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(Citation)
The Kobe journal of the medical sciences,37(4-5):227-243

(Issue Date)
1991

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/E0034253

Kobe J. Med. Sci. 37, 227/243, October 1991

POSSIBLE COLLABORATION BETWEEN C-FOS AND C-MYC PROTO-ONCOGENE PRODUCTS IN IN VIVO LYMPHOMAGENESIS

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INDEXING WORDS

c-fos, c-myc; transgenic mice; immunodeficiency

SYNOPSIS

Transgenic mice carrying the exogenous c-myc gene under regulation of the Ig enhancer (Ig-c-myc) were mated with mice carrying exogenous c-fos gene under control of the H-2K^b promoter (H2-c-fos) to examine their functional collaboration in in vivo lymphomagenesis. Two of the 33 (c-fos x c-myc) mice developed pre-B cell lymphomas within 22 weeks of age.

Received for publication: August 31, 1991

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None of the other F1 progeny expressing c-fos or c-myc alone showed malignant change within 14 months of age, suggesting that the exogenous c-fos and c-myc collaborate in in vivo lymphomagenesis. The exogenous c-myc RNA was overexpressed in the lymphomas, but the amount of exogenous c-fos RNA was not affected, suggesting that the large abundance of c-myc protein is a prerequisite for lymphoma onset or progression and c-fos protein plays a complementary role. C-fos protein induced immunodeficiency in the (c-fos x c-myc) mice like H2-c-fos mice. Natural killer cell activity of (c-fos x c-myc) mice was partially impaired. Therefore, these lymphomas may be a consequence of the synergism of two independent actions caused by the exogenous c-myc (lymphomagenesis) and the exogenous c-fos (low NK activity) in (c-fos x c-myc) mice.

INTRODUCTION

When lymphocytes are stimulated by antigens, two different proto-oncogenes (c-fos and c-myc) are sequentially expressed[1]. However, the role of these oncogene products in the activation of lymphocytes is unknown. Overexpression of the c-myc gene generates a malignant change in cells of the B lineage in transgenic mice[2,3]. Deregulated expression of the c-fos gene does not induce any lymphomas[4,5]. Oncogenesis is a multiplestep process that involves the successive activation of several oncogenes[6]. Therefore, in order to examine the role of the c-fos gene product in in vivo lymphomagenesis generated by the c-myc, we have mated two independent transgenic mice carrying the exogenous c-myc gene with the immunoglobulin (Ig) enhancer (Ig-c-myc)[7] and the exogenous c-fos gene with the H-2K^b promoter (H2-c-

fos)[8] to make double transgenic mice. Ig-c-myc mice, maintained with (C57BL/6 x CD-1) F1 mice, originally developed a high incidence of pre-B cell lymphomas[7]. However, these mice did not develop tumors after backcrossing with C57BL/6 mice for 5 generations. H2-c-fos mice have enlarged spleens and hyperplastic thymuses, but these mice do not develop lymphomas. Lymphocytes in these mice are functionally abnormal. They fail to produce IgG antibodies specific for immunizing antigens[8,9]. In the present study, we show that the exogenous c-fos collaborates with the exogenous c-myc in in vivo lymphomagenesis. The possible mechanism of the collaboration is discussed.

MATERIALS AND METHODS

Animals:

C57BL/6 Cr Slc mice were purchased from the SLC (Hamamatsu, Japan). Transgenic mice carrying the human c-myc gene under the control of mouse Ig heavy chain enhancer and simian virus 40 (SV40) early gene promoter (Ig-c-myc mice) have been made with (C57BL/6 x CD-1) F_1 mice[7] and maintained with C57BL/6 mice.

Transgenic mice carrying the exogenous mouse c-fos gene with the H-2K^b promoter (H2-c-fos mice) have been established and maintained with C57BL/6 mice[8,9].

Antigens:

Ovalbumin (OVA) was purchased from Sigma Chemical Co. 2,4-dinitrophenylated OVA (DNP_4-OVA) was prepared by coupling with 2,4,-dinitrobenzensulfonic acid under alkaline conditions[10].

