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Cyclophosphamide-induced suppressor cells in nude mice

Motomu Shimizu,[CA] Damagoj Sabolovic, Hideki Ozawa and Takao Iwaguchi

M Shimizu, H Ozawa and T Iwaguchi are at the Department of Cancer Therapeutics, Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo, Japan. Tel: 03-3823-2101 ext. 5344. Fax: 03-3823-2965. D Sabolovic is at INSERM Unit 313, Institute de Santé et Développement, 15–21 rue de l'Ecole de Médecine, 75270 Paris, Cedex 06, France.

Lymphocytes regenerated after treatment with a high dose of cyclophosphamide (CY) were characterized in nude mice. Ten days after a single injection of 200 mg/kg CY into nude mice, regenerated spleen cells suppressed *in vitro* primary and second antibody production against sheep red blood cells. The CY-treated spleen cells exhibited normal natural killer (NK) activity, very low B and T cell content, but increases in cell surface charge [electrophoretic mobility (EPM)] and histamine receptors. The suppressor cells could not be removed by treatment with anti-Thy-1 plus complement (C), or treatment with anti-asialo GM_1 (aGM_1) plus C, which abrogated NK activity. It was concluded that CY-treated spleen cells, which exhibited high EPM and histamine receptors, comprise the natural suppressor cells which are Ig^-, Thy-1^- and aGM_1^-.

Key words: Cell electrophoresis, cyclophosphamide, histamine receptor, natural suppressor cells.

Introduction

Cyclophosphamide (CY) is an alkylating agent which acts preferentially on actively dividing cells[1]. CY has been widely used as a potent antitumor drug and exhibits a powerful immunosuppressive effect by depressing the immune system[1]. A burst of lymphatic regeneration occurs after destruction of lymphocytes by CY. However, administration of CY, as well as total lymphoid irradiation (TLI) and chronic graft-versus-host disease (GVHD), induces natural suppressor (NS) cells, which are defined as the unprimed null lymphocytes and suppress the response of lymphocytes to immunogenic and mitogenic stimuli.[2-10] NS cells are also found in neonatal lymphoid tissue and in adult bone marrow.[4-6] NS cells do not exhibit the typical surface markers for B, natural killer (NK) and T cells [Ig^-, Thy-1^- and asialo GM_1^- (aGM_1^-)].[3,4,7] They are shown to suppress antibody production, mitogen-stimulated blastogenesis and the mixed lymphocyte reaction.[3-5,8] Thus, CY-induced NS cells might play an important role in the immunosuppression caused with CY.

Most of the studies of CY-induced NS cells have been carried out in euthymic mice.[9,10] However, since nude mice cogenitally lack thymus,[11] they are a good model for the study of NS cells. The cell surface charge [electrophoretic mobility (EPM)], which is a good parameter for lymphocyte characterization,[12-16] and histamine receptors, which are also expressed in suppressor T cells,[17-20] have not been analyzed in NS cells. Thus, we studied CY-induced splenic suppressor cells in nude mice using different phenotypic markers such as Ig, Thy-1, CD4, CD8, aGM_1, cell surface charge, histamine receptors and inhibition of antibody production against sheep red blood cells (SRBCs).

Materials and methods

Reagents

The following reagents were used: CY (Shionogi Pharmaceutical Co., Osaka, Japan), [2,5-^{3}H]histamine dihydrochloride (specific activity 54 Ci mmol) (Amersham, UK), Lympholyte-M and rabbit serum as complement (C) (Cedarlane, Ontario, Canada), SRBCs (Nissei-zai, Tokyo, Japan), RPMI 1640 and Eagle's minimal essential medium (MEM) (Gibco, NY, USA), FITC–anti-mouse IgG [heavy and light chain specific, $F(ab')_2$ of Goat IgG] (Tago, CA, USA), FITC–anti-rabbit IgG [F_c specific, $F(ab')_2$

[CA] Corresponding Author

of sheep IgG] (Cappel, CA, USA), anti-mouse IgM (μ chain specific, rabbit IgG) and anti-mouse IgG (γ chain specific, rabbit IgG) (MBL, Nagoya, Japan), FITC–anti-Thy-1.2, FITC–anti-L3T4 (CD4) and FITC–anti-Ly-2 (CD8) (monoclonal antibodies, Becton-Dickinson, CA, USA), anti-Thy-1.2 (clone F7D5) (Serotec, UK), and anti-aGM_1 (rabbit IgG) (Wako Pure Chemicals, Osaka, Japan).

