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Thymic lymphomas in interleukin 9 transgenic mice

Jean-Christophe Renauld[1], Nathalie van der Lugt[4], Anne Vink[1], Marian van Roon[4], Catherine Godfraind[2], Guy Warnier[1], Hartmut Merz[3], Alfred Feller[3], Anton Berns[4] & Jacques Van Snick[1]

[1]*Ludwig Institute for Cancer Research, Brussels Branch and Experimental Medicine Unit, Catholic University of Louvain, Brussels, Belgium;* [2]*Department of Pathology, University of Louvain, Brussels, Belgium;* [3]*Institut für Pathologie, University of Lübeck, Lübeck, Germany;* [4]*the Netherlands Cancer Institute, Amsterdam, The Netherlands*

Transgenic mice overexpressing the interleukin 9 gene were generated to study the biological activity of this cytokine *in vivo*. Although no major histological or morphological modifications of the lymphoid system were observed in most animals, ~7% of transgenic mice developed thymic lymphomas at the age of 3–9 months. The tumor cells, which were clonal, with unique T cell rearrangements, were double positive for the expression of CD4 and CD8. The need for additional transforming events, suggested by the low incidence of spontaneous tumors, was further indicated by the high susceptibility of the transgenic animals to injections of low doses of N-methyl-N-nitrosourea, a chemical carcinogen with a thymic tropism. Expression of interleukin 9 was required for optimal tumor growth *in vivo*, as one of the tumors studied, which had lost the transgene, was much more efficiently transplanted into transgenic than in normal mice. Moreover, the *in vitro* proliferative activity of interleukin 9 on cell lines derived from such transgene-negative tumors suggests that an autocrine loop mediates the proliferation of these cells *in vivo*. Taken together, these results indicate that dysregulated IL-9 expression could be involved in the development of some T cell malignancies.

Introduction

Interleukin 9 (IL-9) was originally identified as a T cell growth factor for murine T helper clones (Uyttenhove *et al.*, 1988; Van Snick *et al.*, 1989), an observation later confirmed with human T cells (Renauld *et al.*, 1990b; Houssiau *et al.*, 1993). By contrast, IL-9 seems inactive on freshly isolated T cells (Schmitt *et al.*, 1989; Houssiau *et al.*, 1993) with the exception of mouse day 14 fetal thymocytes that proliferate in response to the combination of IL-9 and IL-2 (Suda *et al.*, 1990).

In addition to this T-cell stimulatory potential, several other activities have been ascribed to this cytokine (Renauld *et al.*, 1993). Murine IL-9 was identified as the protein responsible for a mast cell growth activity termed MEA (Hültner *et al.*, 1990) and further shown to induce the production of IL-6 by these cells (Hültner & Moeller, 1990). Human IL-9 was found to promote the growth of a human megakaryoblastic leukemia (Yang *et al.*, 1989) and a BFU-E promoting activity on erythroid progenitors was described both in human (Donahue *et al.*, 1990) and murine assays (Williams *et al.*, 1990). IL-9 was also shown to potentiate IL-4-induced Ig production by murine and human peripheral B cells (Dugas *et al.*, 1993; Petit-Frère *et al.*, 1993). The recent observation that IL-9 induced the differentiation of mouse immature neural cell lines suggests that this factor could also play some role in the neural ontogeny (Mehler *et al.*, 1993).

Production of IL-9 is restricted to activated T cells (Renauld *et al.*, 1990) and tightly regulated by IL-2 (Houssiau *et al.*, 1992). By contrast, constitutive IL-9 expression has been demonstrated in HTLV-1 infected T cell lines (Yang *et al.*, 1989; Kelleher *et al.*, 1991) and, by *in situ* hybridization, in large cell anaplastic lymphomas and Hodgkin disease (Merz *et al.*, 1991). In the mouse, the involvement of IL-9 in malignancies was also suggested by experiments showing that a T helper cell clone transfected with the IL-9 cDNA formed tumors *in vivo* (Uyttenhove *et al.*, 1991) and by the proliferative response to IL-9 of thymic lymphomas *in vitro* (Vink *et al.*, 1993).