Isolation of total RNA from various organs:

Total RNA was isolated from various organs of mice by the guanidine thiocyanate method[11]. Organs were taken from mice and immediately frozen by liquid nitrogen. They were homogenized in 4M guanidine thiocyanate solution with a Polytron homogenizer (Switzerland). Total RNA was pelleted through 5.7M CsCl cushion by ultracentrifugation at 36,000 rpm in a SW41T rotor for 16 hours at 15°C. The RNA was dissolved in RNase free water and quantified by spectrophotometer.

RNase protection assay:

An RNase protection assay to detect the exogenous and endogenous c-fos and c-myc RNA was performed as described by Rüther et al[8]. Briefly, 10 μg of total RNA was hybridized overnight with a ^{32}P labeled RNA probe at the proper temperature (65°C for c-fos, 60°C for exogenous c-myc and 55°C for endogenous c-myc). Unprotected RNA was digested with RNase A and RNase T_1 at 37°C for 1 hour. The protected RNA fragments were separated by electrophoresis on a 6% polyacrylamide denaturing gel and exposed to X-ray film for 24 to 48 hours at -70°C with intensifier screens.

Immunization protocol:

Naive mice between 20 and 40 weeks of age were injected intraperitoneally with 100 μg of alum-precipitated DNP-OVA and bled 14 days after the immunization.

Determination of serum IgG and specific antibody titers:

The concentrations of serum IgG were determined by enzyme-linked immunosorbent assay (ELISA) as described by Kendall et al[12]. Goat anti-mouse IgG antibodies (Caltag Laboratories Inc.) were coated on the 96-well plastic plates (Corning Glass Works). After serialy diluted sera were applied to the antibody coated wells, the mouse IgG in the wells as determined with biotinylated goat anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham AL) followed by avidin-

peroxidase (Vector Lab. Burlingame, CA). Myeloma protein CBPC101 (IgG_{2a}) was used as a standard protein. Amounts of IgG antibody specific for DNP were determined by ELISA. Pooled sera from C57BL/6 mice immunized with DNP-OVA were used as a standard.

Assay for natural killer (NK) cell activity:

A 4-hour ^{51}Cr release assay was performed using YAC-1 cells for target cells, as described in detail elsewhere[13]. Briefly, single mononuclear cell preparations from spleen were used as effector cells. $5x10^4$ radio-labeled target cells were co-cultured with $2.5x10^5$ (effector/target ratio=50:1), or $1.25x10^5$ (effector/target ratio=25:1) effector cells in 96 wells round bottomed micro titer plates (Corning #25850) with 200µl RPMI1640 medium (GIBCO) containing 10% fetal calf serum (FCS) (FILTRON) in 5% CO_2 at 37°C for 4 hours. The gamma ray radioactivity in 150 µl of a cultured supernatant was counted by an auto-gamma scintillation counter (Aloka, ARC-300). Maximum release of ^{51}Cr from the target cells was examined by treating the cells with 10% Triton X-100. The percent specific lysis for individual assay was calculated from the following formula for triplicate samples.

% specific lysis = (tested release - spontaneous release / maximum release - spontaneous release) X 100

RESULTS

B cell lymphomas in (c-fos x c-myc) mice:

The original Ig-c-myc mice (C57BL/6 x CD-1 strain) developed B cell lymphomas with a high incidence (~50 % within a year). However, the semi-congenic Ig-c-myc mice, after backcrossing with C57BL/6 mice for 5 generations, did not develop any malignancy during more than 2 years of obervation. These semi-congenic Ig-c-myc mice were mated with H2-c-fos

