Specific depletion of the B-cell population induced by aberrant expression of human interferon regulatory factor 1 gene in transgenic mice

(transcription factor/negative growth factor/cytokines/gene regulation)

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ABSTRACT   Interferons (IFNs) are well known both as antiviral proteins and as potent regulators of cell growth and differentiation. In fact, IFNs inhibit growth of various normal and transformed cell types. Previously, a nuclear factor, IRF-1 (interferon regulatory factor 1), which binds to type I IFN and some IFN-inducible gene promoters, was identified and cloned. Since the IRF-1 gene is both virus and IFN inducible, an intriguing issue is raised as to whether the IRF-1 gene is functioning in IFN-mediated regulation of cell growth and differentiation. In this study, we generated transgenic mice carrying the human IRF-1 gene linked to the human immunoglobulin heavy-chain enhancer. In the transgenic mice, all the lymphoid tissues examined showed a dramatic reduction in the number of B lymphocytes (B cells). Preparation and analysis of bone marrow cells from the chimeric mice indicated that the bone marrow is the effective site for specific depletion of the B-cell population. In fact, transgenic bone marrow cells cocultured with a bone marrow-derived stromal cell line revealed an altered B-cell maturation pattern.

Cytokines represent a class of soluble mediators that affect various aspects of cellular responses such as stimulation and inhibition of proliferation and induction of differentiation. Each cytokine delivers signals through specific cognate receptors (1, 2). Despite extensive characterization of the structure and function of many cytokines and their receptors, the molecular mechanism by which cytokine-mediated signal transduction leads to cellular responses still remains unclear, especially with respect to the nuclear events that would control the fate of responding cells.

Interferons (IFNs) have been originally identified in the context of host defense mechanisms against viral infection (3–5). In fact, all IFN species—i.e., type I IFNs (IFN-α and IFN-β), and type II IFN (IFN-γ)—elicit potent antiviral activity of their target cells. On the other hand, they also manifest other biological activities like many other cytokines. In particular, much attention has been focused on IFNs functioning as potent inhibitors of cell proliferation, thus playing a crucial role in the growth control of normal and transformed cells (5–10). Hence, IFNs belong to the family of "negative growth factors." In fact, frequent deletion of the type I IFN loci has been reported in some malignancies (11, 12). Such observations may lend support to the prevailing notion that cells that fail to produce negative growth factors or respond to them are prone to acquire malignant phenotypes (13).

During the course of our study on the regulation of type I IFN gene expression, we identified and cloned nuclear factor, termed IRF-1 (IFN regulatory factor 1), that binds to the upstream cis elements of both IFN-α and IFN-β genes (14–16). A series of IRF-1 cDNA expression studies revealed IRF-1 functioning as a transcriptional activator for the type I IFN genes (17–19). Subsequently, another factor IRF-2 has been identified that apparently antagonizes the IRF-1 effect by competing for the same cis elements (18). It was then found that expression of the IRF-1 gene (as well as the IRF-2 gene) itself is induced not only by viruses, but also by the IFNs; the IRF-1 gene is otherwise expressed only at very low levels (a few mRNA copies per cell; refs. 18 and 20). In fact, IRF binding sites have been identified in a number of IFN-inducible genes (16). The binding sites indeed fall in the sequences that were originally identified as IFN-response sequences within the promoter region of those genes (21, 22). Thus, it has been assumed that cellular responses to IFNs are mediated at least in part by the IFN-inducible IRF-1 (18, 20).

The fact that IFNs function as negative growth factors and that the IRF-1 gene is IFN inducible raises an interesting issue concerning whether IRF-1 plays a role in the IFN-mediated cellular response; that is, the regulation of cell growth. In this context, IRF-1 might cause cell growth inhibition by affecting expression of the IFN genes and/or genes that are directly involved in cell growth inhibition. In this regard, the human IRF-1 gene has recently been assigned to human chromosome 5q23–31 by restriction fragment length polymorphism linkage analysis (S.I., H. Harada, and Y. Nakamura, unpublished data). Interestingly, this region has been termed the "critical region," which is frequently deleted in some myeloid disorders (23). Thus, the observation has raised the possibility that the cells that fail to respond to IFNs include those lacking the IRF-1 gene.