In this report, we have investigated further the biological activity of IL-9 *in vivo* by using transgenic mice constitutively expressing high levels of IL-9. Our results support the hypothesis that dysregulation of IL-9 expression is involved in T cell oncogenesis.

Results

IL-9 transgenic mice constitutively express high levels of IL-9

IL-9 transgenic mice were generated that carried the murine IL-9 gene under the control of the pim-1 promoter, two copies of the Eμ enhancer and the M-MLV LTR (Figure 1). Five transgene-positive mice were obtained (mice no. 5, 25, 54, 83 and 95). Transgenic animals expressed large amounts of IL-9 message in all organs tested (Figure 2). The intensity of the signal suggests that the transgene exhibits no tissue specificity. However, the rather remote possibility that its activity is restricted to a particular cell type present in all organs cannot be formally excluded. High levels of biologically active IL-9, ranging from 0.1 to 2 $\mu g\ ml^{-1}$, were detected in the serum of transgenic but not of control mice. No significant differences were noticed in size, fertility or survival between normal and transgenic animals.

IL-9 transgenic mice spontaneously develop thymic lymphomas

No gross morphological changes were observed in the lymphoid tissues of the IL-9 transgenic mice. FACS

Correspondence: Jean-Christophe Renauld
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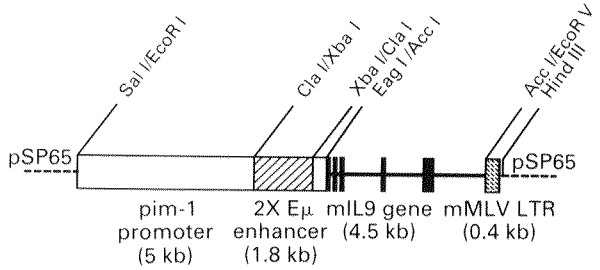

Figure 1 Structure of the IL-9 transgene. The IL-9 gene is shown as wide and narrow boxes corresponding respectively to the exons and introns. The pim-1 promoter, shown as an open rectangle, contains the TATA box and the Cap site. The two copies of the Eμ enhancer as well as the mMLV-LTR are hatched

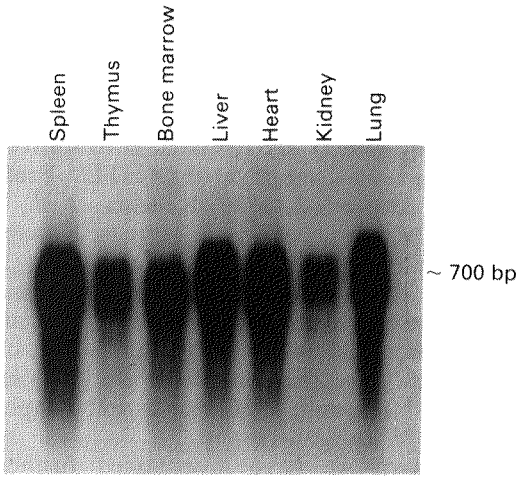

Figure 2 Expression of the transgene. Northern blot analysis of total RNA isolated from tissues from transgenic mice (1 μg per lane) was performed using a ^{32}P-labeled IL-9 cDNA as a probe. Under these conditions, no message was detectable in control mice

analysis of spleen, thymus and lymph node cells did not reveal significant differences in the percentage of CD3, CD4 and CD8 positive cells (data not shown). However, 14 out of 200 transgenic mice followed over a 12 month period developed malignancies involving lymphoid organs, while no control mice ever did so. Most tumors were located primarily in the thymus but also invaded the spleen and the lymph nodes, eventually infiltrating all organs. Tumors arose in mice between 3 to 9 months of age in the progeny of all founders. Microscopic examination indicated that thymic tumors consisted of medium-size lymphocytes with a scanty and moderately basophilic cytoplasma (Figure 3).