Preparation of lymphocytes

The spleen was teased and erythrocytes were removed using Tris-buffered NH_4Cl solution. The lymphocytes were washed 3 times with phosphate-buffered saline (PBS).

Administration of CY and SRBCs

CY was dissolved in sterile water immediately before use. BALB/c nude mice (Charles River Japan Inc., Tokyo) were injected intraperitoneally with 200 mg/kg CY. BALB/c (+/+) mice (Charles River) were injected with 10^8 SRBCs 10–17 weeks before the experiments.

Fluorescence-activated cell sorter (FACS) analyses

The lymphocytes were stained with FITC–anti-mouse IgG, FITC–anti-Thy-1.2, FITC–anti-CD4 or FITC–anti-CD8 antibody for 40 min at 4°C. For the analysis of aGM_1, the lymphocytes were stained with rabbit anti-aGM_1 and then FITC–anti-rabbit IgG antibodies (F_c specific). The lymphocytes (10^4) were analyzed with a FACS IV (Beckon-Dickinson).

NK activity

NK activity was determined by the method of Hashimoto and Sudo.[21] Briefly, 1×10^4 [^{3}H]uridine-labeled YAC-1 cells were incubated with effector cells in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) (Flow Labs, North Ryde, Australia) for 18 h at 37°C in a 5% CO_2 incubator. The radioactivity remaining in the cells was analyzed by a liquid scintillation counter. The percentage lysis was calculated as follows: $\%\text{lysis} = \{[(\text{target})_{\text{c.p.m.}} - (\text{target} + \text{effector})_{\text{c.p.m.}}]/(\text{target})_{\text{c.p.m.}}\} \times 100$ (%).

Cell electrophoresis

The EPM of cells was determined in MEM at 24°C with a fully automated cell electrophoresis instrument (Parmoquant-L), which was capable of reproducible and quantitative analysis of EPM data. Details of the apparatus and the method of measurement have been described elsewhere.[12,13] The principle of automatic electrophoresis is based on image processing. The EPM was expressed in terms of the absolute value and μm/s/V/cm. The EPM of SRBCs (1.00 μm/s/V/cm) was measured before and after each determination to confirm that the system was operating reliably. Each measurement required about 3×10^6 cells and 10 min.

Anti-SRBC response

Spleen cells (4×10^6 cells) from BALB/c (+/+) mice, SRBCs (2×10^6) and CY-induced spleen cells (2×10^6) were suspended in 3 ml of RPMI 1640 medium containing 10% heat-inactivated FBS and 2-mercaptoethanol (5×10^{-5} M) (culture medium) and incubated in a falcon tube (#2054, Becton-Dickinson) for 6 days at 37°C in an atmosphere of 5% CO_2. After culture, cells were centrifuged on a density gradient Lympholyte M for 15 min at 400 *g* to remove dead cells. The viable cells were washed with PBS. The cells suspended in culture medium ($0.9–1.8 \times 10^7$ cell/ml) were restimulated with fresh SRBCs (5×10^5 cell/ml) for 13 h at 37°C. After culture, the supernatant was harvested by centrifugation and the content of anti-SRBC antibody in the supernatant was determined with the FACS IV.

