Oncogene-linked in situ immunotherapy of pre-B lymphoma arising in Ep/ret transgenic mice

M Ichihara1,2, T Iwamoto1, K Isobe1, M Takahashi3, A Nakayama1, M Pu1, Y Dai1, A Parashar1, K Ohkus1, M Kato1, T Hotta2 and I Nakashima1

1Department of Immunology, 21st Department of Internal Medicine and 3Department of Pathology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan.

Summary We attempted to induce anti-tumour immunity for rejecting pre-B lymphoma derived from Ep/ret transgenic mice (TGM). We established pre-B-lymphoma cell lines of C57BL/6 × Balb/c background (H-2b) into which H-2k allantoigen and C3H background were introduced (retL1-6 and retL6-6), and we inoculated BCF mice with these immunising tumour cells. After these tumours were rejected by allantoigen (H-2k/C3H) background-specific effector cells, the mice were challenged with the pre-B-lymphoma cell line derived from the original Ep/ret TGM (ret0-2). All non-immunised control mice died within 80 days, whereas half the immunised mice survived for over 300 days. The immunity was also effective against primary pre-B-lymphoma cells from Ep/ret TGM and the ret-driven melanoma cell line (MEL-ret), but not against the pre-B-lymphoma cell line from Ep/myc TGM. This immunity was at least in part mediated by cell-mediated cytotoxicity that was specific to the ret oncogene product or ret-regulated antigen. Next we immunised Ep/ret TGM by inoculating them with retL6-6 cells once every 2 weeks for the age of 1 month. Interestingly, this immunisation enabled the TGM to survive longer than the non-immunised control group (P<0.05). Moreover, 2 of 11 transgenic mice receiving such immunisation were free from both macroscopic and microscopic tumours at the time when all of the 12 non-immunised control TGM had died from their tumour. This provides a new model for oncogene-linked immunotherapy research.

Keywords: oncogene ret; transgenic mouse; pre-B lymphoma; immunotherapy

Different types of tumour antigens have been found on malignant tumours in humans and laboratory animals (Klein, 1966; Old and Stockert, 1977; Schreiber et al., 1988; Urban and Schreiber, 1992). Many of them are simply recognised by antibodies and helper T cells, and only some of them act in rejecting tumours. The former type of antigens are useful for the diagnosis of tumours and for targeting tumours with toxic reagents, but only the latter are active in the immunological surveillance of tumours and can be best used for therapeutic and prophylactic purposes. Well-known tumour rejection antigens are nuclear antigens of tumour viruses such as SV40 T antigen (Klein, 1966), adenovirus E1A antigen (Dyson et al., 1992) and papillomavirus type 16 nucleoprotein (Chen et al., 1988), proteolysed fragments (peptides) of which can associate with class I major histocompatibility complex (MHC) antigens to be recognised by cytotoxic T lymphocytes (CTL). So-called unique antigens of chemically induced tumours and ultraviolet-induced tumours (Schreiber et al., 1988) also play a role in tumour rejection. Some of them have been characterised as the 85 kDa (Ulrich et al., 1986) and 96 kDa (Srivastava et al., 1986) stress-induced proteins or unique class I MHC antigens (Linsk et al., 1986; Stauss et al., 1986) or P91A (Lurquin et al., 1989) and P198 (Sibille et al., 1990) with mutations of a single nucleotide. Little is known, however, about the potential tumour rejection antigens occurring on spontaneously arising tumours in humans and laboratory animals, although the antigen recognised by autologous CTL on human melanomas has been recently shown to be encoded by the tyrosinase gene (Briehard et al., 1993), and a human homologue of the murine rejection antigen gp96 has been reported (Maki et al., 1990).