By FACS analysis, all tumors were found to express high levels of the Thy-1 antigen. All but one were CD4 and CD8 positive, suggesting that the precursors of the transformed cells were immature thymic T cells. Out of 13 tumors analysed for CD3 expression, three were CD3 negative, seven CD3 low and three CD3 high. HSA/J11d, an antigen carried by immature thymocytes, was expressed by all the tumors tested. B220 and Mac-1 antigens were negative. Southern analysis of DNA extracted from most tumors was consistent with a monoclonal rearrangement of the TCRβ locus

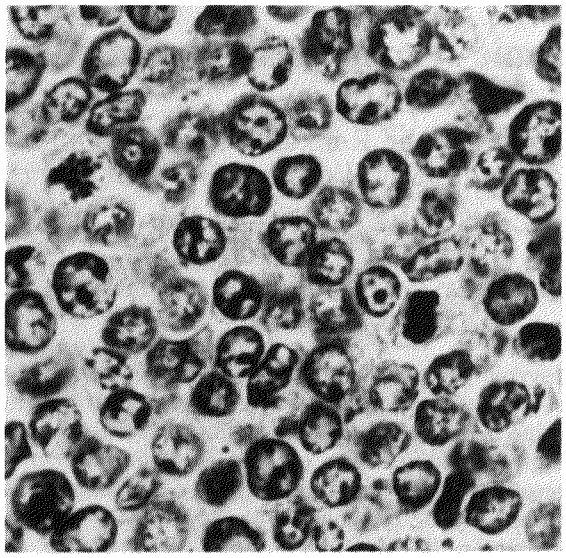

Figure 3 Histology of an IL-9 transgenic thymic lymphoma. Nuclei are polymorphic and contain multiple, small nucleoli. Cytoplasma is hardly visible. Lymphocytes are well separated from another. Fibres are fine and sparse

(Figure 4a). RNA analysis indicated that all tumors expressed TCRβ specific mRNA, with two different transcripts in most tumors (Figure 4b).

High susceptibility of IL-9 transgenic mice to chemical carcinogenesis

Tumor incidence in the IL-9 transgenic mice was about 7%, irrespectively of the integration site of the transgene. This observation suggested that additional transforming events were required for full transformation of the tumoral precursor, as previously demonstrated for pim-1 transgenic mice (Breuer *et al.*, 1989). To test this hypothesis, normal and IL-9 transgenic mice were injected with low doses of N-methyl-N-nitrosourea (MNU), a mutagen known to induce T cell lymphomas. As shown in Figure 5, transgenic mice showed a higher susceptibility to the tumorigenic effect of MNU. As low a dose as 1 μg of MNU was tumorigenic in all transgenic animals but not in any of the control mice. Interestingly, at high doses of mutagen, tumors arose in normal or transgenic animals with similar kinetics. Like the spontaneous tumors, the MNU-induced tumors were CD4 and CD8 positive.

IL-9-dependence of transgenic lymphomas in vivo

All tumors but one were successfully transplanted by intraperitoneal injection (i.p.) into normal syngeneic animals. The exception, 9T4, did not bear the transgene as it arose from a chimeric mouse, where part of the cells had lost the IL-9 transgene. This animal indeed expressed high levels of circulating IL-9 but completely failed to transmit the transgene to its progeny. Southern analysis performed on 9T4 tumor cells did not show any evidence for transgene integration and no IL-9 message was detected in the RNA of these cells by Northern blot hybridization (Figure 6). The finding that 9T4 was readily transplanted into transgenic mice supports the hypothesis that the unability to