Flow cytometric determination of the production of anti-SRBC antibody

The production of anti-SRBC antibody was determined by flow cytometry.[22] SRBCs (10^6) were incubated with 50 μl of the culture supernatant containing anti-SRBC for 45 min at 4°C. After washing, the treated SRBCs (5×10^5) were incubated with 50 μl of anti-mouse IgM (μ chain specific) or anti-mouse IgG (γ chain specific) (rabbit IgG) for 30 min. The fluorescence intensity of antibody-bound SRBCs was determined by incubation with 50 μl of FITC–anti-rabbit IgG (F_c specific). Cells (2×10^4) were measured with the FACS IV equipped with a logarithmic fluorescence signal amplifier (1–255 channels). The channel number of

the fluorescence peak of the SRBCs, which increased with anti-SRBC concentration, was determined as an index of the concentration of anti-SRBC. Since the coefficient of variation of the channel number was less than 2–3% of the mean value, the standard deviation was omitted. For the simple expression of antibody production, the anti-SRBC response was calculated from the peak channel number as follows: anti-SRBC response = {[(peak of culture of spleen cells + SRBCs + sample cells) − (peak of culture of spleen cells alone)]/[(peak of culture of spleen cells + SRBCs) − (peak of culture of spleen cells alone)]} × 100 (%).

Treatment of lymphocytes with antibody and C

The lymphocytes were incubated with 20-fold diluted rabbit anti-aGM_1 or 1000-fold diluted anti-Thy-1.2 for 45 min at 4°C. After centrifugation, the lymphocytes were resuspended in 10-fold diluted rabbit serum as a source of C and incubated for 60 min at 37°C. The treated cells were washed twice and used for the experiments.

Histamine receptors

Spleen cells (10^6 cells/ml) were incubated with 5 μCi of [^{3}H]histamine at 37°C for 2 h. Cells were then washed, the pellet solubilized into 5 ml of scintillation liquid under vigorous agitation and counted. In other experiments, unlabeled histamine, the histamine type 2 (H2) receptor agonist, dimaprit, or the H2 receptor antagonist, ranitidine, were added at a concentration of 10^{-4} M in order to verify the specificity of the binding.

Results

Four days after the injection of 150–250 mg/kg CY the spleen cellularity decreased to about 10^6 cells followed by rapid cell multiplication and then increased for the next 7 days, as confirmed by [^{3}H]thymidine incorporation *in vivo* and *in vitro* (data not shown). At 10–12 days after injection, a number of cells returned to the initial steady state. Thus, we characterized the surface markers and the function of the newly regenerated lymphocytes 10 days after the injection of 200 mg kg CY.

Surface marker

The surface markers of Ig, Thy-1, CD4, CD8 and aGM_1 in CY-treated and untreated spleen cells were determined with the FACS (Figure 1A–E). The percentage of Ig^+ cells in CY-induced spleen cells significantly decreased while that of Thy-1^+, $CD4^+$, $CD8^+$ and aGM_1^+ cells did not increase. This result indicated that CY-induced spleen cells were mainly null cells (Ig^-, Thy-1^- and aGM_1^-).

NK activity

The NK activity was evaluated by using YAC-1 cells as a target (Table 1). The cytotoxic test using [^{3}H]uridine-labeled YAC-1 cells is more sensitive than that using a 4 h ^{51}Cr release test (data not shown). The NK activity was found to be almost same in CY-treated and untreated nude mice. *In vitro* treatment with anti-aGM_1 and C eliminated NK activity in both groups. Thus, the NK activity and percentage of aGM_1^+ cells in CY-induced spleen cells was restored to that in untreated spleen cells.

Suppressor activity in antibody production

The determination of antibody production based on flow cytometry has a higher sensitivity and reproducibility than that based on counting plaque-forming cells (conventional method).[22] Using flow cytometry, the effect of CY-induced spleen cells was evaluated in *in vitro* primary and secondary antibody production against SRBCs. Anti-SRBC production from normal spleen cells plus SRBCs was expressed as 100%, and that from normal spleen cells alone as 0%. As shown in Table 2, anti-SRBC (IgM and IgG) production was not affected by the addition of spleen cells of untreated nude mice. However, CY-induced spleen cells strongly suppressed IgM and IgG antibody production. In addition, *in vitro* treatment with anti-aGM_1 and C had no effect on the suppression of antibody production by CY-induced spleen cells. Moreover, CY-induced spleen cells suppressed *in vitro* secondary antibody production against SRBCs (Table 3). Treatment with anti-Thy-1 and C did not eliminate the suppressor activity of CY-induced spleen cells. These results indicated that suppressor cells were null cells without surface markers such as aGM_1 and Thy-1. The CY-induced spleen cells also inhibited the blastogenesis of lymphocytes stimulated with concanavalin A (data not shown). These results indicated that