As an approach to assess the possible role of IRF-1 in the regulation of cell growth and differentiation, we generated transgenic mice carrying the human IRF-1 gene whose constitutive and high level expression is driven by the juxtaposed human immunoglobulin heavy-chain enhancer (E_μ). We report that such transgenic mice show a dramatic reduction in B lymphocyte (B cell) population.

MATERIALS AND METHODS

Construction of the E_μ IRF-1 Gene and Production of Transgenic Mice. The 2.1-kilobase (kb) Xba I fragment con-

Abbreviations: IFN, interferon; IRF, IFN regulatory factor; E_μ, immunoglobulin heavy-chain enhancer; FACS, fluorescence-activated cell sorter; PFC, plaque-forming cell; SRBC, sheep erythrocyte; CFU, colony-forming unit; IL, interleukin; PBL, peripheral blood lymphocyte.
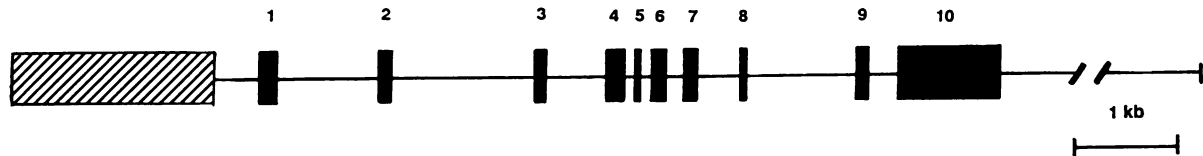
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FIG. 1.   Schematic representation of the injected transgene fragment carrying the human IRF-1 gene and the human E_μ (24). Solid boxes represent exons of the human IRF-1 gene with the number of each exon given above. The human IRF-1 gene contains an 11.5-kb sequence downstream of its last exon.

taining E_μ (derived from cU23; ref. 24) and the 19-kb human IRF-1 gene *Eco*RI fragment (derived from λ human IRF-1B; S.I., H. Harada, and M. Maruyama, unpublished data) were subsequently subcloned into the *Xba* I and *Sma* I sites of pUC19, respectively. (The *Eco*RI site was converted to a blunt end prior to ligation and the site was not restored after ligation.) The resultant plasmid was digested by *Eco*RI and *Sal* I to obtain the entire transgene fragment (E_μ-IRF-1) and was used for microinjection.

The schematic construction of the E_μ-IRF-1 gene is shown in Fig. 1. Transgenic mice were produced as described (25) by using C57BL/6 mice purchased from Nippon Clea (Osaka).

RNA Blotting Analysis. RNA blotting analysis was carried out following described procedures (26). The *Sac* I/*Kpn* I fragment from the human IRF-1 cDNA clone pHIRF31 (27) was used as a probe.

Fluorescence-Activated Cell Sorter (FACS) Analysis. Half a million cells from various lymphoid tissues were stained with either fluorescein-conjugated or phycocyanin-conjugated monoclonal antibodies RA3-6B2 (anti-B220; ref. 28), 30H12 (anti-Thy1.2; ref. 29), 53-6 (anti-Lyt-2; ref. 30), GK1.5 (anti-L3T4; ref. 31) and analyzed by a dual-laser FACS 440 (Becton Dickinson) equipped with an argon ion laser and a dye laser (32).

In Vitro **Assay of Mitogen-Induced Spleen Cell Proliferation and Plaque-Forming Cell (PFC) Assay.** Spleen cells were isolated and cultured with RPMI 1640 medium containing 10% fetal calf serum plus ConA (Pharmacia) or lipopolysaccharide (Difco) with the cell concentration of 2×10^5 cells per well in 96-well flat-bottomed microculture plates. After 36 hr of culture, 1 μCi (1 Ci = 37 GBq) of [³H]thymidine was added to each well, incubation was continued for another 12 hr, and [³H]thymidine incorporation was measured. The measurement of the PFC was as described (33). Eight days after immunization of sheep erythrocytes (SRBCs) (500 μl; 10% packed vol) and after another 2 days of booster injections, spleen cells were isolated and tested for the number of anti-SRBC PFCs by a direct method.