Recent progress in oncology has revealed the multistep actions of oncogenes in oncogenesis (Cory and Adams, 1988). The products of these oncogenes may be antigenic because of the occurrence of point mutations, deletion mutations or chromosomal translocation (Hellstrom and Hellstrom, 1989; Urban and Schreiber, 1992). There are many reported examples of tumour-specific antigens closely linked to oncogene proteins, such as mutated ras p21 (Ferramisco et al., 1985; Pullano et al., 1989; Jung and Schlessinger, 1991; Peace et al., 1991), the bcr–abl fusion protein (Van Denderen et al., 1989), mutated p53 (Gannon et al., 1990) and the deletion mutants of epidermal growth factor receptor (Humphrey et al., 1990). These tumour antigens are, in most cases, recognised by antibodies and helper T cells for antibody production. Exceptions are mutated p53, which is a nuclear suppressor gene product (Yanuck et al., 1993), and mutated ras proteins produced by recombinant vaccina viruses (Skipper and Stauss, 1993), which are recognised by CTL. So far, it is not known whether non-nuclear oncogene proteins in native tumour cells will act as tumour rejection antigens and whether any restricted mutational changes of the proteins can induce tumour rejection immunity.

We recently established Ep/ret (Iwamoto et al., 1991a) and MT/ret (Iwamoto et al., 1991b) transgenic mice (TGM), in which pre-B lymphoma and melanocytic tumours developed respectively. The ret oncogene, introduced into the TGM, is a fusion gene of the protein tyrosine kinase proto-ret, whose sequence coding the receptor domain is replaced with another gene named rfp (Takahashi et al., 1985), and the Ret protein is expressed in association with cell membrane (Taniguchi et al., 1992). In order to determine the answers to the two questions posed above, we tried to induce rejection immunity against these tumours. A new method was used to modify the transgenic tumour for immunisation; modified tumour cell lines were established from the tumour arising in the transgenic mice into which allantoigenes were introduced by crossing. The results show that the ret oncogene is active in inducing anti-tumour immunity which is effective in rejecting both transgenic tumours transplanted into otherwise genetically compatible hosts and tumours arising in transgenic mice in situ.
**Materials and methods**

**Mice**

C57BL/6 × Balb/c F1 (BCF1) (H-2<sup>b</sup>) and Balb/c females were bred in the Institute for Animal Research, Nagoya University School of Medicine, or purchased from Shizuoka Agricultural Center, Hamamatsu, Shizuoka. The Ep/ret TGM were previously established from BCF1 mice (Iwamoto et al., 1991a). Ep/ret TGM were bred with Balb/c, and the progeny containing the transgene were selected as previously described (Iwamoto et al., 1991a).

**Cells**

retO-2 was established from lymphoma developed in an Ep/ret TGM. MEL-ret (Taniguchi et al., 1992), a melanoma cell line, was derived from a metallothionen/ret TGM (Iwamoto et al., 1991b). An Ep/Myc pre-B-lymphoma cell line of C57BL/6 background (Yukawa et al., 1989) was kindly donated by Dr Yukawa (Institute for Molecular and Cellular Biology, Osaka University). These cell lines, which are listed in Table 1 together with their H-2 haplotypes, were used as challenging tumours to the previously immunised BCF1 mice.

**Establishment of immunising tumour cell lines**

In our previous study, we induced a strong anti-tumour immunity to original tumour cells by immunisation with the tumour cells xenografted by introducing allogeneic MHC Class I gene (Isobe et al., 1989). Instead of transfecting with the allogeneic MHC class I gene, we bred the Ep/ret TGM with C3H/HeJ mice expressing H-2<sup>b</sup> antigen to obtain lymphoma cell lines possessing the same character as retO-2 except for the H-2<sup>b</sup>/C3H background expression. We established two lymphoma cell lines (H-2<sup>ab</sup>), retLl-6 and retL6-6, as immunising tumour cells (Table 1).

**Immunisation procedure**

BCF1 male mice (2–3 months old) received i.p. injections of 1 × 10<sup>7</sup> immunising tumour cells twice in a month. Two weeks after the last i.p. injection, challenging tumour cells were injected i.p. into the mice and the survival time was estimated as compared with that of control mice that did not receive immunising tumour cells.

We also evaluated the effects of immunisation on tumour development in Ep/ret TGM. A group of Ep/ret TGM received repeated i.p. injections of 1 × 10<sup>7</sup> immunising tumour cells once every 2 weeks from the age of 1 month. Survival times from their birth were compared with those of control TGM that did not receive immunising tumour cells. Some mice were sacrificed and examined pathologically.

