation	–	±	–	+
DTH response	+	–	–	ND
Antibody response	++++	+	+++	ND
TIM infiltration: F4/80+	+++	+++	++++	++
Class II MHC+	+	+	+	+
TIL infiltration				
CD3+	+	++++	+	+
CD4+	++	+++	++	+
CD8+	++	+++	+	+
PMN infiltration	–	+++	–	–
Tumour cell production of cytokines				
IL-6	±	++++	–	+++
TGFβ	+	±	++++	++++
GM-CSF/IL-3	–	–	–	–
IL-1	–	–	–	–
TNFα	–	–	–	–

IL, interleukin; TGF, transforming growth factor; GM-CSF, granulocyte/macrophage-colony-stimulating factor; TNF, tumour necrosis factor

specific antibody response with typical kinetics developed following subcutaneous MM cell inoculation (data not shown); however, it has as yet not been possible to confirm this finding by Western blotting or enzyme-linked immunosorbent assay, using MM cell-membrane preparations, possibly because of destruction of the epitope(s) during membrane preparation. Of the three MM lines examined only AB1 induced a detectable, specific DTH response.

Cytokine production during MM development in vivo

The clinical as well as pathological findings suggested that factors other than, or in addition to, the tumour bulk *per se* contributed to the characteristic manifestations. In addition, the *in situ* phenotypic features of TIL and TIM suggested down-regulating signals received from the tumour microenvironment. The changes in peripheral lymphoid organs, detectable prior to visible tumour growth or clinical symptoms, likewise suggested the influence of systemic mediators. In related studies several of the murine MM cell lines have been found to produce high levels of TGFβ and/

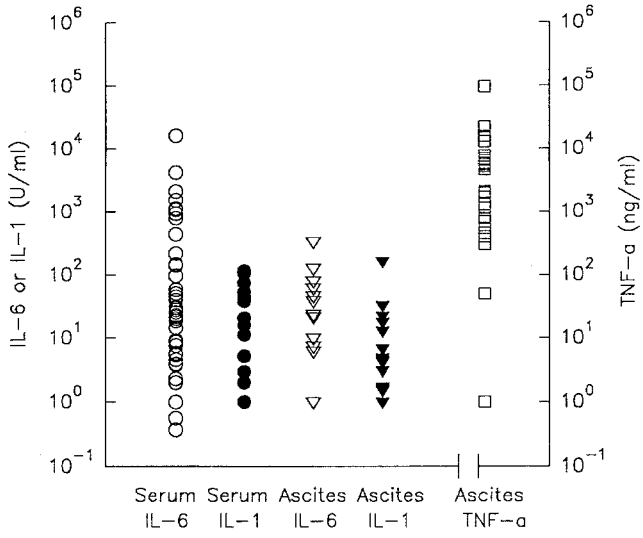

Fig. 7. Levels of bioactive interleukin-6 (*IL-6*), IL-1, and tumour necrosis factor α (*TNFα*) in sera and ascites from animals with intraperitoneally growing AC29 MM tumours

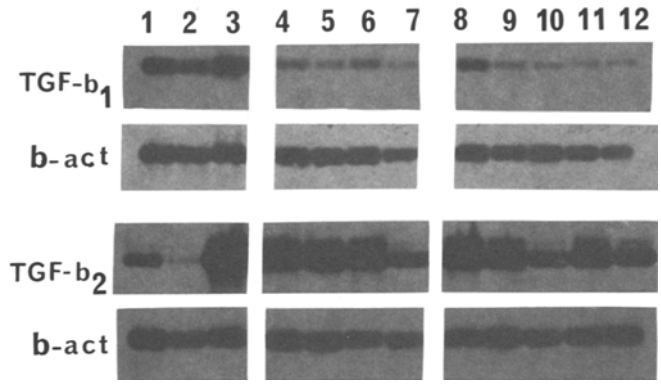

Fig. 8. Southern blot analysis of reverse-transcriptase/polymerase-chain-reaction-amplified transforming growth factor β1 (TGFβ1) and -β2 mRNA in s.c. (lanes *4–7*) and i.p. (lanes *8–12*) AC29 MM tumours. Total RNA extracted from cryosections of tumours was reverse-transcribed into cDNA followed by co-amplification with β-actin-specific primers plus either TGFβ1 or -β2 specific primers in 27 cycles. Products were separated on 2% agarose gel, blotted, probed with biotinylated probes and detected by enhanced chemoluminescence. Control samples included RNA extracted from in vitro grown AC29 tumour cells (lane *1*), normal murine spleen (lane *2*) and liver (lane *3*)