Preparation of Bone Marrow Chimeric Mice. Bone marrow cells were isolated from either transgenic or littermate mice and 8×10^6 cells were injected intravenously into lethally irradiated (880 rad by a Cs source; 1 rad = 0.01 Gy) 8-week-old C57BL/6 mice; the lymphoid cells were analyzed for surface marker expression 8 weeks after reconstitution.

Analysis of B-Cell Precursor Frequency and Colony-Forming Unit–Interleukin 7 (CFU–IL-7) Frequency from Bone Marrow Cells. Analysis of B-cell precursor frequency and CFU–IL-7 frequency was as described (34, 35). Briefly, a 96-well microculture plate seeded with a subconfluent layer of stromal cell line PA6 was prepared and 10^2 bone marrow cells were overlaid with IL-7 (20 units/ml) and cultured for 12 days. Wells containing pre-B cells (identified as B220-positive cells by FACS analysis) were scored and frequency was determined based on a Poisson distribution (34, 35). CFU–IL-7 or CFU–IL-3 colonies were obtained after 7 days of culture of 2×10^4 bone marrow cells seeded on methylcellulose plates with IL-7 (10 units/ml) or IL-3 (200 units/ml). Recombinant IL-3 was kindly provided by Y. Sudo (Biomaterial Research Institute, Yokohama, Japan) and recombinant IL-7 was prepared as described (35).

RESULTS

Establishment of Transgenic Mice Containing the Human IRF-1 Gene Linked with the Human E_μ. The structure of the injected DNA fragment is illustrated in Fig. 1. It comprises the entire human IRF-1 gene with its 10 exons and 5' flanking sequence extending 455 base pairs from the major cap site (S.I., unpublished data). The IRF-1 gene was linked with the human E_μ (24) with the expectation that the IRF-1 gene would be specifically expressed in lymphoid cells. The resulting DNA fragment encompassing 21 kb was microinjected into C57BL/6-derived fertilized eggs and transferred to oviducts of pseudopregnant ICR mice. Among the 41 offspring, 3 mice were shown to be positive for transgene integration (data not shown). Line 7 (about a 3-copy integrant) was mainly used for
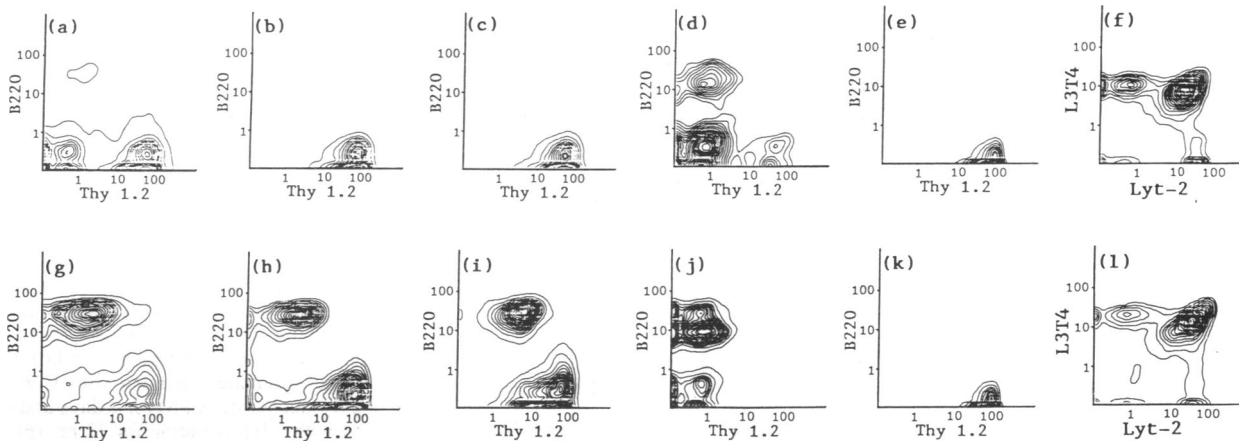

