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Prethymic Nylon Wool-Passed Bone Marrow Cells Can Make Distinction between Self and Non-Self X-Chromosome-Linked Gene Products (Xir Antigens) on the Stimulator Cells, Resulting in Regulation of the Generation of Cytotoxic T Lymphocytes in Mixed Lymphocyte Cultures

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ABSTRACT

We have previously reported that nylon wool-passed bone marrow cells treated with anti-Thy.1 antibody and complement (Thy.1⁻ NW-BM cells) had helper-like activity which could augment the generation of cytotoxic T lymphocytes (CTL)[1]. In this study, we determined the antigens to which these NW-BM cells responded and recognized. When a few responder lymph node (LN) cells and an excess of NW-BM responder cells from B10BR ($H\text{-}2^k$, B10 background) mice were cultured with stimulator spleen cells from either B10D2 ($H\text{-}2^d$, B10 background) or BALB/c ($H\text{-}2^d$, BALB/c background) mice, the number of CTL induced by the stimulation with BALB/c spleen cells was significantly higher than in those from B10D2 mice. When the cells from BALB.K ($H\text{-}2^k$, BALB/c background) mice were used as the responder, the results showed that B10D2 spleen cells were better stimulators than those of BALB/c. These results showed that NW-BM cells responded to the non-self allogeneic background gene products on the stimulator cells, but not to those of self, and that these responder NW-BM cells augmented CTL generation. We genetically analyzed the antigens that NW-BM cells recognized among many products derived from background genes. B10BR NW-BM cells were well stimulated by (BALB.K×B10D2) F1 male spleen cells, but not by those of (B10D2×BALB.K) F1 males. On the contrary, BALB.K NW-BM cells were highly stimulated by (B10D2×BALB.K) F1 males, but not by (BALB.K×B10D2) F1 males. These results showed that NW-BM cells had a strict preference for X-chromosome linked gene products (Xir antigens). We also showed that the augmenting ability of NW-BM cells was attributed to the $Thy.1^-$ population, and that BALB/c NW-BM cells possessing the $H\text{-}2^d$ haplotype also responded preferentially to non-self Xir antigens. To conclude, NW-BM cells could distinguish between self and non-self Xir antigens, resulting in regulation of the CTL response. *Ryukyu Med. J., 14 (1) 33 ~ 41, 1994*

Key words : X-chromosome, bone marrow cells, self-nonself discrimination, cytotoxic T lymphocytes

INTRODUCTION

The T cells acquire the repertoire of antigen specific receptors (TcR) as a result of positive- or negative-selection during ontogenesis in the thymus[2]. Responding to self major histocompatibility complex (MHC) antigens on the surface of thymic epithelial cells, immature thymocytes survive and maturate, resulting in the establishment of antigenic recognition, that is, in a self MHC restricted manner (positive selection)[3,4]. On the other hand, responding to either self peptides in the context of the self MHC antigens or self MHC antigens alone on the surface of thymic dendritic cells or macrophages, immature thymocytes are deleted, resulting in the establishment of self tolerance (negative selection)[5,6]. The T cells distinguish between self and non-self antigens by using the repertoire of TcR prepared in the thymus. The essence is that T cells have the TcR for non-self antigens but not for self antigens.

The helper T (Th) cells recognize allo MHC class II antigens or non-self antigen peptides in the context of self MHC class II antigens[7-9], in order to produce some helper

factors which augment the generation of CTL[10-12]. We previously reported that Thy.1⁻ NW-BM cells also have helper-like activity which augments the generation of CTL specific for the allo MHC antigens[1]. When Thy.1⁻ NW-BM cells were added to the culture, the CTL which were specific for minor histocompatibility (minor-H) antigens[13] and tumor specific transplantation antigens (TSTA)[14] in a self-MHC restricted manner, were also induced easily in primary cultures *in vitro*.

A low number of responder LN cells were cultured with stimulator spleen cells having allo MHC antigens. These cultures were supplemented with the minimum but sufficient dose of T cell growth factor (TCGF). The CTL were however, induced only slightly under these culture condition. When Thy.1⁻ NW-BM cells from the same mice with responder cells were poured into these cultures, the CTL specific to allo MHC antigens were induced vigorously[1].

These Thy.1⁻ NW-BM cells had some characteristics that differed from the Th cells. i) Thy.1⁻ NW-BM cells require the identity of the MHC antigens with CTL precursor cells in responder cells to augment CTL generation[1]. On the other hand, the Th cells do not have this requirement because the TCGF that they produce is non-specifically active beyond the MHC barrier[15]. ii) The potential cell population in the Thy.1⁻ NW-BM cells express Thy.1^{low}, CD3⁻, TcR⁻, CD4⁻ and CD8⁻ on their cell surfaces, according to analyses using a fluorescence activated cell sorter. Thy.1⁻ NW-BM cells indeed do not have the TcR (data not shown). iii) Immature T cells do not possess any activities until they acquire the TcR in the thymus or periphery, as proven with studies using *scid*-mice[16,17]. Thus, these facts suggested that Thy.1⁻ NW-BM cells do not belong to the T cell lineage.