or IL-6 in vitro, but no IL-1, TNFα, IL-3/GM-CSF or CSF-1 (summarised in Table 4) [18] (unpubl. results). In addition to cytokine production by the tumour cells, leucocytes and parenchymal cells may respond to the tumour, directly or indirectly, by cytokine production. As demonstrated in Fig. 7, high levels of IL-6, TNFα and IL-1 were detected in sera and cell-free ascitic fluids of mice succumbing to i.p. MM growth. Time-course studies showed that measurable cytokine bioactivities appear in the body fluids up to 8 days prior to overt clinical symptoms (unpubl. results). CSF-1 and IL-10 were also demonstrated in ascites and/or in sera in a limited number of animals tested, the latter perhaps caused indirectly by the severe haemorrhage [1]. No cytokines have been detected in sera of normal control mice or mice with s.c. tumours (data not shown). By RNA analysis, high-level expression of TGFβ1 and TGFβ2 mRNA in AC29 tumours growing both s.c. and i.p. has been confirmed (Fig. 8, and [18]), however contributions from TIM and stromal cells, in addition to the MM cells, to these levels cannot be excluded.

Discussion

Some of the most notable immunobiological features of the murine MM model, described in this and the previous papers [13, 18, 31], are summarised in Table 4. From the series of experiments described in this report, i.p. tumour growth in the murine model appeared best to reflect the clinical, pathophysiological and pathological phenomena characteristic of MM in human patients [22, 36]. In mice bearing i.p. tumours, cytokines with potentially adverse effects on tissue integrity and immune function were detected in body fluids, and pathology compatible with IL-1, IL-6, TGFβ and/or TNFα effects was seen, including cachexia, diarrhoea, depressed activity, degenerative changes in the liver, and sudden shock-like death [38, 40, 41, 52]. Thus, it is probably not the tumour bulk *per se* that is the causative factor for the pathophysiological phenomena, even when the tumour cells are known to produce IL-6 and/or TGFβ (Table 4). Rather it may be the induction of host responses to the growth of MM cells in a serosal cavity. This hypothesis is supported by preliminary data in studies of the effects of reducing systemic IL-6 levels demonstrating ameliorating effects of such treatments (unpublished results). This augurs well for the possibility of designing new treatment strategies for clinical evaluation.

A number of findings presented here favour the hypothesis that functional deactivation of T cells and macrophages occurs locally in the growing tumours. Several of the murine MM cell lines, and in particular lines AC29 and AC31, used in this study, are avid producers of TGFβ [18]. TGFβ has been shown to be the major factor in induction of either anergy or immune deviation in other tumour systems [16, 42, 48]. Furthermore, the *in situ* phenotyping of leucocytes in MM tumours demonstrated a selective down-regulation of CD3 and CD2 expression on TIL and of MHC class II, CD11b and possibly CD18 on TIM (Fig. 2). The former phenomenon is found in anergised $CD4^+$ T cells [51], and may be suggestive of an impaired function of these cells. However, stimulation can also cause transient down-regulation of CD3 [14], and clonal selection of T cells with $CD3\delta^{low}/CD3\gamma^{high}$ phenotype can occur [2]. In a polyclonal population, stimulation usually causes down-regulation of the CD3γ and -ε chains, whereas the CD3δ, and -ζ chains are up-regulated [14]. In the case of MM-infiltrating TIL a selective down-regulation of CD3δ, relative to CD3ε expression, was demonstrated on the mRNA level [24], and the cells appeared conspicuously unresponsive to most stimuli in vitro. This down-regulation could be partially reversed by antisense-RNA-mediated inhibition of tumour-derived TGFβ expression [18]. Since it has been shown that the CD3δ chain is essential either for