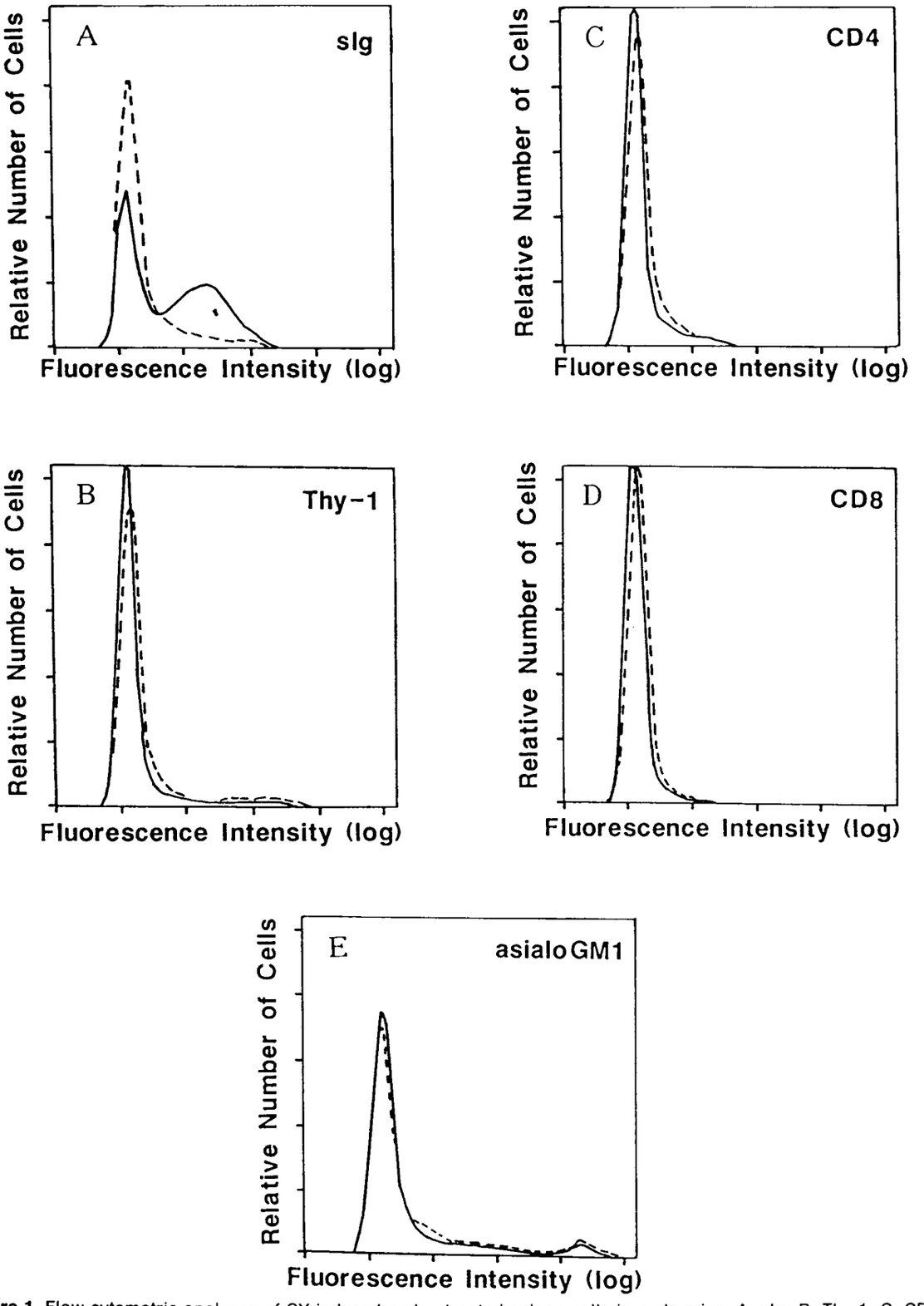

Figure 1. Flow cytometric analyses of CY-induced and untreated spleen cells in nude mice. A, sIg; B, Thy-1; C, CD4; D, CD8; E, asialo GM_1. CY-induced (— — —) and untreated (———) spleen cells.

