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ANTI-ASIALO GM1 ANTIBODY SUPPRESSION OF CYCLOPHOSPHAMIDE-INDUCED DIABETES IN NOD MICE

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SUMMARY To elucidate the role of natural killer (NK) cells in the pathogenesis of diabetes in the non-obese diabetic (NOD) mouse, we examined whether or not cyclophosphamide-induced diabetes occurs in NOD mice intraperitoneally (i.p.) injected with anti-asialo GM1 antibody. Two weeks after a single intraperitoneal injection of cyclophosphamide, none of the 24 NOD mice which had previously been treated with anti-asialo GM1 antibody, 2–3 times per week for either 2 or 3 weeks, had developed indications of diabetes such as glycosuria or a high plasma glucose level. On the other hand, signs of diabetes were found in 10 of 24 control NOD mice injected with normal rabbit Ig instead of anti-asialo GM1 antibody ($p < 0.01$). The NK cell activities of spleen cells from anti-asialo GM1 antibody-treated mice were significantly lower than those of control mice ($p < 0.01$). Flowcytometry analysis demonstrated that anti-asialo GM1 antibody-positive cells had disappeared from the spleens of anti-asialo GM1 antibody-injected mice but no suppression of $CD8^+$ and $CD4^+$ cells could be demonstrated. These observations suggest that NK cells are involved in the development of diabetes in NOD mice.

Key words: NOD mouse, NK-cell, anti-asialo GM1 antibody, prevention, cyclophosphamide

INTRODUCTION

THE non-obese diabetic (NOD) mouse is regarded as one of the most suitable animal models of insulin-dependent diabetes mellitus because ketosis-prone diabetes develops after the appearance of lymphocytic infiltration of the islets (insulitis), just as in human beings (1–4). There is increasing evidence that the development of insulin-dependent diabetes is mediated by an autoreactive immune response associated with several genes including those of the MHC class II (5). In the NOD mouse, the main subset of infiltrating lymphocytes has been reported to be CD4-positive T-cells reacting to the host's own islet cells (6, 7). It has also been suggested that cytotoxic T cells and macrophages, which are detectable around islets, are activated to be effecter cells (8, 9). However, little is known about the role of natural killer (NK) cells in the development of developing diabetes in NOD mice.

To address this issue, we examined whether or not cyclophosphamide-induced diabetes developed in NOD mice lacking NK activity. The NOD mice used for these experiments were prepared by pre-treating with anti-asialo GM1 antibody which is known to reduce NK cell activity *in vivo*.

MATERIALS AND METHODS

Animals

Female NOD mice were obtained from Clea Japan, Inc. (Tokyo, Japan). Twelve-week-old mice were used for these experiments.

Administration of Anti-asialo GM1 Antibody and Cyclophosphamide

A single cyclophosphamide injection (Sionogi Pharmaceutical Co. Ltd, Osaka, Japan) of 200 mg/kg body weight was given three days after the initial treatment with either rabbit polyclonal antibody against asialo GM1 (Wako Pharmaceutical, Co., Tokyo, Japan, 0·7 mg/mouse) or rabbit Ig 2–3 times per week for either 2 or 3 weeks.

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Plasma Glucose Determination

Two and three weeks after cyclophosphamide injection, the mice were sacrificed by aspirating blood from the heart. Blood glucose levels were determined by the glucose oxidase method and individual mice were classified as diabetic when the values exceeded 250 mg/dl.

NK Activity Assay

Seven days after cyclophosphamide injection, spleen cells were obtained from the mice and used as effecter cells. The suspensions of YAC-1 cells were incubated at 37°C for 60 min in 1 ml RPMI 1640 with fetal bovine serum (FBS) and 0·05 mCi $Na_2{}^{51}CrO_4$. After incubation, the cells were washed three times, by centrifugation (450 rev for 20 min) in RPMI with FBS, and used for target cells. The suspensions of 2×10^4 target cells were incubated at 37°C in 5% CO_2 for 5 h with 1×10^6 effecter cells. After incubation, released ^{51}Cr was counted by a well type gamma counter and specific release was calculated using the following formula:

$$\text{specific release} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100\%$$

Flow Cytometry Analysis of Cell Surface Markers

Fourteen days after cyclophosphamide injection, spleen and thymus cell analyses were performed. Spleen cells obtained from the NOD mice were incubated with polyclonal anti-asialo GM1 for 20 minutes and enhanced with goat anti-rabbit IgG. After washing 3 times, the goat anti-rabbit IgG (Cartego, USA) was added to the cell pellets which were then incubated for a further 20 min on ice. After three additional washings with PBS, the cells were fixed with 1% paraformaldehyde.

For dual staining of anti-L3T4 and anti-Lyt2, spleen cells or thymus cells were added to the antibody mixture (fluorescence-conjugated anti-Lyt2 and biotinylated anti-L3T4, Becton Dickinson, California, USA). After 20 min of incubation on ice, the cells were washed three times. PE-streptoavidin (Biomeda, California, USA) was added and the cells were incubated on ice for 20 min. After three additional washings with PBS, the cells were fixed with 1% paraformaldehyde.

Profiles of stained cells were analyzed with FACSan (Becton Dickinson, California, USA). For each sample, data from a volume of 10,000 viable cells were collected.