In this study, we determined the antigens that Thy.1⁻ NW-BM cells recognized and responded to on the stimulator cells. Thy.1⁻ NW-BM cells responded to allogeneic, non-self, X-chromosome linked gene products (Xir antigens), and completely ignored allo MHC antigens as an object of the recognition. Namely, Thy.1⁻ NW-BM cells could distinguish between self and non-self Xir antigens. This resulted in the regulation of the immune response, such as the CTL generation, through the recognition of Xir antigens. These results showed that there is a novel immune regulatory system, which is controlled by Thy.1⁻ NW-BM cells, and that it differs from those regulated by Th cells[7-9]. We propose that there are two immune regulatory systems that control the generation of CTL. One is dependent upon the Th cells, recognizing the non-self antigens restricted with MHC via TcR[7-9]. The other is dependent on Thy.1⁻ NW-BM cells, recognizing non-self Xir antigens via unknown receptors.

MATERIALS AND METHODS

Mice

We used 8- to 16-week-old male mice of B10BR (H-2^k), B10D2 (H-2^d), BALB.K (H-2^k) and BALB/c (H-2^d) inbred strains, and hybrid (B10D2XBALB.K) F1, (BALB.KXB10D2) F1, (B10BRXBALB/c) F1 and (BALB/cXB10BR) F1 male mice, which were bred in the Institute for Animal Experiments, Faculty of Medicine, University of the Ryukyus. The experimental plan in this report was approved by the Animal Care and Use Committee, University of the Ryukyus.

Mixed lymphocyte cultures (MLC)

The procedures have been described in detail previously[1]. In brief, cells from lymph nodes (LN) (inguinal, axillary, and brachial) or spleens were suspended in standard culture medium (α -minimum essential medium [α -MEM]; Flow Laboratories, Inc., Rockville, Md.) containing 10% fetal calf serum (FCS; Lot No. 2M1073, Bio-Whittaker, Walkersville, Md.), 10mM HEPES buffer, and 5X10^{-5}M 2-mercaptoethanol. Cell suspensions of bone marrow (BM) cells obtained from the femur were prepared by passing them through syringe needles. Two milliliters (2.5X10^7 cells/ml) of BM cell suspensions were loaded onto a column containing nylon wool (Wako Pure Chemical, Inc., Osaka, Japan). After incubation for 45 minutes at 37℃, nonadherent cells were eluted, washed once by centrifugation, resuspended in the culture medium as described previously[18], then referred to as NW-BM cells. Several doses of LN cells (responders), NW-BM cells (helpers[1]) and 3X10^5 mitomycin-c treated spleen cells (stimulators) were co-cultured in V-shaped microculture wells (Cat. No. 76-023-05, Flow Laboratories, Inc., McLean, Virginia) containing 0.2 ml of culture medium supplemented with several doses of rat T cell growth factor (TCGF) (Cat. No. 40116, Collaborative Research, Inc., Oak Park Bedford, Mass.) as described previously[19]. Control cultures, from which the spontaneous value were obtained, did not contain responder LN cells. These cultures were incubated for 5 days at 37℃ in an atmosphere of 5% CO_2 in air.

Antiserum treatment

Cells were incubated with anti-Thy.1.2 monoclonal antibody (HO-13) for 30 minutes at 4℃, washed once in cytotoxicity medium (Cedarlane Laboratories, Inc., Hornby, Ontario, Canada), and incubated in a water bath with rabbit complement (Low-Tox-M; Cedarlane Laboratories), at a final dilution of 1:10, for 45 minutes at 37℃. Cells were then washed three times in culture medium.

Cytotoxicity assay

After five days in culture, MLC were assayed for cytotoxic activity by using ^{51}Cr-labeled concanavalin A (con A) spleen blast target cells (3X10^3 cells/well) from a stimulator strain, as previously described[20]. After four hours in culture, the specific ^{51}Cr release, p, was defined as the observed counts minus spontaneous count / total releasable counts minus spontaneous counts. The results can be re-expressed in terms of cytotoxic activity, which is proportional to the number of CTL generated, accord-