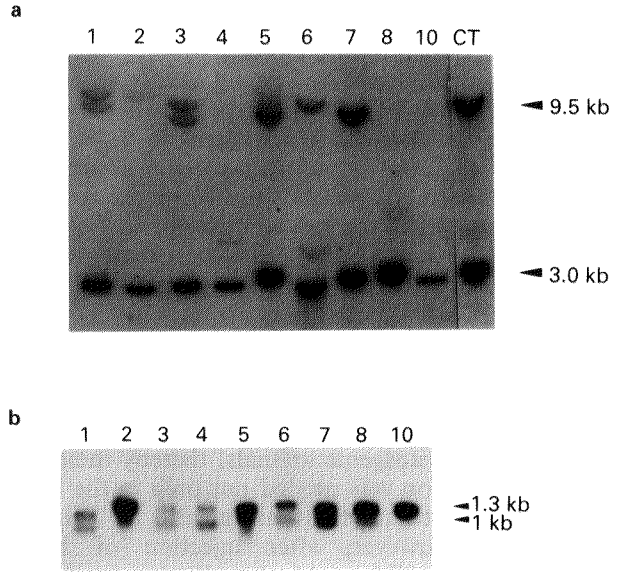

Figure 4 TCRβ genomic rearrangements and mRNA expression in IL-9 transgenic lymphomas. (**a**) Genomic DNA was extracted from the tumors, digested with HindIII, electrophoresed through 0.7% agarose and blotted onto nylon membrane before hybridization with a TCRβ cDNA probe. The arrows indicate the 3 kb and 9.5 kb bands containing the Cβ2 and Cβ1 respectively. Rearrangement to either locus results in a disappearance of the 9.5 kb band either by deletion or by alteration of the fragment size, whereas the 3 kb band stays unchanged. Numbering refers to the tumors 9T1–8 and 9T10; CT correspond to liver genomic DNA (germline conformation). Genomic rearrangements have been confirmed with another restriction enzyme (data not shown). (**b**) Northern blot analysis of the same tumors using 1 μg of total RNA and a TCRβ cDNA probe. Arrows indicate the two different transcripts detected. The largest one (1.3 kb) is thought to correspond to mature form of TCRβ-RNA transcribed after complete rearrangement of the β locus and the small one (1 kb) is thought to be transcribed from partially rearranged TCRβ-genes from a promoter located between V and D regions and activated by the DJ rearrangements (Siu *et al*, 1984)

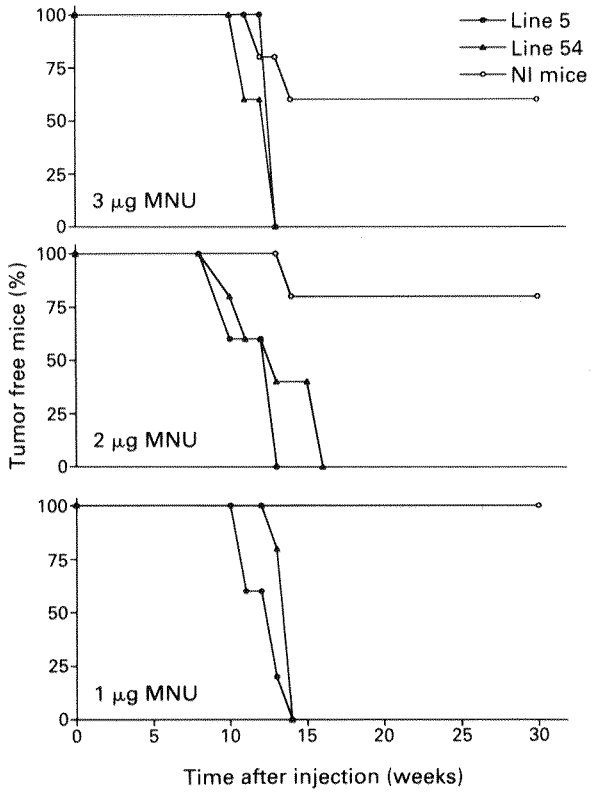

Figure 5 MNU-induced lymphoma incidence in IL-9 transgenic and control mice. Six week old mice (5 per group) were injected i.p. with 1, 2 or 3 μg of MNU and were examined every week for lymphoma development over a 5 month period. Mice were killed when moribund and lymphomas were collected. All tumors arose between 3 and 4 months after MNU injection