surface routing or stable expression of the TCRαβ [8], the CD3δ down-regulation in MM-infiltrating TIL may be indicative of a profound functional inactivation of these cells in the local tumour milieu. This may be exacerbated by the absence of detectable CD2 expression in these cells, since CD2 signal transduction appears to play a role in both antigen- and mitogen-dependent stimulation of proliferation and cytokine production, and stimulation via the CD2 pathway may depend in part upon the expression of the TCR-CD3 complex (reviewed in [6]). To our knowledge selective down-regulation of either CD3δ or CD2 in tumour-infiltrating T lymphocytes has not been previously described, but a parallel may be found in the report on selective down-regulation of CD3γ and -ζ in peripheral T cells from colon- and renal-carcinoma-bearing mice [34]. The mechanism(s) controlling these selective changes in signal-transducing cell-surface molecules are unknown, but work by others [34], as well as preliminary in vitro studies in our lab [18] (and unpublished data) suggest that tumour-cell-derived soluble factor(s), including TGFβ, may play an important role.

TGFβ may likewise play an important role in recruitment and subsequent modulation of macrophage effector functions. MM-derived TGFβ is a strong chemoattractant for monocytes/macrophages [46]. Once in the tumour environment, MM-derived TGFβ could stimulate the macrophages to produce additional TGFβ, as well as platelet-derived growth factor, a growth-factor for mesothelioma cells [28], IL-1, IL-6 and TNFα [10, 46, 47]. In contrast, TGFβ may, over time, down-regulate the expression of MHC class II antigens and integrins by TIM, as seen in the present study, thereby compromising their antigen-presenting capacity [48, 50]. Thus, if TGFβ production or receptor binding and signal transduction could be abrogated [7], it might be possible to break the circuit and allow immune effector functions to prevail – or, at the least, allow for greater efficacy of conventional treatments such as surgery. Using antisense technology, our recent studies suggest that this is indeed a realistic possibility [18].

Whether TNFα is involved in the often extensive necrosis seen even in well-vascularized tumours remains to be investigated. However, at least the AC29 tumour cell line is highly sensitive to the cytotoxic effect of TNFα in vitro (Bielefeldt-Ohmann, unpublished data). In that regard it may also be noteworthy that the only leucocytes detected by immunocytochemistry near or in the edge of necrotic foci were CD11b$^+$ macrophage-like cells and CD4$^+$ cells. Thus, MM tumour necrosis may in some cases be mediated, at least in part, by newly recruited CD11b$^+$ TIM, unaccompanied by cachexia [15, 43]. Macrophages procured from the peritoneal cavity of mice with i.p. tumour growth produced high levels of IL-6 and IL-1 spontaneously and following stimulation (Table 3), corroborating the hypothesis that continued recruitment of macrophages and production of high levels of "proinflammatory" cytokines may ultimately lead to systemic effects [38], and that it is these effects rather than the tumour bulk *per se* that ultimately cause the demise of the host.

The cause of the disappearance or functional depression of cells retained in the peripheral lymphoid organs around the time of clinical manifestation of MM development is unknown, although several possibilities could be considered, including (a) chronic exposure to tumour antigens at inappropriate levels [47], (b) cytokine-induced changes in lymphocyte trafficking and cell activities, i.e., immunodeviation [37], (c) corticosteroid- or cytokine-induced apoptosis of activated lymphocytes [27]. Those cells not yet deleted from or trafficked out of the organ may nevertheless have been adversely affected and therefore showing decreased reactivity, even to non-specific stimuli such as a mitogen. If the MM-antigen-specific cells are the first to be affected by inhibitory signals, then that may explain the difficulties in demonstrating a MM-specific T cell response in vitro and in vivo, in the latter case using the Winn assay (Bielefeldt-Ohmann, unpublished data) or DTH responsiveness (Table 4), although at least the latter phenomenon could also be due to inhibition by TGFβ [33].