**Assay for cell-mediated cytotoxicity**

Spleen cells suspended in RPMI-1640 medium containing 10% fetal calf serum were sensitised in vitro with tumour cells irradiated with 1500 rad at a stimulator-to-target ratio of 1:10 for 4 days. The cytotoxicity of these cells was measured by the ⁵¹Cr-release assay. Target cells were labelled with ⁵¹Cr, mixed with effector cells and incubated for 4 h at 37°C. The supernatant was collected for measurement of radioactivity by a gamma scintillation counter. The percentage of specific lysis was calculated as follows:

\[
\text{Specific lysis (\%)} = \frac{(\text{experimental c.p.m.} - \text{spontaneous c.p.m.})}{(\text{maximum c.p.m.} - \text{spontaneous c.p.m.})} \times 100
\]

**Statistical analysis**

The survival rate was calculated using the Kaplan–Meier method. Statistical analysis was based on generalised Wilcoxon tests. Values were expressed as the mean ± standard deviation (s.d.).

**Results**

**Characterisation of the immunising/challenging tumour cell lines**

We first examined the characteristics of the immunising/challenging tumour cell lines. The negative cell-surface Ig expression and the selective rearrangement of the immunoglobulin heavy-chain genes indicated that the cell lines retO-2, retL1-6 and retL6-6 had the pre-B-lymphoma phenotype. A difference in the rearranged band size of the immunoglobulin gene between retL1-6 and retL6-6 showed that these lymphoma cell lines were different clones (data not shown). As might be anticipated, these two cell lines, which expressed H-2<sup>b</sup> antigen, were completely rejected in BCF1 mice after i.p. inoculation (data not shown).

**Induction of anti-tumour immunity rejecting TGM-derived tumour cells**

Figure 1 shows the survival time of mice previously immunised with retL1-6 or retL6-6 after challenging with 2 × 10⁶ retO-2 cells. Both retL1-6 and retL6-6 cell lines induced anti-tumour immunity, rejecting retO-2 cells. One-half to two-thirds of the mice survived more than 300 days after challenge. On the other hand, all the mice that had not been immunised died within 100 days. This difference was statistically significant (P < 0.01). These data demonstrated that two different lymphoma cell lines (retL1-6, retL6-6) independently induced anti-tumour immunity to the other lymphoma cell line (retO-2). When we inoculated 1 × 10⁷ of retL0-2 cells into the immunised mice, however, there was no statistically significant difference in the survival time between immunised and non-immunised mice, although the survival time of immunised mice was slightly longer than that of the non-immunised control mice. Survival rate was recorded in days post tumour challenge.

**Table 1** A list of transgenic tumour cell lines used in this study

| Cell line | Transgenic oncogene | H-2 | Used for |
|-----------|---------------------|-----|----------|
| retO-2    | ret                 | b/d | Challenge in vivo |
| retL1-6   | ret                 | k/d | Target of in vitro killing |
| retL6-6   | ret                 | k/d | Immunisation |
| MEL-ret   | ret                 | b   | Target of in vitro killing |
| Ep/MyC pre-B | myc              | b   | Challenge in vivo |
|           |                     |     | Target of in vitro killing |
non-immunised mice (data not shown). This result showed that the immunity induced was not very strong.

Next, we evaluated the specificity of the anti-tumour immunity induced by retL6-6 (Figure 2). Even when challenged by primary lymphoma cells from Epi/ret TGM, immunised mice survived longer ($P<0.05$) than non-immunised mice. Interestingly, the anti-tumour immunity induced by immunisation with Epi/ret B-lymphoma cells was also directed against MEL-ret melanoma cells, while no significant effect was observed on Epi/myc TGM-derived pre-B-lymphoma cells. These data suggested that the induced anti-tumour immunity was specific to the ret oncogene product or its closely related antigen.

A further study was conducted to determine whether cell-mediated cytotoxicity was induced by the immunising protocol. After inoculation of retL6-6 cells and ret0-2 cells into mice and in vitro secondary sensitisation of spleen cells from these mice with ret0-2 cells, the effector activity developed to kill ret0-2 cells in addition to retL6-6 cells. The effector cells also killed MEL-ret cells, but not Epi/myc TGM-derived pre-B-lymphoma cells (Table II). These results confirmed the specificity of the induced immunity against ret TGM-derived tumours.