FIG. 2.   Two-color FACS analysis for cell-surface marker expression (B220, Thy1.2, L3T4, Lyt-2 antigens) of various lymphoid cells from transgenic (*a–f*) or littermate (*g–l*) mice. (*a* and *g*) Staining pattern spleen cells. (*b* and *h*) PBLs. (*c* and *i*) Lymph node cells. (*d* and *j*) Bone marrow cells. (*e, f, k*, and *l*) Thymocytes.
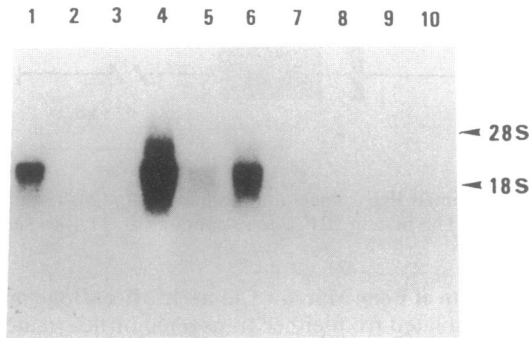
FIG. 3. Analysis of the human IRF-1-specific transcript in various tissues of transgenic (lanes 3–6) and littermate (lanes 7–10) mice. Each lane contains 5 μg of total RNA from the following cells: Lane 1, FL cells treated with Newcastle disease virus (NDV) for 15 hr; lane 2, L929 cells treated with NDV virus for 9 hr; lane 3, liver; lane 4, thymus; lane 5, spleen; lane 6, bone marrow; lane 7, liver; lane 8, thymus; lane 9, spleen; lane 10, bone marrow.

this study. However, another line, 5-2 (about a 1-copy integrant) gave essentially the same results (data not shown).

B-Cell Population Is Dramatically Reduced in All Lymphoid Tissues of Transgenic Mice. The lymphocyte subset of these transgenic and littermate mice was examined by two-color FACS analysis. Fig. 2 shows the results of the two-color FACS analysis of the various lymphoid tissue cells stained with the pan B-cell marker anti-B220, and the pan T-cell marker anti-Thy1.2. The B220-positive B-cell fraction was dramatically reduced in spleen (Fig. 2a), peripheral blood lymphocytes (PBLs) (Fig. 2b), lymph node (mesenchymal) (Fig. 2c), and bone marrow (Fig. 2d) of the transgenic mice. In the spleen, B220-positive B cells constituted only ≈1% of the total cell population in the transgenic line, whereas in littermates 68% were B220 positive (Fig. 2g). In PBLs and lymph nodes, the B220-positive cells constituted <1% in the transgenic line. However, in bone marrow, a significant number of cells (21%) were B220 positive. The observation that the transgenic mice are devoid of most B220-positive B cells in all the lymphoid tissues except bone marrow suggests that bone marrow may be primarily responsible for the reduction, since this is where B-cell differentiation initiates (36, 37). Analysis of bone marrow chimeric mice reconstituted by the transgenic bone marrow cells also supported this view (see below and Fig. 5).

Subsequently, 14 F₁ transgenic offspring and littermates from line 7 were analyzed for their lymphocyte subset

composition, revealing that the B-cell-specific reduction is perfectly concordant with the transmission of the transgene. Moreover, the other transgenic mouse line 5-2 gave similar findings (data not shown). These results indicate that the specific reduction in the B-cell population is not due to the effect of integration of the transgene into a specific site on host chromosomes.

No particular change in the T-cell subpopulations expressing surface markers such as Thy1.2, L3T4 (CD4), and Lyt-2 (CD8) were observed in the thymus of transgenic mice when compared to those from littermates (Fig. 2).