In summary, we have shown that a range of cytokines with potentially adverse effects on immune effector functions and tissue cell integrity are produced during MM development and, perhaps most important, that T lymphocytes in the tumour microenvironment show profound alterations in signal transduction molecules. Additional adverse effects on peripheral lymphoid organs occur, all of which may contribute to the escape of MM cells from immuno-mediated destruction. Further elucidation of the mechanisms governing all of these phenomena might enhance the chances for developing treatment strategies to reverse or diminish the impact and thus improve the prognosis for patients with this type of cancer.

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References

1. Abraham E, Chang Y-H (1992) Haemorrhage-induced alterations in function and cytokine production of T cells and T cell subpopulations. Clin Exp Immunol 90: 497
2. Alarcon B, Ley SC, Sanchez-Madrid F, Blumberg RS, Ju ST, Fresno M, Terhorst C (1991) The CD3-γ and CD3-δ subunits of the T cell antigen receptor can be expressed within distinct functional TCR/CD3 complexes. EMBO J 10: 903
3. Bielefeldt-Ohmann H (1987) Double immuno-labeling systems for phenotyping of immune cells harboring bovine viral diarrhoea virus. J Histochem Cytochem 35: 627
4. Bielefeldt-Ohmann H, Babiuk LA (1986) Bovine alveolar macrophages: functional heterogeneity among subpopulations defined by differential centrifugation on a discontinuous density gradient. J Leukoc Biol 39: 167
5. Reference deleted
6. Bierer BE, Sleckman BP, Ratnofsky SE, Burakoff SJ (1989) The biological roles of CD2, CD4, and CD8 in T-cell activation. Annu Rev Immunol 7: 579
7. Brandes ME, Wakefield LM, Wahl SM (1991) Modulation of monocyte type I transforming growth factor-β receptors by inflammatory stimuli. J Biol Chem 266: 19697