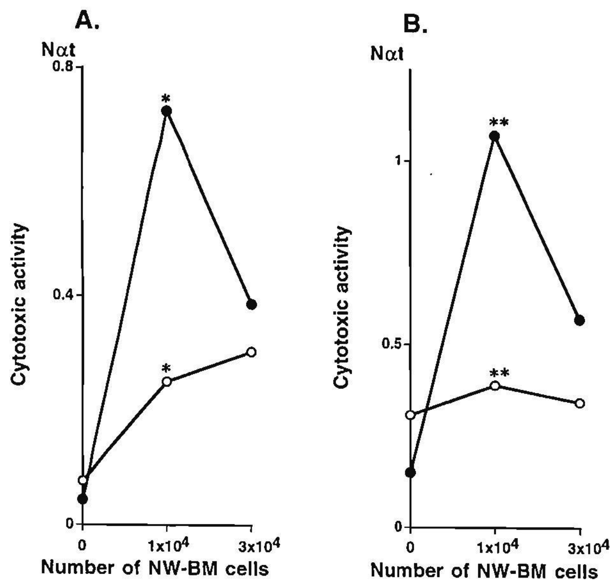

Fig. 1. B10BR NW-BM cells responded to allogeneic background gene products from BALB/c but not syngeneic ones from B10D2. Six thousand (A) or 1×10^4(B) B10BR LN cells/well were cultured with 3×10^5 mitomycin-c treated spleen cells/well from either B10D2 (○) or BALB/c (●) mice in replicate cultures supplemented with 0.5 units/ml of rat TCGF. Several doses of B10BR NW-BM cells were added to these cultures as indicated. Cultures were incubated for 5 days and assayed for cytotoxic activity, using 3×10^3 ^{51}Cr-labeled spleen Con A blast cells from each stimulator strain. The results are expressed as cytotoxic activity (Nαt), as described in 'Materials and Methods'. The Mann-Whitney U test was used to assess whether cytotoxic activity was significantly different between both stimulator groups. *; $p<0.01$, **; $p<0.01$.

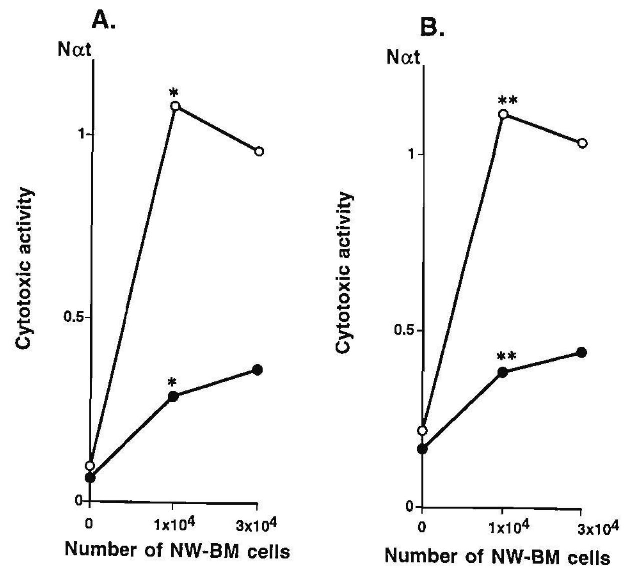

Fig. 2. BALB.K NW-BM cells responded to allogeneic background gene products from B10D2 but not syngeneic ones from BALB/c. Six thousand (A) or 1×10^4 (B) BALB.K LN cells/well were cultured with 3×10^5 mitomycin-c treated spleen cells/well from either B10D2 (○) or BALB/c (●) mice in replicate cultures supplemented with 0.67 units/ml (A) or 0.5 units/ml (B) of rat TCGF. Several doses of BALB.K NW-BM cells were added to these cultures as indicated. The cytotoxic activity was assayed and its significance was estimated as described in the legend to Fig. 1. *; $p<0.01$, **; $p<0.01$.

ing to the method developed by Miller[21], namely, $N\alpha t = -\ln(1-p)$, where N is the total number of sensitized cells, α is a constant proportional to the frequency of CTL, and t is the assay time in hours. Nαt is directly proportional to the number of cytotoxic lymphocytes produced. Nαt=0.1 is equivalent to 10% specific ^{51}Cr release and Nαt=1.0 is equivalent to 63% specific ^{51}Cr release. Each experimental group contained 8 replicate cultures. The significance of the differences between the cytotoxic activities of each experimental group was estimated by the Mann-Whitney U test[22].

RESULTS

NW-BM cells responded to allogeneic background gene products, but not to allo MHC

We reported that NW-BM cells could augment the generation of CTL specific to allo MHC antigens in primary cultures *in vitro*[1]. Here we intended to clarify which antigens NW-BM cells recognized and responded to. Firstly, we examined whether NW-BM cells preferentially recognized MHC or some other antigens. A few LN cells from B10BR (H-2^k, B10 background) mice as responder cells were cultured with mitomycin-c treated spleen cells from either B10D2 (H-2^d, B10 background) or BALB/c (H-2^d, BALB/c background) mice as stimulators. Several NW-BM cells from B10BR mice and a minimum but sufficient dose of TCGF were also added.