### Suppression of tumour development in Epi-ret TGM by immunisation

As the final goal of this immunisation, we tested whether this immunisation procedure could suppress tumour development in Epi/ret TGM in situ. Figure 3 shows the survival time of the Epi/ret TGM which had or had not been immunised with retL6-6 cells. This immunisation enabled the TGM to survive longer than the non-immunised control group ($P<0.05$).

Autopsies were performed on all mice tested. In the non-immunised control group, all 12 TGM died from progression of lymphoma by day 174 after birth. In the immunised group, however, 8 of 11 (73%) TGM survived until day 174, three of which showed no evidence of lymphoma development on superficial examination. Two of these TGM were sacrificed, and another died of severe general emaciation, for reasons unknown. Pathological examination revealed that one of them had a lymphomatous mass in the abdomen, but the other two were pathologically free of lymphoma development (Figure 4a–c). This contrasted with extensive proliferation of lymphoma cells observed in the lymph nodes (Figure 4d and e) and bone marrow (Figure 4f) of all mice in the control group. Transmission of the ret gene into those lymphoma-free mice was confirmed by repeated testing from tail DNA.

### Discussion

This study shows that pre-B-lymphoma cells arising in the Epi/ret transgenic mouse carry tumour antigens which induce anti-tumour immunity for both in vivo tumour rejection and in vitro tumour cell killing. This immunity was not as strong as that produced by virally or chemically induced tumours. However, the immunity was effective not only in prolonging the survival of mice transplanted with the tumour, but also in suppressing the primary development of the tumour in the TGM in situ (Figure 3). The latter finding is particularly notable because it for the first time provides evidence that cellular oncogene-induced tumours may be subject to oncogene product-linked immunological surveillance.

Analysis using four different types of ret-transgenic tumour cells as the target of the immunity, all of which except the transgenic ret were genetically compatible with the host immune system, demonstrated that the immunity was ret-specific or ret-linked. Three types of ret transgenic tumour
cells from different transgenic individuals, the \( E\mu/ret \) pre-B-lymphoma cell line (H-2\(^b\)), \( E\mu/ret \) primary pre-B lymphoma (H-2\(^b\)) and the MEL-ret melanoma cell line (H-2\(^d\)), were susceptible to the anti-tumour immunity. This suggested that the induced immunity was directed against the ret-linked antigen or the ret protein itself, which should be the only potential candidate immunogenic element shared by the three tumour cells as all other genetically determined antigens (H-2 and minor) of these tumours of B6 \( \times \) Balb/c origin should not be antigenic for the host F\(_1\) mice of B6 and Balb/c strains. In agreement with this conclusion, the induced immunity was not directed to \( E\mu/myc \) pre-B lymphoma (H-2\(^d\)), which differs only at the transgenic oncogene from \( E\mu/ret \) primary pre-B lymphoma (H-2\(^b\)), except for some background genes (B6 for \( E\mu/myc \) and B6 \( \times \) Balb/c for \( E\mu/ret \)) whose products should not be antigenic in F\(_1\) mice according to the accepted transplantation immunology rule.

The ret-linked anti-tumour immunity was induced by priming with allantigen (H-2\(^d\)) bearing ret-transgenic cells (retL1-6 or retL6-6). However, the induced immunity was not restricted by the H-2 of the cells for priming, protecting against the challenge of both H-2\(^b\) and H-2\(^d\) ret-transgenic tumours. This finding corresponded to our previous result that priming for the secondary CTL responses to non-H-2 cellular antigens \textit{in vivo} (Mizoguchi et al., 1988) and \textit{in vitro} (Ando et al., 1988) is not restricted by the H-2 of the immunising cells for priming, suggesting effective processing of the tumour antigen by host cells for priming.