Table 1. NK activity of spleen cells of CY-treated and untreated nude mice

Nude spleen cells		YAC-1 Lysis (%)		
treatment		E:T ratio		
in vivo	*in vitro*	100:1	50:1	25:1
CY	anti-aGM1 + C	14 ± 2[a]	13 ± 3[a]	5 ± 1[a]
CY	C	39 ± 2	33 ± 3	31 ± 4
None	anti-aGM1 + C	9 ± 2[a]	0 ± 9[a]	0 ± 3[a]
None	C	46 ± 2	23 ± 5	17 ± 5

[a] Statistically significant versus C control by Student's *t*-test ($p < 0.05$) ($n = 3$).

Table 2. Suppression of anti-SRBC response by spleen cells of CY-treated nude mice[a]

Normal spleen cells	SRBCs	Nude spleen cells treatment		Anti-SRBC per culture (%)	
		in vivo	*in vitro*	IgM	IgG
+	+	CY	anti-aGM1 + C	5[b]	17[b]
+	+	CY	C	5[b]	11[b]
+	+	none	anti-aGM1 + C	105	111
+	+	none	C	105	95
+	+	—		100	100
+	—	—		0	0

[a] Normal spleen cells, SRBCs and nude spleen cells were incubated for 6 days in RPMI 1640 containing 10% FBS and 2-mercaptoethanol (5×10^{-5} M).
[b] Statistically significant versus normal spleen cells plus SRBCs (100%) by Student's *t*-test ($p < 0.05$). See Materials and methods for detailed description of statistical analysis. One of two experiments.

Table 3. Suppression of anti-SRBC response of SRBC-immunized spleen cells by spleen cells of CY-treated nude mice

SRBC-immunized spleen cells[a]	SRBCs	CY-treated nude spleen cells	Anti-SRBC per culture (%)	
			IgM	IgG
+	+	anti-Thy-1 + C	0[b]	80[b]
+	+	C	23[b]	76[b]
+	+	—	100	100
+	—	—	0	0

[a] SRBC-immunized mice: SRBC 10^8 cells, i.p.
[b] Statistically significant versus SRBC-immunized spleen cells plus SRBCs (100%) by Student's *t*-test ($p < 0.05$). One of two experiments.

CY-induced spleen cells contained NS cells without surface markers.

EPM histograms of CY-induced spleen cells

We have already reported that the EPM histogram of spleen cells in nude mice shows a major peak of B cells with low EPM and a minor peak of NK cells with high EPM.[13] Ten days after the treatment with CY, the low EPM peak of the B cells decreased while the high EPM peak significantly increased (Figure 2). Thus, CY-induced spleen cells were found to be a high EPM cell type.

Histamine receptors

Following treatment with CY, the histamine receptors of spleen cells increased more than 37 times that of control mice (Table 4). Since [^{3}H]histamine binding was inhibited by addition of cold histamine (88%), a H2 agonist dimaprit (68%), or a H2 antagonist, ranitidine (45%), the binding of [^{3}H]-histamine was specific for the histamine receptors. Autoradiography of CY-induced spleen cells with [^{3}H]histamine showed that individual cells were very charged with histamine (data not shown), indicating that the cells with histamine receptors increased the number of the receptors.

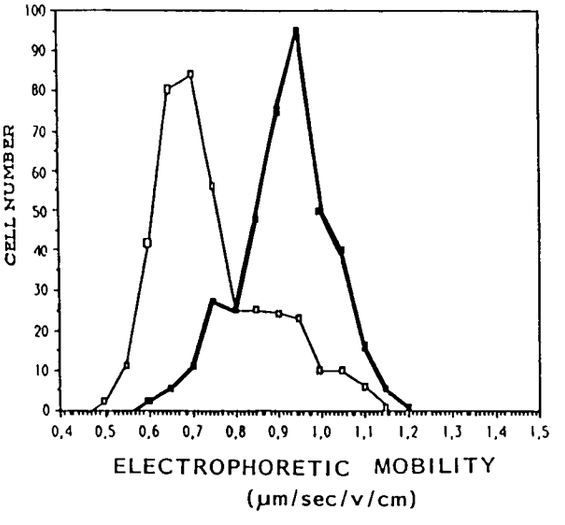

Figure 2. EPM histogram of CY-induced and untreated spleen cells in nude mice. Four hundred cells were measured with an automatic cell electrophoretic instrument. CY-induced (■——■) and untreated (□——□) spleen cells.