When no NW-BM cells were added to these cultures, the CTL specific for the MHC antigens of stimulator cells, H-2^d, were induced at extremely low level (Fig. 1). Only a few Th and CTL precursor cells, each of which were specific for class II^d or class I^d antigens of MHC, were present in 6×10^3 (Fig. 1A) or 10^4 (Fig. 1B) responder LN cells, because the frequency of these cells having specificity was about one cell per 10^3 LN cells[23]. The added amount of TCGF was also not very high. Thus, the level of CTL induction was very low. When 10^4 or 3×10^4 NW-BM cells were added into these cultures, the CTL were markedly induced (Fig. 1). The NW-BM cells activated by the stimulus synergistically augmented the activity of CTL precursor cells among the responder LN cells.

When 10^4 B10BR NW-BM cells were added to these cultures, significantly more CTL were induced in the cultures containing BALB/c stimulator spleen cells than in

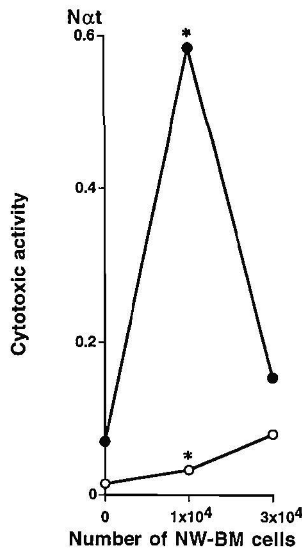

Fig. 3. The ability to augment the generation of CTL was attributed to the Thy.1⁻ population in NW-BM cells. Six thousand B10BR LN cells/well were cultured with 3×10^5 mitomycin-c treated spleen cells/well from either B10D2 (○) or BALB/c (●) mice in replicate cultures supplemented with 0.67 units/ml of rat TCGF. Several doses of B10BR NW-BM cells treated with anti-Thy.1 monoclonal antibody and complement were added to these cultures as indicated. The cytotoxic activity was assayed and its significance was estimated as described in Fig. 1. *; $p<0.01$.

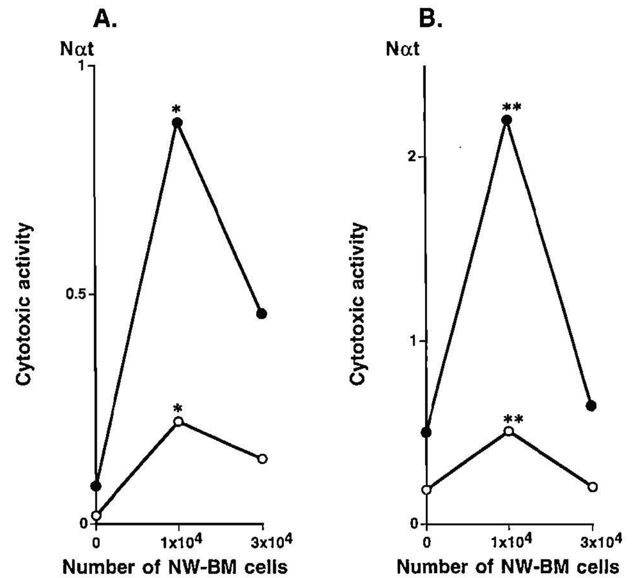

Fig. 4. B10BR NW-BM cells responded to allogeneic, non-self, but not syngeneic self Xir antigens. Six thousand (A) or 1×10^4 (B) B10BR LN cells/well were cultured with 3×10^5 mitomycin-c treated spleen cells/well from either (B10D2XBALB.K) F1 male (○) or (BALB.KXB10D2) F1 male (●) mice in replicate cultures supplemented with 0.67 units/ml of rat TCGF. Several doses of B10BR NW-BM cells were added to these cultures as indicated. The cytotoxic activity and its significance was estimated as described in Fig. 1. *; $p<0.05$, **; $p<0.01$.

those containing B10D2 spleen cells, although both stimulator cells had common MHC antigens (Fig. 1). These results showed that B10BR NW-BM cells could respond better to stimulator cells from BALB/c mice, having different background genes from B10BR mice, than to B10D2, to which they were identical. This meant that NW-BM cells from B10BR mice responded to the allogeneic background gene products on the surface of stimulator cells from BALB/c mice but not to self background ones from B10D2 mice. Furthermore, NW-BM cells ignored allo MHC antigens on the stimulator cells because both stimulator cells possessing the common MHC antigens had different ability to stimulate NW-BM cells.