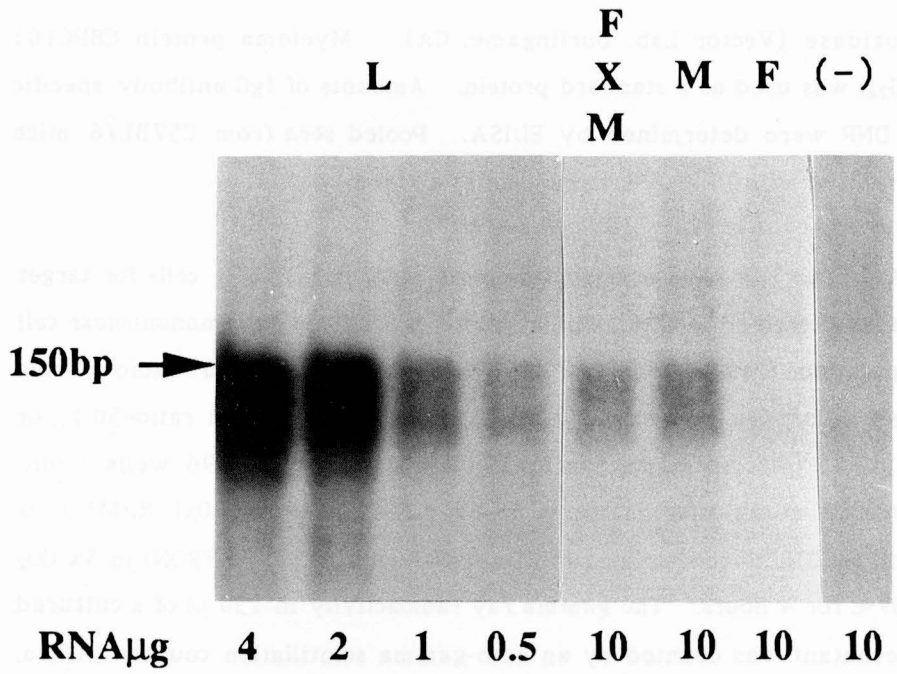

Figure 1. Expression analysis of exogenous c-myc RNA in the lymphoma from (c-fos x c-myc) mice. The amount of exogenous c-myc RNA in total RNA from the lymphoma and spleens of the progeny were analyzed by an RNase protection assay. The amount of total RNA (μg) used in this assay is written under each lane. L, lymphoma ; F x M, (c-fos x c-myc) mouse without lymphoma ; F, H2-c-fos mouse ; M, Ig-c-myc mouse ; (-), negative litter mate.

mice. We obtained 107 F1 progeny (33 of (c-fos x c-myc), 29 of H2-c-fos, 30 of Ig-c-myc, and 15 of negative litter mates). Two of the (c-fos x c-myc) mice developed lymphomas at 12 and 22 weeks of age. These mice had the enlarged lymph-nodes, spleens and thymuses filled with neoplastically proliferating lymphoblasts (data not shown). Cell lineage analysis of the lymphomas indicated the cytoplasmic IgM-positive and surface IgM-negative cells, consistent with characteristics of pre B cells (data not shown). In contrast, none of the other F1 progeny showed malignant change during 14