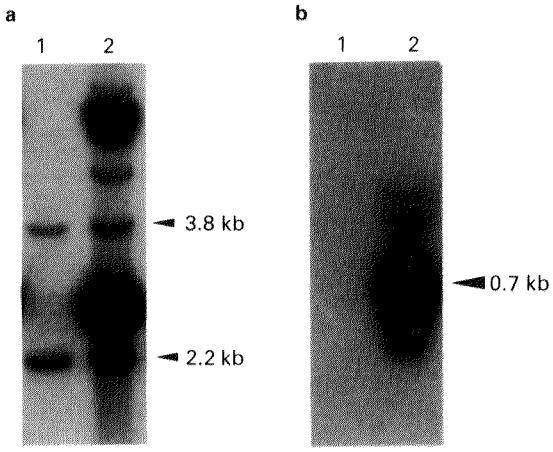

Figure 6 Absence of the IL-9 transgene in the 9T4 tumor. (**a**) Southern blot analysis of genomic DNA from tumors 9T4 (lane 1) and 9T5 (lane 2). DNA was digested with EcoRI and hybridization was performed with the IL-9 cDNA probe. Arrows indicate the 3.8 kb and 2.2 kb bands corresponding to the endogeneous IL-9 genes; all additional bands correspond to the transgene. (**b**) Northern blot analysis of the same tumors using 1 μg of total RNA and the IL-9 cDNA probe

grow in normal syngeneic animals was due to the loss of the transgene.

Interestingly, when high doses of 9T4 tumor cells were injected i.p. into normal mice (10^7 cells per mouse), tumors developed in four out of five animals. However, the growth of these tumors was significantly delayed from less than 5 up to 14 weeks in control *vs* transgenic mice (Figure 7). DNA and RNA analyses of 9T4 tumors arising in normal mice indicated that they did not result from selection of a transgene-positive or IL-9 expressing subpopulation. However, subsequent injection of two of these tumors into normal and transgenic animals showed that they had acquired the ability to grow indistinctly in a transgenic or normal environment (data not shown).

In vitro *growth promoting activity of IL-9 on transgenic lymphomas*

The observation that transgenic lymphomas were dependent on IL-9 for *in vivo* growth raised the question whether IL-9 acted directly or indirectly on these cells. To address this issue, we took advantage of transgene-negative tumors, such as 9T4, arising in chimeric mice sporadically generated in the progeny of one of the founders. Cell lines were derived *in vitro* from the transgene-negative 9T4 and 9T7 tumors in the presence of IL-2, IL-7 and IL-9, three potent growth factors for thymic lymphomas (Vink *et al.*, 1993). When stable cell lines were established, their proliferative responses to IL-9 were evaluated. We found

that IL-9 stimulated the proliferation of these cells in short term assays, either alone or in synergy with IL-2 (Figure 8). IL-9 (or the combination of IL-9 and IL-2) also sustained the long-term proliferation of cell lines derived from these tumors (data not shown).

Discussion

The present study demonstrates that constitutive overexpression of IL-9 results in the development of thymic lymphoblastic lymphomas in ~7% of IL-9 transgenic mice. This tumor incidence is increased to 100% after injection of low doses of mutagen that are totally innocuous in normal animals. Analysis of the lymphoid organs of tumor-free IL-9 transgenic mice did not suggest the existence of a pretransformed stage. These data contrast with those reported for other cytokines possibly involved in malignancies. In IL-7 transgenic mice, a reduction in double positive thymocytes and a polyclonal lymphoproliferation in the skin preceed the development of B or T cell lymphomas which occur within 4 months of life (Rich *et al.*, 1993). Similarly, IL-6 transgenic mice develop a non-tumoral plasmacytosis that could be further transformed after c-*myc* rearrangement in a particular genetic background (Suematsu *et al.*, 1992). The absence of such a pre-neoplastic state in the IL-9 transgenic mice suggests that this factor is involved in later stages of the transformation process.