There was however, the possibility that BALB/c stimulator spleen cells had intrinsically stronger antigenicity than B10D2 cells. To rule out this possibility, both responder LN and NW-BM cells derived from BALB.K ($H\text{-}2^k$, BALB/c background) mice were cultured with stimulator spleen cells either from B10D2 or BALB/c mice (Fig. 2). In contrast to the results shown in Fig. 1, significantly more CTL were induced by stimulation with B10D2 spleen cells than with those from BALB/c mice. This confirmed the previous conclusion that NW-BM cells from BALB.K mice responded to allogeneic but not self background gene products, and also suggested that both B10D2 and BALB/c spleen cells had a similar level of stimulating activity.

The ability of NW-BM cells to augment the generation of CTL is attributable to their Thy.1⁻ population

NW-BM cells contained a few mature T cells, such as Th and CTL precursor cells. It was possible that these mature T cells contributed to the increased CTL generation as shown in Figs. 1 and 2. We examined whether the Thy.1⁻ population in NW-BM cells could augment the generation of CTL. Thy.1⁺ mature T cells were depleted from NW-BM cells using anti-Thy.1 monoclonal antibody and complement. These Thy.1⁻ NW-BM cells also could augment the generation of CTL (Fig. 3). When responder LN and Thy.1⁻ NW-BM cells from B10BR mice were stimulated with spleen cells either from B10D2 or BALB/c mice, the same results were obtained as seen in Fig. 1; significantly more CTL were induced by stimulation with BALB/c spleen than by B10D2 cells. These results suggested that the Thy.1⁻ population in NW-BM cells were almost totally responsible for augmenting CTL generation.

NW-BM cells had a strict preference for X-chromosome linked gene products (Xir antigens)

NW-BM cells responded to allogeneic but not self background gene products on the surface of stimulator cells, as described in Figs. 1, 2 and 3. We next examined which antigens NW-BM cells recognized among the many products derived from background genes. First of all, we studied whether the antigens recognized by NW-BM cells

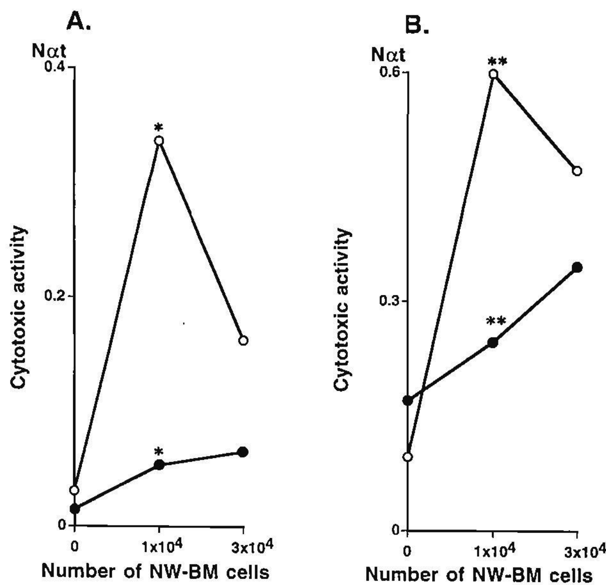

Fig. 5. BALB.K NW-BM cells also responded to non-self but not to self Xir antigens. Six thousand (A) or $1X10^4$ (B) BALB.K LN cells/well were cultured with $3X 10^5$ mitomycin-c treated spleen cells/well from either (B10D2X BALB.K) F1 male (○) or (BALB.KXB10D2) F1 male (●) mice in replicate cultures supplemented with 0.4 units/ml (A) or 0.5 units/ml (B) of rat TCGF. Several doses of BALB.K NW-BM cells were added to these cultures as indicated. The cytotoxic activity and its significance was estimated as described in Fig. 1. *; $p<0.05$, **; $p<0.05$.

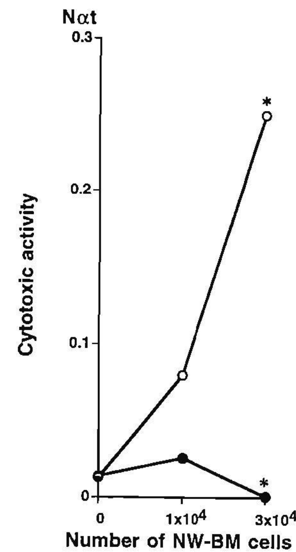

Fig. 6. Thy.1 population in NW-BM cells was responsible for most of the ability to distinguish between self and non-self Xir Antigen. Six thousand BALB.K LN cells/well were cultured with $3X 10^5$ mitomycin-c treated spleen cells/well from either (B10BRXBALB/c) F1 male (○) or (BALB/c X B10BR) F1 male (●) mice in replicate cultures supplemented with 0.5 units/ml of rat TCGF. Several doses of BALB.K NW-BM cells treated with anti-Thy.1 monoclonal antibody and complement were added to these cultures as indicated. The cytotoxic activity and its significance was estimated as described in Fig. 1. *; $p<0.01$.