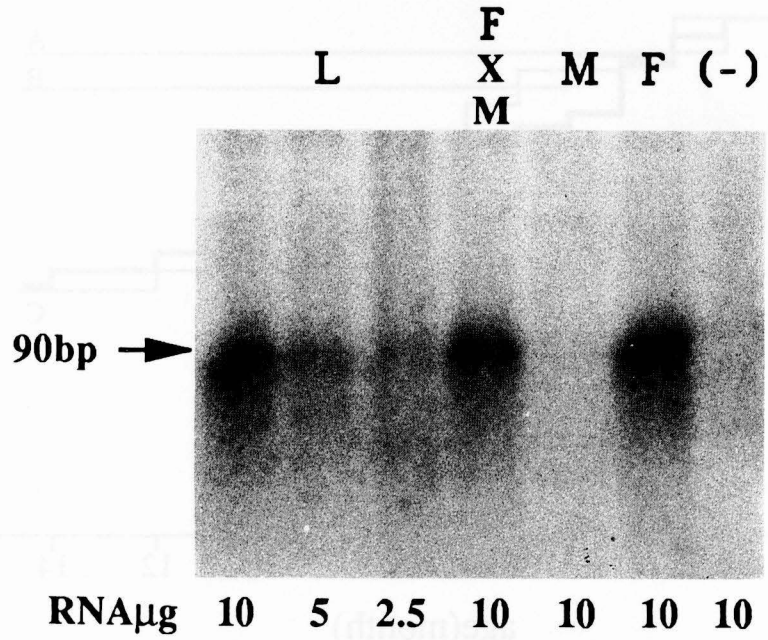

Figure 2. Expression analysis of exogenous c-fos RNA in the lymphoma from (c-fos x c-myc) mice. The amount of exogenous c-fos RNA in total RNA from the lymphoma and spleens of the progeny were analyzed by an RNase protection assay. The amount of total RNA (μg) used in this assay is written under the each lane. L, lymphoma ; F x M, (c-fos x c-myc) mouse without lymphoma ; F, H2-c-fos mouse ; M, Ig-c-myc mouse ; (-), negative litter mate.

months of observation. These results suggest that the exogenous c-fos collaborates with the exogenous c-myc in in vivo lymphomagenesis.

The amounts of c-fos and c-myc RNA in total RNA isolated from the lymphomas were examined by an RNase protection assay. As shown in Fig.1, the amount of exogenous c-myc RNA in the lymphomas was twenty times more abundant than in spleens from (c-fos x c-myc) and Ig-c-myc mice. In contrast, the amount of exogenous c-fos (see Fig.2) and endogenous c-myc (data not shown) RNA in spleens was almost identical in the

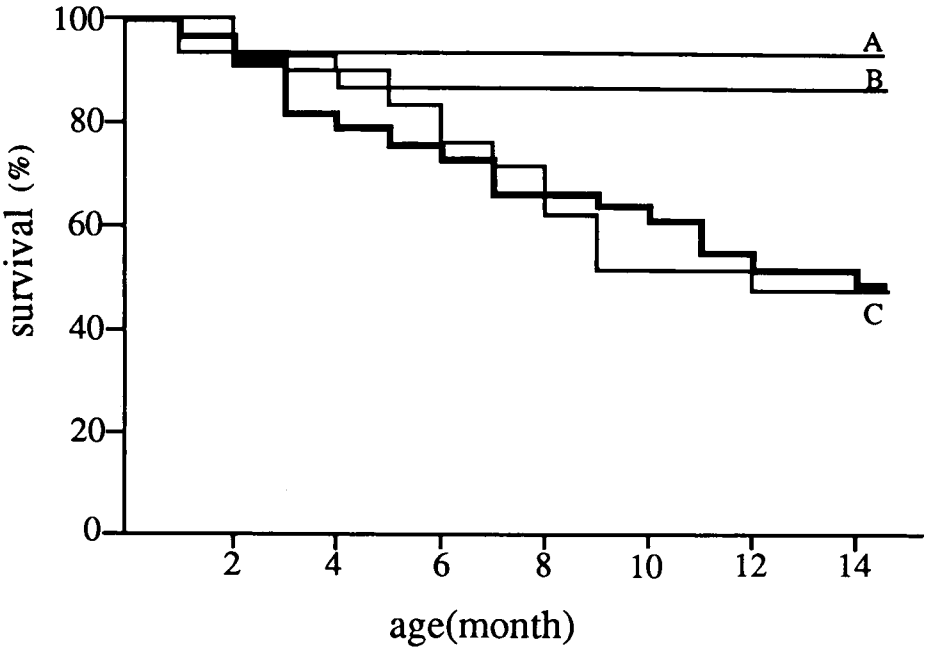

Figure 3. Survival curves of F1 progeny. Thirty three (c-fos x c-myc) mice, 29 H2-c-fos mice, 30 Ig-c-myc mice and 15 negative litter mates were observed for fourteen months. The survival curves of (c-fos x c-myc) mice (-) and their litter mates (-) (A; negative litter mates, B; Ig-c-myc mice, C; H2-c-fos mice) are shown. Survival rates of (c-fos x c-myc), H2-c-fos, Ig-c-myc mice and negative litter mates were 49%,48%,87% and 93% respectively.

lymphomas, c-fos and (c-fos x c-myc) mice. Endogenous c-fos RNA could not be detected in those RNA samples (data not shown). These results suggest that large amounts of the exogenous c-myc but not the c-fos RNA are required for the lymphomagenesis.