8. Buferne M, Luton F, Letourneur F, Hoeveler A, Couez D, Barad M, Malissen B, Schmitt-Verhulst A-M, Boyer C (1992) Role of CD3δ in surface expression of the TCR/CD3 complex and in activation for killing analyzed with a CD3δ-negative cytotoxic T lymphocyte variant. J Immunol 148: 657
9. Burger R, Gramatzki M (1993) Responsiveness of the interleukin (IL)-6-dependent cell line B9 to IL11. J Immunol Methods 158: 147
10. Chantry D, Turner M, Abney E, Feldmann M (1989) Modulation of cytokine production by transforming growth factor-β. J Immunol 142: 4295
11. Cherwinsky HM, Schumacher JH, Brown KD, Mosmann TR (1987) Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J Exp Med 166: 1229
12. Christmas T, Manning LS, Garlepp MJ, Musk AW, Robinson BWS (1993) Effect of interferon alpha-2a on malignant mesothelioma. J Interferon Res 13: 9
13. Davis MR, Manning LS, Whitaker D, Garlepp MJ, Robinson BWS (1992) Establishment of a murine model of malignant mesothelioma. Int J Cancer 52: 881
14. Doskow JR, Wilkinson MF (1992) CD3-γ, -δ, -ε, -ζ, T-cell receptor-α and -β transcripts are independently regulated during thymocyte ontogeny and T-cell activation. Immunology 77: 465
15. Fan S-T, Edgington TS (1993) Integrin regulation of leukocyte inflammatory functions. CD11b/CD18 enhancement of the tumour necrosis factor-α responses of monocytes. J Immunol 150: 2972
16. Fargeas C, Wu CY, Nakajima T, Cox D, Nutman T, Delespesse G (1992) Differential effect of transforming growth factor β on the synthesis of T_h1- and T_h2-like lymphokines by human T lymphocytes. Eur J Immunol 22: 2173
17. Fitzpatrick DR, Wie J, Webb D, Bonfiglioli R, Gardner ID, Mathews JD, Bielefeldt-Ohmann H (1991) Preferential binding of *Chlamydia trachomatis* to subsets of human lymphocytes and induction of interleukin-6 and interferon-gamma. Immunol Cell Biol 69: 337
18. Fitzpatrick DR, Bielefeldt-Ohmann H, Himbeck RP, Jarnicki AG, Marzo AL, Robinson BWS (1994) Transforming growth factor-beta: antisense RNA-mediated inhibition affects anchorage-independent growth, tumourigenicity and T-cell infiltration in malignant mesothelioma. Growth Factors 10 (in press)
19. Fulton AM (1980) Tumour-associated macrophages. In: Heppner GH, and Fulton AM (eds) Macrophages and cancer. CRC Press, Baton Rouge, pp 97–112
20. Green LM, Reade JL, Ware CF (1984) Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. J Immunol Methods 70: 257
21. Haskill JS, Yamamura Y, Radov L, Parthenais E (1976) Are peripheral and in situ tumour immunity related? Ann NY Acad Sci 276: 373
22. Henderson DW, Shilkin KB, Whitaker D, Attwood HD, Constance TJ, Steele RH, Leppard PJ (1992) The pathology of malignant mesothelioma, including immunohistology and ultrastructure. In: Henderson DW, Shilkin KB, Langlois SLP, Whitaker D (eds) Malignant mesothelioma. Hemisphere, New York, p 69
23. Hurliman J, Sarage P (1985) Mononuclear cells infiltrating human mammary carcinomas: immunohistochemical analysis with monoclonal antibodies. Int J Cancer 35: 753
24. Jarnicki AG, Fitzpatrick DR, Marzo A, Robinson B, Bielefeldt-Ohmann H (1993) Surface antigen and cytokine profile of tumour–infiltrating lymphocytes (TIL) in malignant mesothelioma. J Leukoc Biol 54[Suppl]: 90
25. Kay J, Porcelli S, Tite J, Jones B, Janeway CA (1983) Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. J Exp Med 158: 836
26. Kelso A (1990) Frequency analysis of lymphokine-secreting $CD4^+$ and $CD8^+$ T cells activated in a graft-versus-host reaction. J Immunol 145: 2167
27. King LB, Ashwell JD (1993) Signaling for death of lymphoid cells. Curr Opin Immunol 5: 368
28. Langerak AW, Vietsch H, Bouts MJ, Hagemeijer A, Versnel MA (1993) A spontaneously in vitro transformed mesothelial cell line has a similar pattern of PDGF chain and PDGF receptor expression to malignant mesothelioma cell lines. Eur Respir Rev 3: 170
29. Leo O, Foo M, Sachs DH, Samuelsen LE, Bluestone JA (1987) Identification of a monoclonal antibody specific for murine T3. Proc Natl Acad Sci USA 84: 1374
30. Martin MS, Caignard A, Hammann A, Pelletier H, Martin F (1987) An immunohistological study of cells infiltrating progressive and regressive tumours induced by two variant subpopulations of a rat colon cancer cell line. Int J Cancer 40: 87
31. Manning LS, Davis MR, Bielefeldt-Ohmann H, Marzo AL, Garlepp MJ, Whitaker D, Robinson BWS (1993) Evaluation of immunogenicity of murine mesothelioma cells by immunization. Eur Resp Rev 3: 234
32. Manning LS, Rose AH, Bowman RV, Christmas TI, Garlepp MJ, Robinson BWS (1992) Immune function related to asbestos exposure and mesothelioma, and immunotherapy of mesothelioma. In: Henderson DW, Shilkin KB, Langlois SLP, Whitaker D (eds) Malignant mesothelioma. Hemisphere, New York, p 278
33. Meade R, Askenase PW, Geba GP, Neddermann K, Jacoby RO, Pasternak RD (1992) Transforming growth factor-β_1 inhibits murine immediate and delayed type hypersensitivity. J Immunol 149: 521
34. Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC (1992) Alterations in signal transduction molecules in T lymphocytes from tumour-bearing mice. Science 258: 1795
35. Mosman TR, Schumacher J, Fiorentino DF, Leverah J, Moore KW, Bond MW (1990) Isolation of mAb specific for IL4, IL5, IL6, and a new Th2-specific cytokine synthesis inhibitory factor (CSIF; IL10) using a solid phase radioimmunosorbent assay: blocking and non-blocking anti-CSIF mAb. J Immunol 145: 2938
36. Musk AW, Bowman RV, Christmas TI, Robinson BWS (1992) Management of malignant mesothelioma. In: Henderson DW, Shilkin KB, Langlois SLP, Whitaker D (eds) Malignant mesothelioma. Hemisphere, New York, p 292
37. Pabst R, Binns RM (1989) Heterogeneity of lymphocyte homing physiology. Several mechanisms operate in the control of migration to lymphoid and non-lymphoid organs in vivo. Immunol Rev 108: 83
38. Pinsky MR, Vincent J-L, Deviere J, Alegre M, Kahn RJ, Dupont E (1993) Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. Chest 103: 565
39. Rao M, Lee WT, Conrad DH (1987) Characterization of a monoclonal antibody directed against the murine B lymphocyte receptor for IgE. J Immunol 138: 1845
40. Strassman G, Jacob CO, Evans R, Beall D, Fong M (1992) Mechanisms of experimental cancer cachexia. Interaction between mononuclear phagocytes and colon-26 carcinoma and its relevance to IL-6-mediated cancer cachexia. J Immunol 148: 3674
41. Strassmann G, Masui Y, Chizzonite R, Fong M (1993) Mechanisms of experimental cancer cachexia. Local involvement of IL-1 in colon-26 tumour. J Immunol 150: 2341
42. Tada T, Ohzeki S, Utsumi K, Takiuchi H, Muramatsu M, Li X-F, Shimizu J, Fujiwara H, Hamaoka T (1991) Transforming growth factor-β-induced inhibition of T cell function. Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumour-bearing state. J Immunol 146: 1077
43. Teng MN, Park BH, Koeppen HKW, Tracey KJ, Fendley BM, Schreiber H (1991) Long-term inhibition of tumour growth by tumour necrosis factor in the absence of cachexia or T-cell immunity. Proc Natl Acad Sci USA 88: 3535
44. Vink A, Coulie P, Warnier G, Renauld J-C, Stevens M, Donckers D, Snick J van (1992) Mouse plasmacytoma growth in vivo: enhancement by interleukin 6 (IL-6) and inhibition by antibodies directed against IL-6 or its receptor. J Exp Med 172: 997
45. Vose BM, Moore M (1985) Human tumour–infiltrating lymphocytes. Semin Hematol 22: 27

46. Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl LM, Roberts AB, Sporn MB (1987) Transforming growth factor type β induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci USA 84: 5788
47. Wang R, Murphy KM, Loh DY, Weaver C, Russell JH (1993) Differential activation of antigen-stimulated suicide and cytokine production pathways in CD4+ T cells is regulated by the antigen-presenting cell. J Immunol 150: 3832
48. Wilbanks GA, Streilein JW (1992) Fluids from immune previleged sites endow macrophages with the capacity to induce antigen-specific immune deviation via a mechanism involving transforming growth factor-β. Eur J Immunol 22: 1031
49. Yagita H, Nakamura T, Karasuyama H, Okumura K (1989) Monoclonal antibodies specific for murine CD2 reveal its presence on B as well as T cells. Proc Natl Acad Sci USA 86: 645
50. Yorochko AD, Burger CJ, Elgert KD (1990) Tumour modulation of autoreactivity: decreased macrophage and autoreactive T cell interactions. Cell Immunol 127: 105
51. Zanders ED, Lamb JR, Feldmann M, Green N, Beverley PCL (1983) Tolerance of T-cell clones is associated with membrane antigen changes. Nature 303: 625
52. Zugmaier G, Paik S, Wilding G, Knabbe C, Bano M, Lupu R, Deschauer B, Simpson S, Dickson RB, Lippman M (1991) Transforming growth factor β1 induces cachexia and systemic fibrosis without an antitumour effect in nude mice. Cancer Res 51: 3590