were derived from sex chromosome or autosome linked gene products. Thus, we used (B10D2XBALB.K) F1 male and (BALB.KXB10D2) F1 male spleen cells as stimulators. Both F1 male mice had the same autosomes but reciprocally different sex chromosomes derived from either parental strain. Both the responder LN and the NW-BM cells derived from B10BR mice were cultured with either (B10D2XBALB.K) F1 male or (BALB. KXB10D2) F1 male spleen cells as stimulators (Fig. 4). The number of CTL induced by stimulation with (BALB.KXB10D2) F1 male spleen cells was significantly higher than that by (B10D2XBALB.K) F1 male ones. These results showed that NW-BM cells responded to the antigens from sex chromosome linked gene products, because both F1 male stimulators had different sex chromosomes, but identical autosomes. Furthermore, these results suggested that NW-BM cells were stimulated by the antigens derived from the non-self X-chromosome linked gene products (Xir antigens). B10BR mice, that were the source of NW-BM cells, had a different X-chromosome from the good stimulator (BALB.KXB10D2) F1 male mice. However, they had an identical X-chromosome to the poor stimulator (B10D2XBALB.K) F1 male mice.

When both responder LN and NW-BM cells from BALB.K mice were cultured with the same stimulators used in Fig. 4, the inverse results were obtained, that is, (B10D2XBALB.K) F1 male spleen cells, having different Xir antigens from BALB.K NW-BM cells, were good stimulators, but (BALB.KX B10D2) F1 males, possessing identical Xir antigens with BALB.K NW-BM cells, were poor (Fig. 5). These results again confirmed that NW-BM cells responded to non-self Xir antigens, as shown in Fig. 4.

We showed in Fig. 3 that $Thy.1^-$ population in NW-BM cells owed the main part of the activity augmenting the CTL generation. The same conclusion was presented in Fig. 6. $Thy.1^-$ NW-BM cells and responder LN cells from BALB.K mice were stimulated with either (B10BR X BALB/c) F1 male or (BALB/cXB10BR) F1 male spleen cells. The CTL induced by the stimulation with (B10BRX BALB/c) F1 male spleen cells were significantly higher than that by (BALB/cXB10BR) F1 male ones (Fig. 6). Thus, we concluded from the results of Figs. 4, 5 and 6 that $Thy.1^-$ NW-BM cells could distinguish between the Xir antigens on the stimulator cells as self or non-self.

BALB/c NW-BM cells, having $H\text{-}2^d$ haplotype, could also respond to Xir antigens

In all the experiments described above, we used cells having an $H\text{-}2^k$ haplotype as the responders. That is, responders were LN and NW-BM cells, and those with an $H\text{-}2^d$ haplotype were the stimulator spleen cells. We studied whether the NW-BM cells could also respond to Xir antigens when the cultures were arranged with a reverse

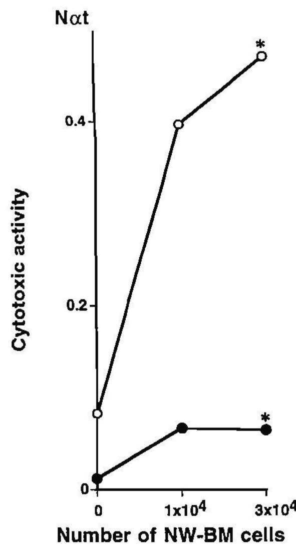

Fig. 7. BALB/c NW-BM cells, having H-2[d] haplotype, could also respond to non-self Xir antigens. Ten thousand BALB/c LN cells/well were cultured with 3X 10^5 mitomycin-c treated spleen cells/well from either (B10D2X BALB.K) F1 male (○) or (BALB.KXB10D2) F1 male (●) mice in replicate cultures supplemented with 0.67 units/ml of rat TCGF. Several doses of BALB.K NW-BM cells were added to these cultures as indicated. The cytotoxic activity and its significance was estimated as described in Fig. 1. *; $p<0.01$.

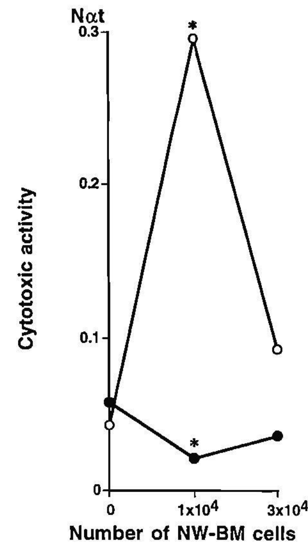

Fig. 8. Thy.1⁻ population in BALB/c NW-BM cells was responsible for most of the ability to respond to non-self Xir antigens. Six thousand BALB/c LN cells/well were cultured with 3X 10^5 mitomycin-c treated spleen cells/well from either (B10D2XBALB.K) F1 male (○) or (BALB.KXB10D2) F1 male (●) mice in replicate cultures supplemented with 0.67 units/ml of rat TCGF. Several doses of BALB.K NW-BM cells treated with anti-Thy.1 monoclonal antibody and complement were added to these cultures as indicated. The cytotoxic activity and its significance was estimated as described in Fig. 1. *; $p<0.01$.