Immunodeficiency in (c-fos x c-myc) mice:

When we maintained the F1 progeny in our conventional animal room, more than 50% of H2-c-fos and (c-fos x c-myc) mice died within a year (see

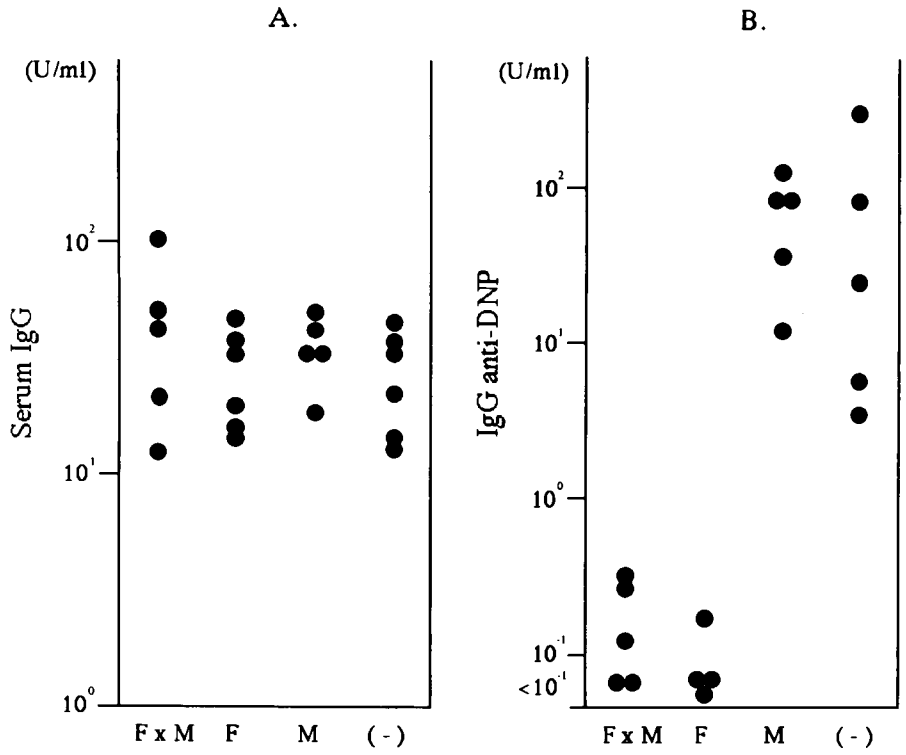

Figure 4. IgG productions of F1 progeny . A: Naive mice were bled between 20 and 40 weeks of age. Each closed circle indicates individual mouse. The titer of IgG in the sera were measured by ELISA. 40 μg/ml of IgG standard myeloma protein (CBPC101) was arbitarily taken as 1 unit. B: Naive mice were immunized with 100 μg of DNP-OVA on Alum and the sera were isolated 14 days after the immunization. The titers of IgG anti-DNP antibodies in the sera were measured by ELISA. Each closed circle indicates an individual mouse. The antibody titer of the standard anti-DNP sera was arbitrarily taken as 100 units. FxM, (c-fos x c-myc) mouse; F, H2-c-fos mouse; M, Ig-c-myc mouse; (-), negative litter mate.

Fig.3). The survival curves of the H2-c-fos and (c-fos x c-myc) mice were similar. Pathological analysis of lungs from the dead mice showed interstitial pneumonia with pulmonary abscess (data not shown), suggesting that the mice are immune deficient. H2-c-fos mice, with normal levels of