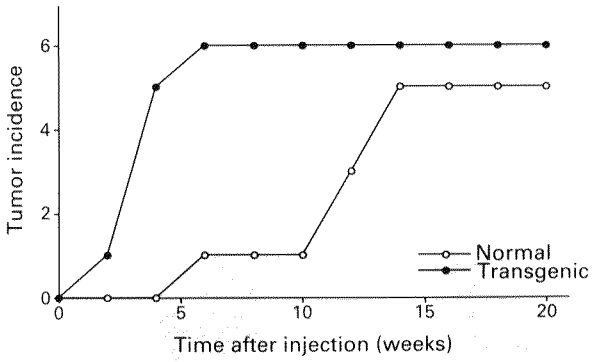

Figure 7 *In vivo* development of 9T4 tumors in normal or IL-9 transgenic mice. Freshly isolated 9T4 tumor cells (10^7 cells) were injected i.p. into normal or transgenic syngeneic mice ($n = 6$)

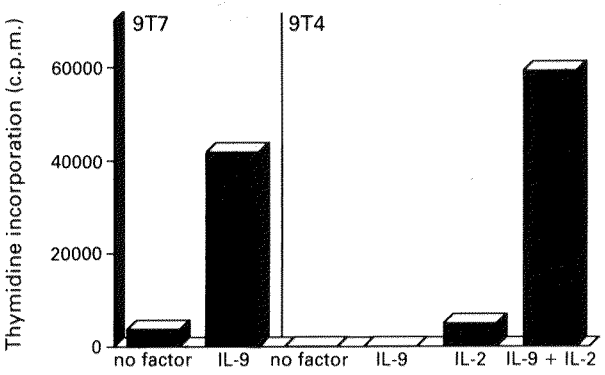

Figure 8 IL-9 dependent proliferation of transgene-defective tumors *in vitro*. Lymphoma cells were incubated at 10^3 per well (9T7) or two 10^3 per well (9T4) in the presence or absence of IL-9 (500 U ml^{-1}) or IL-2 (100 U ml^{-1}) as indicated. Thymidine incorporations were measured in triplicate cultures on day 7

The requirement for transforming events other than overexpression of IL-9 was suggested by the low incidence of spontaneous lymphomas in IL-9 transgenics and was further confirmed by the experiments with MNU-induced carcinogenesis. While the transforming capacity of N-methyl-N-nitrosourea has been associated with *ras* activating mutations, the mutations involved in IL-9 transgenic tumors remain to be elucidated.

The occurrence of transgene-negative tumors in chimeric animals gave us the opportunity to study the role of IL-9 in the development of thymic lymphomas. The transgene-negative tumor 9T4 was found to grow preferentially in transgenic animals, thereby indicating that IL-9 expression was needed not only in the initial steps involved in transformation but also for actual *in vivo* growth. However, after injection of higher numbers of cells, 9T4 could also develop in normal animals, though after a much longer delay. Interestingly, the tumor cells which finally grew in normal animals no longer needed the transgenic environment as they subsequently grew equally well in normal and transgenic mice. As no rearrangement nor re-expression of the IL-9 gene was noticed in these cells, one might conclude that other pathways are used to escape from the dependence on IL-9.

Permanent cell lines derived *in vitro* from transgene negative tumors were used to provide evidence of a direct growth activity of IL-9, either alone or in synergy with IL-2. These experiments demonstrated a direct proliferative effect of IL-9 on the tumor cells. However, they did not formally demonstrate that this effect is responsible for the *in vivo* tumor growth nor rule out indirect effects *in vivo* on accessory cells or via a modulation of putative anti-tumor immune responses.