Table 4. [^{3}H]histamine binding activity in spleen cells of CY-treated and untreated nude mice

Spleen cells from mice treated with	[^{3}H]histamine binding activity (c.p.m./10^6 cells)
CY	45000 ± 5184[a]
none	1200 ± 657

Mean ± standard deviation of four experiments ([a]$p < 0.001$ by Student's *t*-test).

Discussion

NS cells are found in neonatal lymphoid tissue, adult bone marrow, and in the spleen of mice after CY or TLI treatment, or undergoing chronic GVHD.[2,10] Bone marrow may be a site of some tolerance induction throughout adult life. TLI and CY treatments are practical means of induction of a general immunosuppressive environment where tolerance induction is possible. These results suggest that CY-induced suppressor cells may be involved in the establishment and maintenance of self-tolerance and immunosuppression.

Cytological examination of spleen cell smears 10 days after treatment with CY showed a decrease in lymphoid cell types and a significant increase of blastoid cells with a large nucleus and small cytoplasm (data not shown). The morphology of CY-induced spleen cells agreed with that of NS cells.[3,23] The spleen cellularity reached a normal value at the time of assay. These results indicated that CY-induced spleen cells mainly consisted of regenerating lymphocytes. The CY-induced spleen cells were found to be null lymphocytes (Ig^-, Thy-1^- and aGM_1^-) with strong suppressor activity (Figure 1, and Tables 2 and 3). Thus, we confirmed that NS cells are induced by treatment with CY in athymic nude mice as well as euthymic mice.

In vivo treatment with CY in euthymic mice is known to kill B, T and NK cells.[24-26] It is reported that during regeneration, the euthymic spleen recovered between 5 and 11 days after CY treatment and NK cells recovered later (9–12 days after CY).[10,24-26] The recovery of B cells in nude mice needed more than 10 days[27] (Figure 1A). NS cells induced with CY in nude mice were enriched 10 days after the injection because of slow recovering B cells and the lack of fast recovering T cells[26] (Figures 1 and 2, and Tables 2 and 3). Thus, nude mice are a good model for the study of NS cells.

In this report, we have examined in detail some properties such as the surface charge (EPM) (Figure 2) and histamine receptors (Table 4) in CY-induced spleen cells of nude mice.

The EPM reflects the overall chemical composition of ionized groups on the cell surface. The surface charge depends on five types of chemical groups: amino, sulfhydryl, carboxyl, phosphate and other groups.[28] The carboxyl group of sialic acid is mainly responsible for the negative surface charge in lymphocytes.[28,29] Cell surface charge was easily measured by a full automatic procedure in a highly reproducible way.[12,13] Thus, the EPM is a good parameter for the characterization of lymphocytes.[12,16] We have already reported that the EPM increases in the following order: B, NK and T cells.[13,14] However, the EPM of NS cells was not clarified. Thus, we have studied CY-induced spleen cells as NS cells in nude mice because of the congenital lack of any thymus. NS cells were found to be the NK cell type with high EPM (Figure 2). Thus, it is suggested that NK and NS cells were not separated into two distinct EPM cell populations on the basis of cell surface charge.

The CY-treated spleen cells were found to bind radioactive histamine up to 37-fold (Table 4). The presence of H2 receptors has been described as one of the characteristics of suppressor T cells and as a way by which histamine modulates the immune response.[17-20] The binding of [^{3}H]histamine in CY-induced spleen cells was also inhibited by H2 agonists and antagonists. These results suggested that NS cells bore histamine receptors. However, we need to clarify the function of the H2 receptor in CY-induced spleen cells.

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