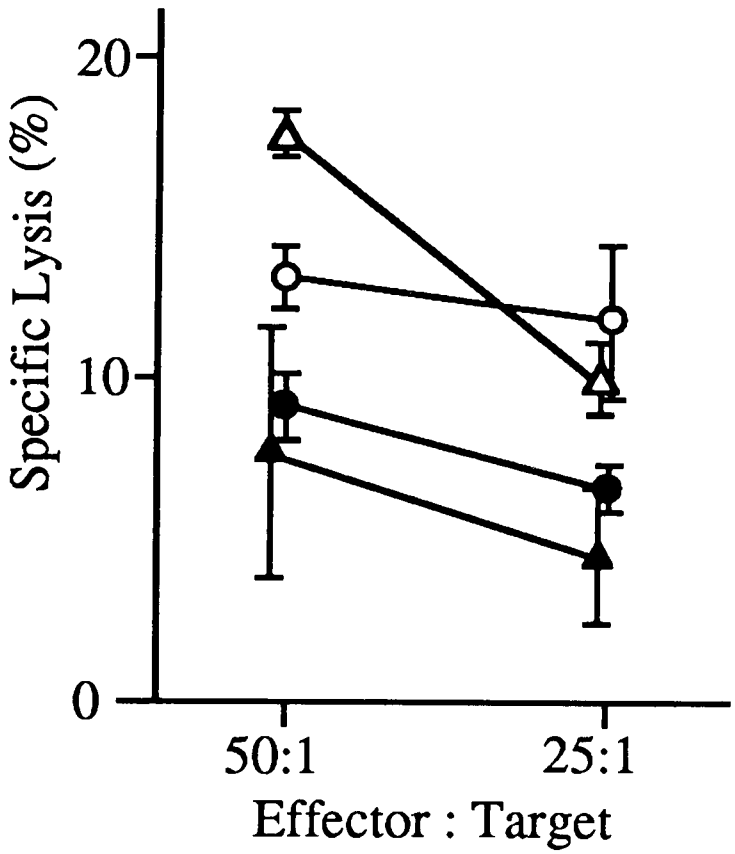

Figure 5. Natural killer (NK) cell activity of F1 progeny. ^{51}Cr-labelled YAC-1 cells were used as target cells. A preparation of single mononuclear spleen cells as used for effector cells. Target cells ($5x10^4$) were mixed with 2.5 $x10^5$ (effector : target - 50:1), or 1.25 $x10^5$ (effector : target - 25:1) effector cells and incubated for 4 hours. The released isotope into culture medium was monitored and the percent specific lysis was calculated as described in materials and methods. Means and standard deviations were calculated from triplicate samples.

▲; (c-fos x c-myc) mouse, ●; H2-c-fos mouse, △; Ig-c-myc mouse, ○; negative litter mate

serum IgG, can not produce IgG antibodies specific for immunizing antigens[9]. In order to examine the influence of the c-myc in the abnormal IgG production caused by the exogenous c-fos, (c-fos x c-myc) mice were immunized with alum-precipitated DNP-OVA antigen. The primary IgG anti-DNP responses were examined in the sera 14 days after the immunization by ELISA. As shown in Fig.4, IgG anti-DNP responses of (c-fos x c-myc) and H2-c-fos mice were approximately two to three orders of magnitude lower than those of Ig-c-myc mice and negative litter mates. We could not detect any infuence of the exogenous c-myc in these immune abnormalities.

Activity of natural killer (NK) cells in (c-fos x c-myc) mice:

The mechanism of the collaboration between c-fos and c-myc in in vivo lymphomagenesis is controversial. Tumor cells have been thought to be recognized by immune competent cells which are potentially active in limiting or eliminating those tumor cells[14,15]. Natural killer (NK) cells are one of the effector cells involved in this immunosurveillance[16,17]. Since (c-fos x c-myc) mice are immune deficient, NK cell activity of (c-fos x c-myc) mice was examined against YAC-1 cells using a 4 hours ^{51}Cr release assay. Fig.5 shows that the cytolytic activity of spleen cells from (c-fos x c-myc) and H2-c-fos mice was significantly lower (less than 50%) than that from the other F1 progeny. This partial impairment of immunosurveillance may promote in vivo lymphomagenesis.