**Figure 4** Complete suppression of tumour development in some TGM of the immunised group. The histology of the lymph node and bone marrow from one of the two TGM sacrificed on day 174 (see Figure 3) and a control non-immunised TGM that died on that day is shown. Note that there is no evidence of lymphoma development in the normal structure of the peripheral lymph node with primary and secondary follicles (d and e) and bone marrow (f) from the immunised TGM, which contrasted with extensive proliferation of lymphoma cells in the peripheral lymph node (a and b) and bone marrow (c) from the non-immunised control TGM, destroying the normal structure. Stained with haematoxylin–eosin. a and d, \( \times 17; \) b, c, e and f, \( \times 170. \)
Our conclusion that the induced immunity was ret-specific may not be surprising by itself, because the transgenic ret of human origin as a model of homologous ret with extensive mutation is expected to produce ret protein with xenogenic epitopes that must be immunogenic to conventional mice. Actually, we succeeded in demonstrating T-cell proliferation response to recombinant ret protein that was injected with Freund's adjuvant into BCF, mice (Dai et al., 1994). However, it was rather surprising that the immunity induced with either recombinant ret protein or recombinant ret protein (Dai et al., 1994) was active in tumour rejection (in both studies) and tumour cell killing (in this study only), probably including helper type (in the other study) and cytotoxic (in this study) anti-tumour T-cell immunity.

Human proto-ret has 83% sequence homology to mouse proto-ret (Iwamoto et al., 1993). The present results suggest that such an oncogene product with extensive molecular modification from the native one still works as antigen for anti-tumour immunity. The ret protein was localised on cell membranes (Tanguchi et al., 1992), and was not therefore expected to work as a strong tumour rejection antigen. The successful induction of tumour rejection immunity to this antigen supports the view that oncogene products with molecular modifications can induce tumour rejection immunity. Note that what the change in the molecular structure or the cellular location of the oncogene products. However, the level of the immunity induced in the present study was not very strong, even when the molecular variation of the oncogene product (human vs mouse) as antigen was extensive and a potentially powerful method of immunisation was used. This may suggest the limitation of the immunity specific to non-nuclear oncogene products.

Even though all the results suggest that the anti-tumour immunity was induced by the transgenic human ret protein bearing xenogenic epitopes or by another transgenic ret-linked antigen, we do not know what peptide sequences of the ret protein or ret-linked antigen are the target epitopes of the tumour-rejecting lymphocytes. Use of transfectants of Eμ/myc pre-B lymphoma with different segments of ret cDNA might be effective for further characterisation of the ret or ret-linked immunogenic peptide(s) for the anti-tumour immunity. Studies are therefore in progress to establish a hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)-defective mutant of the Eμ/myc pre-B lymphoma for selection of such transfectants and to prepare a number of suitable gene constructs for transfection.

The epitopes that should be recognised by tumour-specific T cells in TGM where the tumour arose might, however, be different from those seen by the T cells in the conventional mice transplanted with the tumour. This is because immunological tolerance would be established against the xenogenic epitopes on human ret protein in the T lymphocytes of the former but not the latter mice. For this reason, the transgenic human ret protein might behave like a self antigen, providing a model in which the immunology of the self ret protein can be studied. Why, then, did tumour-rejecting immunity develop in TGM as a result of injecting immunising tumour cells in our study? It might be that tolerance was incomplete. It should be remembered that, in 2 of 11 TGM in the immunised group, no tumours developed during the whole period examined when all TGM in the unimmunised control group died of tumours. There might exist a variation in the level of tolerance among individual TGM, as was previously reported among different lines of SV40 large T antigen TGM (Faas et al., 1987). Alternatively, some mutation might appear in the ret oncogene during the development of a tumour, creating a new epitope to which TGM are not tolerant. Germline mutations of the ret proto-oncogene have been reported in human multiple endocrine neoplasia type IIa (Mulligan et al., 1993). It could be that immunisation with retL6-6 potentially bearing a type of mutation was fully protective against the tumour in which the same type of mutation occurred, but was only partially suppressive against the tumours bearing different types of mutations. Present results do not have any evidence for or against either of these alternative views.

Finally some points should be considered regarding the new method of tumour modification used in this study. We introduced H-2 alloantigen into the transgenic tumour by breeding the TGM with an allogene strain. This trial was an extension of our previous experiments in which we induced tumour rejection immunity against a chemically induced tumour by using tumour cells transfected with an allogene H-2K gene (Isobe et al., 1989). The effectiveness of introduction of the allogeneic gene in inducing tumour rejection immunity has also been reported in a human system (Plautz et al., 1993). Our present results further confirm the effectiveness of this method of tumour modification, and justify the trial of immunotherapy by introducing the MHC gene into local tumours (Nabel et al., 1992). However, it still remains unanswered whether or not these methods are superior to others.

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