Lymphocyte-Specific Expression of mRNA for the Transgene. We next examined the expression of the mRNA from the transgene in various tissues by Northern blotting analysis. Total RNA was isolated from various tissues and hybridized with a human IRF-1-specific probe. As shown in Fig. 3, RNA specific for the transgene was expressed constitutively in lymphoid tissues, with levels highest in thymus, followed by bone marrow and spleen (Fig. 3).

Mitogenic Response of the Lymphocytes and Measurement of the PFCs in Spleen Cells. To gain insights into the alterations of lymphocyte function in transgenic mice, responses of the spleen cells to B-cell-specific mitogen lipopolysaccharide and T-cell-specific mitogen (ConA) were examined. Spleen cells were isolated from a transgenic or littermate mouse and the response to each mitogen was measured by incorporation of [³H]thymidine. As shown in Fig. 4a, the magnitude of response to lipopolysaccharide by cells from the transgenic mouse was reduced by 94%, compared to that of similarly treated cells from the littermate. On the other hand, no evident difference was detected for the ConA response between the two groups.

To assess the functional status of the humoral immune response, numbers of antibody-producing cells for SRBCs were measured by hemolytic PFC assay in the spleen cells from SRBC immunized or nonimmunized, transgenic or littermate mice. As shown in Fig. 4b, the numbers of anti-SRBC antibody-producing cells were dramatically lower in the transgenic line compared to the littermates.

Bone Marrow Is Primarily Responsible for the Reduction in the B-Cell Fraction. As the human Eμ was linked to the transgene, we assumed that the B-cell-specific reduction was primarily mediated in the bone marrow. To directly examine this possibility, bone marrow chimeric mice were made and analyzed for the composition of their lymphocyte subset. Lethally irradiated (880 rad) syngeneic C57BL/6 mice were injected intravenously with bone marrow cells (8 × 10⁶ cells per mouse) derived from either a transgenic or a littermate
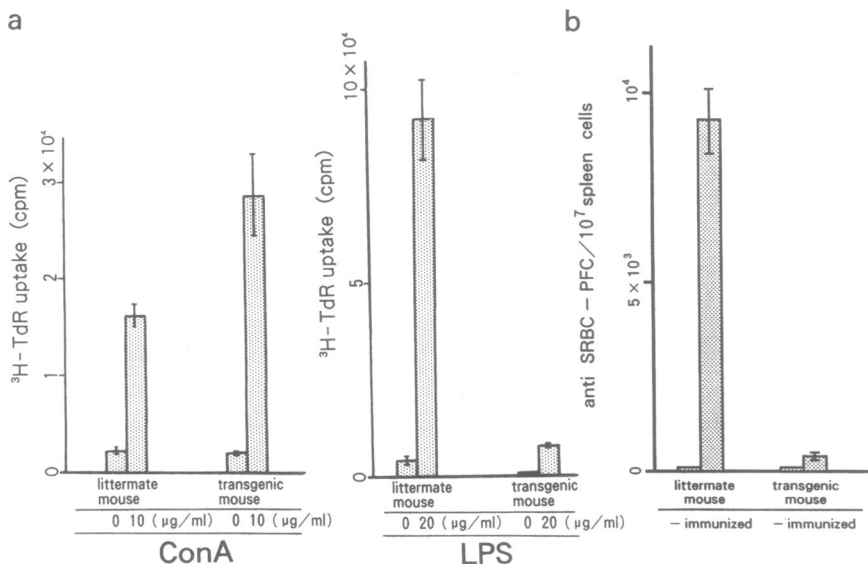