DISCUSSION

The continuous expression of c-myc oncogene generates malignant change in transgenic mice[2,3]. The original Ig-c-myc mice, maintained with (C57BL/6 x CD-1) F_1 mice, also developed pre B cell lymphomas at

high incidence[7]. However, semi-congenic Ig-c-myc mice, backcrossed with C57BL/6 mice for 5 generations, did not show any malignancy. Although the strain restriction on oncogenesis is not fully understood, conceivably the genetic background of the mice (CD-1 strain) might have some role. We thought that B lineage cells from the semi-congenic Ig-c-myc mice, expressing detectable amounts of the exogenous c-myc RNA, were at a pre-neoplastic stage. Since oncogenesis is derived from a multiplestep process of deregulated events, the Ig-c-myc mice could be used for analyzing an additional signal for lymphomagenesis of B lineage cells.

In this study, we have examined the possibility that c-fos oncogene functions as an additional signal for lymphomagenesis, since c-fos protein is sequentially expessed with c-myc protein in antigen-stimulated lymphocytes and is also oncogenic. The results suggest that c-fos collaborates with the c-myc in in vivo lymphomagenesis, since (c-fos x c-myc) mice that simultaneously produce exogenous c-fos and c-myc RNA developed pre-B cell lymphomas. No lymphomas developed in any c-fos transgenic mice[4,5,8] and the type and onset of tumors in (c-fos x c-myc) mice were similar to those in the original Ig-c-myc mice, suggesting that the c-fos protein plays a complementary role in the in vivo lymphomagenesis generated by the c-myc protein. The incidence of lymphoma development in (c-fos x c-myc) mice was very low (6%). One reason may be the high mortality of (c-fos x c-myc) mice (see Fig.1). Many (c-fos x c-myc) mice die of pneumonia before developing lymphomas.

The amount of exogenous c-myc RNA in the lymphomas was twenty times more abundant than in spleens from Ig-c-myc mice (Fig. 3). Rosenbaum et al also showed that leukemic cells contained a 20 fold excess

of exogenous c-myc RNA compared with preleukemic tissues from their c-myc/v-abl double transgenic mice[18]. Furthermore, in most human Burkitt lymphomas and murine plasmacytomas, the c-myc gene has been activated by translocation to the IgH locus[19,20]. Therefore, the large abundance of c-myc protein is a prerequisite for lymphoma onset or progression.

The amount of c-fos RNA in the lymphomas was the same as in spleens from (c-fos x c-myc) mice. This raises the question of how the c-fos collaborates with the c-myc in in vivo lymphomagenesis. The amounts of c-fos and c-myc RNA in spleens from (c-fos x c-myc) mice were the same as in spleens from H2-c-fos and Ig-c-myc mice, respectively. The survival curves and the abnormal production of IgG antibodies to antigens were also similar between H2-c-fos and (c-fos x c-myc) mice. Thus, we cannot detect any collaboration between exogenous c-fos and c-myc in the amount of their RNA and in immune abnormalities. Immunosurveillance is thought to be important to repress tumor development in its early stage[14]. Killer T cells and natural killer (NK) cells have been proposed as a first level of defense against the initiation and spread of tumors[21]. Killer T cells provide specific cytotoxic activity of tumor-bearing individuals against autologous tumor cells[15]. NK cells recognize a variety of target-cell structures, and may play a more important role in natural host resistance against tumors at the tumor initiation step. For example, the 'beige' mouse that lacks NK cells is also more susceptible to primary tumor development by chemical carcinogenesis[16]. The NK cell activity was partially impaired in (c-fos x c-myc) mice compared to that in Ig-c-myc mice(see fig.5). Therefore, B lineage cells newly transformed by the exogenous c-myc protein may be killed by the surveillance system at an early stage in Ig-c-myc mice. The partial impairment of

immunosurveillance induced by the exogenous c-fos protein may contribute to the development of lymphomas by c-myc in (c-fos x c-myc) mice.

ACKNOWLEDGMENT

We would like to extend our sincere thanks to Drs. U. Ruther, K. Hanioka, H. Itoh, K. Yamamoto, Y. Saito, Praseno, M. Hatano, K. Wakita, N. Miki and T. Koizumi for their helpful discussions and collaborations. We express our appreciation to Miss Aya Maruyama for her excellent secretarial assistance.

This work is partially supported by a Grant-in-Aid-for Cancer Research from the Ministry of Education, Culture and Science, Japan and the Sagawa foundation for promotion of cancer research.

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