combination of MHC haplotypes between the responder and stimulator cells, that is, H-2[d] anti H-2[k]. As shown in Figs. 7 and 8, intact NW-BM cells (Fig. 7) and Thy.1⁻ NW-BM cells (Fig. 8) from BALB/c mice could also respond to allogeneic, non-self, Xir antigens of (B10D2X BALB.K) F1 male stimulator spleen cells, but not to syngeneic, self, Xir antigens of (BALB.KX B10D2) F1 males.

DISCUSSION

We have previously reported that prethymic Thy.1⁻ NW-BM cells could augment the generation of CTL specific for allo MHC antigens in primary culture *in vitro*[1]. In this report, the same results were obtained using intact NW-BM cells as well as Thy.1⁻ NW-BM cells (Figs. 1-8). We also reported that Thy.1⁻ NW-BM cells augmented the induction of the CTL specific to allo minor-H antigens[13] or tumor specific transplantation antigens (TSTA)[14] in primary cultures *in vitro*.

The potential population in the NW-BM cells, which augmented the generation of CTL specific to allo MHC antigens, was analyzed using a fluorescence activated cell sorter (FACS). These cells expressed $Thy.1^{low}$, CD3⁻, TcR-α^-/β^-, γ^-/δ^-, CD4⁻ and CD8⁻ on their cell surface (data not shown). Though multipotent haematopoietic stem cells also expressed $Thy.1^{low}$ on their cell surface[24], $Thy.1^{low}$ NW-BM cells might belong to the more mature lymphoid lineage-committed cells, because their function was extremely essential for the immunological response.

Several cell populations among the lymphoid lineage cells augmented the generation of CTL. TcR-α^+/β^+ Th cells were classified into Th1 and Th2 cells according to their patterns of cytokine secretion[25-27]. They augmented the generation of CTL via these cytokines[28-32]. Thy.1⁺, CD3⁺, TcR-γ^+/δ^+ T cells also had the helper activity because they produced interleukin-2 (IL-2) upon antigenic stimulation[33,34]. However, these T cells could not acquire the helper activity during their immature stage in the bone marrow because TcR was not yet present on their surface, as it was acquired lately in the thymus or the periphery[35]. Thus, the immature T cells were unlikely to be the potential population in $Thy.1^{low}$ NW-BM cells. As NK cells were present in the bone marrow cells and could produce IL-2[36], it was supposed that NK cells helped to induce the CTL. NW-BM cells directly contacted CTL precursor cells then activated them, resulting in the generation of CTL, because the identity of MHC antigens between NW-BM cells and CTL precursor cells was required for this augmentation, as reported previously[1]. Thus, the augmentation by the NW-BM cells was not mediated by a non-specific factor, such as IL-2 or other cytokines. These results meant that IL-2 production was not sufficient for CTL induction. We could not, however, exclude the NK cells, until we proved that they

had no augmenting activity in our culture.

In this study, we clarified the antigens recognized by NW-BM cells. Initially, we studied whether NW-BM cells preferentially recognized MHC or some other antigens. Both a low number of responder LN cells and intact NW-BM cells, from B10BR mice ($H\text{-}2^k$, B10 background), were cultured with the stimulator spleen cells from either B10D2 ($H\text{-}2^d$, B10 background) or BALB/c ($H\text{-}2^d$, BALB/c background) mice. The number of CTL induced by stimulation with the BALB/c spleen cells was significantly higher than by B10D2 cells (Fig. 1). The reverse results were obtained when the responder LN and NW-BM cells from BALB.K ($H\text{-}2^k$, BALB/c background) mice were cultured with the same stimulator cells as used in Fig. 1. Namely, B10D2 spleen cells were better stimulators than BALB/c cells (Fig. 2). The augmentation of CTL generation was attributed to the $Thy.1^-$ population in NW-BM cells (Fig. 3). These results showed that NW-BM cells responded better to stimulator cells having different background genes, than to those having identical genes.