FIG. 4. (a) Mitogen-induced spleen cell proliferation was measured by uptake of [³H]thymidine (³H-TαR). Spleen cells were isolated from either transgenic or littermate mice and stimulated *in vitro* with ConA or lypopolysaccharide (LPS) for 2 days and then pulsed with [³H]thymidine for 12 hr. (b) The direct PFC assay was performed following described procedures (33) and using unimmunized or immunized spleen cells from either transgenic or littermate mice.
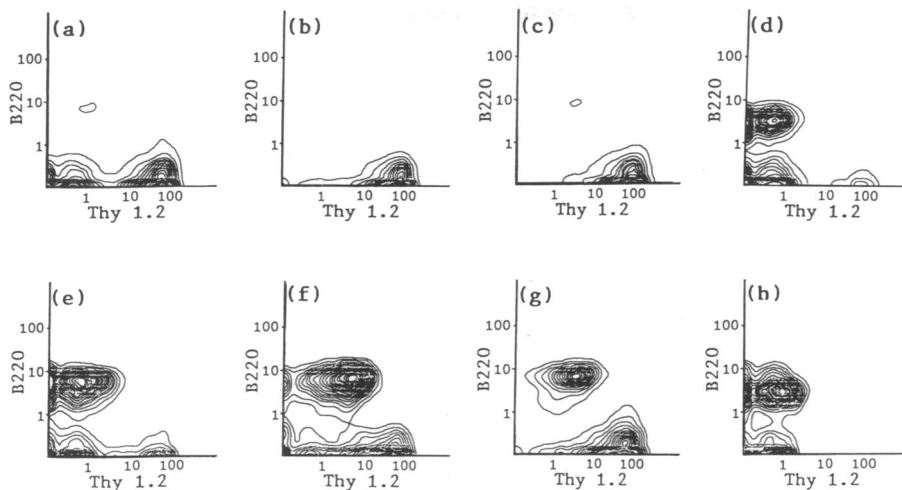
FIG. 5. Two-color FACS analysis for cell-surface marker expression (B220 and Thy1.2 antigen) of various lymphoid cells from chimeric mice reconstituted by either transgenic (*a–d*) or littermate (*e–h*) bone marrow cells. (*a* and *e*) Spleen cells. (*b* and *f*) PBLs. (*c* and *g*) Lymph node cells. (*d* and *h*) Bone marrow cells.

mouse. Eight weeks later, cells from lymphoid tissues were analyzed by two-color FACS analysis (Fig. 5). The chimeric mouse reconstituted with transgenic bone marrow cells showed a similar staining pattern as the transgenic donor. As shown in Fig. 5, the B-cell fraction was dramatically reduced in the spleen, PBLs, and lymph nodes. These observations indicate that transgenic bone marrow is primarily responsible for the depletion of B-lineage cells.

In Vitro **Culture of Transgenic Bone Marrow Cells with a Bone Marrow-Derived Stromal Cell Line.** Recently, bone marrow-derived stromal cell lines were established by several groups and, by utilizing those cell lines, at least part of the B-cell differentiation could be reproduced *in vitro* by coculture with bone marrow cells (34, 38–40). To test whether the B-cell differentiation program is affected in transgenic bone marrow, bone marrow cells were cocultivated with the stromal cell line PA6 (34) and IL-7.

As summarized in Table 1, the frequency of pre-B-cell generation is dramatically lower in transgenic bone marrow compared with that of the littermate mouse. The altered B-cell maturation was also illustrated by the CFU–IL-7-dependent colony formation assay (Table 1; ref. 41). The CFU–IL-7 colony formation was undetectable in the transgenic line, whereas the number of cells for multicolony formation by IL-3 (CFU–IL-3) was similar in transgenic and littermate mice, indicating that hematopoiesis is not affected in transgenic mice (Table 1).

DISCUSSION

Much attention has been focused on IFNs for their effects on growth or differentiation in various cell types. In the case of hematopoietic cells, IFN treatment of normal hematopoietic cells results in inhibition of growth or differentiation (5–10). In fact, aberrant production of IFNs was found to be accompanied by the human pathological state aplastic anemia, suggesting further the negative effects of IFNs in cell growth

and differentiation (42, 43). In a similar context, reports that several transformed cells of various origins were accompanied with chromosomal deletions in the IFN (IFN-α1 and IFN-β) gene loci are intriguing as to the role of IFNs in controlling aberrant cell growth (11, 12).