The hypothesis that IL-9 overexpression could result in tumoral proliferation was previously raised by the observation that a murine T helper clone could be rendered tumorigenic upon transfection with the IL-9 cDNA (Uyttenhove *et al.*, 1991). Moreover, IL-9 was recently reported to stimulate the growth of chemically- or radiation-induced thymic lymphomas *in vitro* (Vink *et al.*, 1993). In the human, constitutive IL-9 expression was found in lymph nodes from patients with Hodgkin disease or large cell anaplastic lymphoma (Merz *et al.*, 1991). Furthermore, the hypothesis of an autocrine loop in some of these malignancies was recently supported by the observation that an anti-IL-9 antibody could block the *in vitro* proliferation of one Hodgkin cell line (Gruss *et al.*, 1992). Taken together, these results demonstrate that IL-9 can act as an oncogene *in vivo* and suggest that IL-9 overexpression could be involved in the development of T cell malignancies.

Materials and methods

Transgene construct and generation of transgenic mice

A 4.5 kb AccI fragment of the IL-9 gene was obtained by partial digestion of the genomic clone previously described (Renauld *et al.*, 1990a). This region contained all the coding sequences, 1.5 kb of the 3′ untranslated region including two

consensus sequences for the polyadenylation signal, and 28 bp of the 5′ untranslated region. The fragment was isolated and inserted into the pSP65pim1 vector, a plasmid containing the promoter of the murine pim-1 oncogene (5 kb SalI-EcoRI fragment), including the TATA box and the cap site, followed by two copies of the Eμ enhancer (0.9 kb SalI fragment) and one copy of the mMLV LTR. This construct was micro-injected into the pronuclei of fertilized eggs of FVB/N mice (Taketo *et al.*, 1991). For the screening of transgenic mice, tail DNA was analysed as described below or serum IL-9 activity was measured on TS1 cells (Uyttenhove *et al.*, 1988).

Southern and Northern blot analyses

Total RNAs were isolated by the guanidine isothiocyanate/CsCl method and 1 μg of total RNA was analysed by Northern blotting as described (Ausubel *et al.*, 1993). High molecular weight DNA was prepared from the tails or tissue fragments and subjected to Southern blot analysis as described (Southern, 1975). Hybridisations were performed using the mouse IL-9 cDNA P40.2B4 (Van Snick *et al.*, 1989) or a 0.8 kb EcoRI cDNA fragment encoding the Cβ2 region of the TCR β (Hedrick *et al.*, 1984) as ^{32}P-labeled probes.

Microscopic and flow cytometry analyses

Tumor specimens were fixed in 4% buffered paraformaldehyde or Bouin fixative and paraffin embedded. 3–5 mm sections were cut and placed on glass slides. Tissue specimens were dehydrated, dewaxed and stained for H + E, Giemsa, PAS and with Gomorri silver stain.

For FACS analysis, a cell suspension was prepared from fresh tumor fragments and labeled with the following antibodies: D803E4 (anti-Thy-1.2), 145-2C11 (anti-CD3) (Leo *et al.*, 1987), GK1.5 (anti-CD4) (Wilde *et al.*, 1982), 53-6.72 (anti-CD8) (Ledbetter & Herzenberg, 1979), RA3-3A1 (anti-B220) (Coffman & Weissman, 1981) J11d (anti-HSA) (Bruce *et al.*, 1981).

In vitro *cell culture and cytokines*

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine. Recombinant murine IL-9 was produced in the baculovirus system and purified as previously described (Druez *et al.*, 1990). IL-9 units were calculated in the TS1 bioassay (Uyttenhove *et al.*, 1988), 1 U corresponding to 10 pg of recombinant protein.

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