We next examined which antigens NW-BM cells recognized among the many products derived from background genes. We used (B10D2XBALB.K) F1 male and (BALB.KX B10D2) F1 male spleen cells as stimulators. Both F1 male mice had the same autosomes but reciprocally different sex chromosomes derived from either parental strain. B10BR NW-BM cells responded well to (BALB.KX B10D2), but not to (B10D2X BALB.K) F1 male spleen cells (Fig. 4). These data meant that NW-BM cells responded to non-self sex chromosome linked gene products, and that they responded to the non-self X-chromosome linked gene products (Xir antigens). This was because B10BR mice had a different X-chromosome from good stimulators (BALB.KXB10D2) F1 male mice, but an identical one with poor stimulator (B10D2XBALB.K) F1 male mice. On the contrary, BALB.K NW-BM cells responded well to the non-self Xir antigens from (B10D2X BALB.K) F1 male spleen cells, but not to the self Xir antigens from (BALB.KXB10D2) F1 males (Fig. 5). In all the experiments described above, augmenting activity of NW-BM cells were shown within the fixed MLC, that is, $H\text{-}2^k$ anti $H\text{-}2^d$. Their activity was also shown in the reverse MLC, that is, $H\text{-}2^d$ anti $H\text{-}2^k$. BALB/c NW-BM cells also responded to non-self Xir antigens alone (Fig. 7). The ability which responded to non-self Xir antigens and augmented the generation of CTL, was attributed to the $Thy.1^-$ population in NW-BM cells (Fig. 6 and 8). We concluded that $Thy.1^-$ NW-BM cells could discriminate between the Xir antigens on stimulator cells as self or non-self, and responded to non-self Xir antigens but not to self ones. Thus, Xir antigens were the immune response regulatory gene products and they could determine whether or not NW-BM cells responded.

There are three possible mechanisms to explain how NW-BM cells distinguished between self and non-self Xir antigens. i) NW-BM cells recognize non-self Xir antigens, but not self antigens. ii) NW-BM cells recognize both self Xir antigens and non-self ones regardless of their difference. Non-self Xir antigens assist the NW-BM cells into the active state, which could augment the CTL generation, whereas self Xir antigens induce a suppressive state, which could nonspecifically downregulate the immune response by some produced factor, such as TGF-β etc[37]. iii) NW-BM cells recognize and respond to both self and non-self Xir antigens. However, self reactive NW-BM cells recognize the self Xir antigens on the suppressor veto cells as well as on the stimulator cells, and then are suppressed by the veto cells[38,39]. We explain the third possibility in detail, because it is very complex. As suppressor veto cells are present in BM cells[20], they always express the self Xir antigens in common with NW-BM cells. If NW-BM cells respond to the self Xir antigens on the stimulator cells, these self reactive NW-BM cells also recognize the self ones on the veto cells. We could not decide on which possibility was correct. Nevertheless, NW-BM cells recognized only the Xir antigens, that is, they could respond to non-self Xir antigen alone initially, then to both self and non-self Xir antigens at the second or third instance.

The Th cells recognized foreign antigens peptides in the context of self MHC class II antigens via their T cell receptors[7-9]. The response of the Th cells depended upon the interaction between TcR and MHC antigens. On the other hand, NW-BM cells recognized allogeneic, non-self, Xir antigens via some unknown receptors, but not TcR. The response of NW-BM cells depended on the interaction between unknown receptors and Xir antigens. As described above, NW-BM cells had some characteristics that differed from those of Th cells. i) As NW-BM cells expressed $Thy.1^{low}$, $CD3^-$, TcR^-, $CD4^-$ and $CD8^-$ on the cell surface, they had no TcR (data not shown). ii) NW-BM cells recognized Xir antigens, which were different from the MHC antigens recognized via TcR. iii) NW-BM cells directly contacted CTL precursor cells and augmented the generation of CTL[1]. Until now, it was unknown that Th cells augmented CTL precursor cells by direct cell to cell interaction[15]. From these three facts, we concluded that NW-BM cells belong to a different lineage from that of T cells and that their regulation of the immune response is independent of the regulation by Th cells. We thus propose the working hypothesis that the immune response is regulated by two systems, that is, a Th cell (TcR)-MHC antigen system and a NW-BM cell (unknown receptor)-Xir antigen system.

We determined whether there was a population having activity equivalent to that of NW-BM cells in the periphery. We found that the $Thy.1^-$ population in spleen cells had similar activity to that of NW-BM cells (data not shown). We are now intensively studying this issue.

We reported that NW-BM cells could augment the generation of anti minor-H specific CTL in primary cultures *in vitro*[13]. These CTL were specific only to Xir antigens restricted by MHC antigens. The CTL specific to many

other minor-H antigens were not induced at all. The Xir antigens were the absolute dominant antigen over many other minor-H antigens. These results suggested that Xir antigens were important for the universal immune responses as well as the object of the recognition by NW-BM cells.

Several reports have described that various immunodeficiency diseases are closely related to X-chromosome linked genes in human and mice[40-44]. There are many important genes involved in immune responses on the X-chromosome. It is important to define the relationship between our Xir genes and these X-chromosome linked immunodeficiency genes.

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