We have shown previously that expression of the IRF-1 gene is strongly induced not only by viruses but also by IFNs (18, 20). Thus, an intriguing issue has been raised concerning the role of IRF-1 in IFN-induced cellular events, in particular for inhibition of cell growth. In this study, we created the experimental condition in which the human IRF-1 gene is constitutively expressed at high levels by the human E_μ in transgenic mice. The IRF-1 transgene was expressed in a lymphoid tissue-specific manner and specific depletion of the B-cell population was observed in such transgenic mice. Essentially the same observation was made with another line of transgenic mouse (data not shown). Thus, it is likely that B-cell depletion is primarily caused by the IRF-1 encoded by the transgene.

At present, it is not clear by which mechanism IRF-1 causes B-cell depletion. Although the transgene is also expressed in the thymus, no significant alternations in T-cell population and function were observed. It is possible that the thymic T cells do not express the IRF-1 sufficient enough to perturb their growth. Alternatively, the IRF-1 protein might exert its function for negative cell growth through regulating the expression of cellular genes, such as IFN-β and -α genes whose expression levels may depend on cell types. So far, we have been unable to detect any IFN or IFN mRNA induction in lymphoid tissues. In fact, we have provided evidence that production of IRF-1 *per se* is insufficient for IFN gene induction in differentiated cells (19). Although, we cannot strictly rule out a very low level induction of IFN genes, it is more likely that IRF-1 affects cell growth directly rather than by inducing expression of IFNs. Whatever the mechanisms, this may be a unique observation that cells of mice expressing the transgene for a cytokine-inducible nuclear factor are depleted.

Table 1. Decreased B-cell precursor frequency and CFU–IL-7 frequency in IRF-1 transgenic mice bone marrow cells

	B-cell precursor frequency analysis*		CFU–IL-7, IL-3 frequency analysis†	
	Positive well	Frequency	IL-7 (10 units/ml)	IL-3 (200 units/ml)
Littermate mouse	17/96	1/513	12 (±2)	70 (±10)
IRF-1 transgenic mouse	0/96	<1/9550	0 (±0)	99 (±6)

*Bone marrow cells (10²) were overlaid on a subconfluent layer of PA6 cells in a 96-well microculture plate and 12 days after the culture wells containing B220-positive cells were scored.
†Number of CFUs (±SD) obtained per 2 × 10⁴ bone marrow cells.

Recently, several groups showed the significant roles of IL-7 as well as bone marrow-derived stromal cell lines in their functions of supporting B-cell development (34, 38–40, 44). Nishikawa and colleagues (34, 35) proposed that the B-cell differentiation pathways in bone marrow cells can be divided into three stages by utilizing recombinant IL-7 and bone marrow-derived stromal cell lines. They are (*i*) the pro-B-cell stage, which is independent of the cytokine IL-7 (41), and (*ii*) the early pre-B-cell stage requiring both IL-7 and stromal cells as a necessary environment, and (*iii*) the mature (late) early pre-B-cell stage in which the cells can proliferate in response to IL-7 alone. In the present studies, the frequency of generating pre-B cells (i.e., during or after stage *ii*) of transgenic mice was significantly low (Table 1). Hence, it is likely that B-cell growth is affected as soon as the E_μ starts affecting expression of the transgene. It has been shown that the murine IL-7 receptor (IL-7R) is expressed on pre-B cells but not on mature B cells (45). Interestingly, the murine IL-7R gene contains IRF-1 sites within its promoter region (Immunex, S. F. Ziegler, personal communication). Thus, overexpression of IRF-1 might affect the expression of murine IL-7R.

Recent studies indicate that IRF-2, a factor structurally related to IRF-1, antagonizes the IRF-1 effect by competing for the same cis elements (18, 19). In view of our findings with IRF-1 transgenic mice, it will be interesting to examine whether B-cell depletion can be reversed by expressing IRF-2 at high levels.

Up to now, several model mice have been reported to have various immune abnormalities in function and/or cell composition in their immune systems. Studies with these mice have greatly contributed to understanding the functions of the specific subset of immune cells. Similar applications with these IRF-1 transgenic mice might provide a means of studying various immunological problems.

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