Histology

Histological examination of pancreatic islets was performed 14 days after cyclophosphamide injection. Pancreata were removed, fixed in Bouin's solution, embedded in paraffin, stained with hematoxilyn-eosin, and examined light microscopically.

Results

As shown in Table 1, 10 of 24 NOD mice pretreated only with rabbit Ig showed hyperglycemia two weeks after the single injection of cyclophosphamide and 4 of 8 were hyperglycemic three weeks after the cyclophosphamide injection. However, none of the 24 which had been treated with anti-asialo GM1 for 2 weeks nor any of the 8 mice treated for 3 weeks developed hyperglycemia. The incidence of clinical diabetes in anti-asialo GM1-treated mice was significantly lower than in control mice ($X^2 = 12{\cdot}6$, $p < 0{\cdot}01$, $X^2 = 5{\cdot}3$, $p < 0{\cdot}05$, respectively).

The NK cell activities of spleen cells from NOD mice 7 days after cyclophosphamide injection are shown in Table 2. Spleen cells from cyclophosphamide-injected NOD mice without anti-asialo GM1 antibody pre-treatment showed a stronger tendency for splenic lysis of YAC-1 cells than did those from untreated mice. Spleen

Table 1 Frequency of clinical diabetes in NOD mice two and three weeks after cyclophosphamide injection

	Anti asialo GM1-treated mice		*Rabbit Ig-treated mice*
frequency rate (two weeks after CY-injection)	0/24(0%)	*	10/24(41·7%)
frequency rate (three weeks after CY-injection)	0/8(0%)	**	4/8(50·0%)

* $p < 0{\cdot}01$, ** $p < 0{\cdot}05$

cells from cyclophosphamide-injected NOD mice with anti-asialo GM1 antibody treatment exhibited a significant decrease in YAC-1 target cell lysis as compared with untreated controls and cyclophosphamide-injected NOD mice without anti-asialo GM1 antibody treatment ($p < 0{\cdot}01$, $p < 0{\cdot}05$, respectively).

With regards to flowcytometry analysis of these spleen cells, anti-asialo GM1 positive cells were significantly reduced in anti-asialo GM1 antibody-treated mice (Figure 1). However, there were no significant differences between the anti-asialo GM1 treated mice and controls in the percentage of Lyt2- and L3T4-positive cells (Figure 2). In addition, thymus cell analysis revealed no significant differences between these two groups in terms of their Lyt2- and L3T4-positive cell populations (Figure 3).

Insulitis was seen in all NOD mice. The degree of insulitis seemed to be less severe in anti-asialo GM1 antibody-treated mice than control mice but the difference was not significant.

Discussion

Our results demonstrate that anti-asialo GM1 antibody suppresses the development of cyclophosphamide-induced diabetes in NOD mice and that the NK-cell

Table 2 NK activity of spleen cells from NOD mice 7 days after cyclophosphamide injection

	Specific cytotoxicity (%)		
anti asialo GM1 antibody and cyclophosphamide treated mice	3·98 ± 1·52	*	**
rabbit Ig and cyclophosphamide treated mice	9·78 ± 3·90		
untreated control	7·72 ± 1·47		

Data expressed as mean ± SD.
* $p < 0{\cdot}05$, ** $p < 0{\cdot}01$

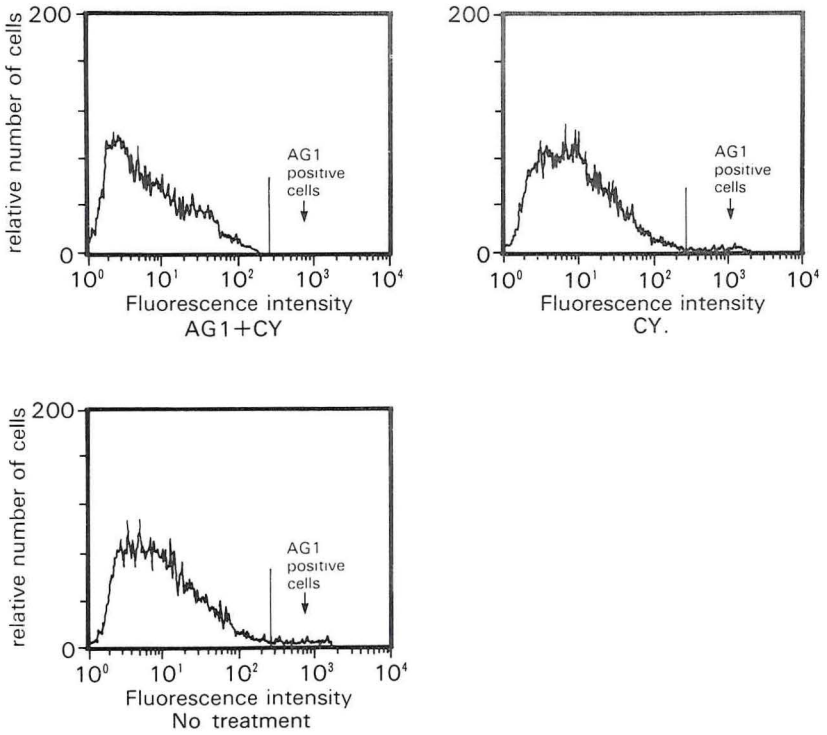
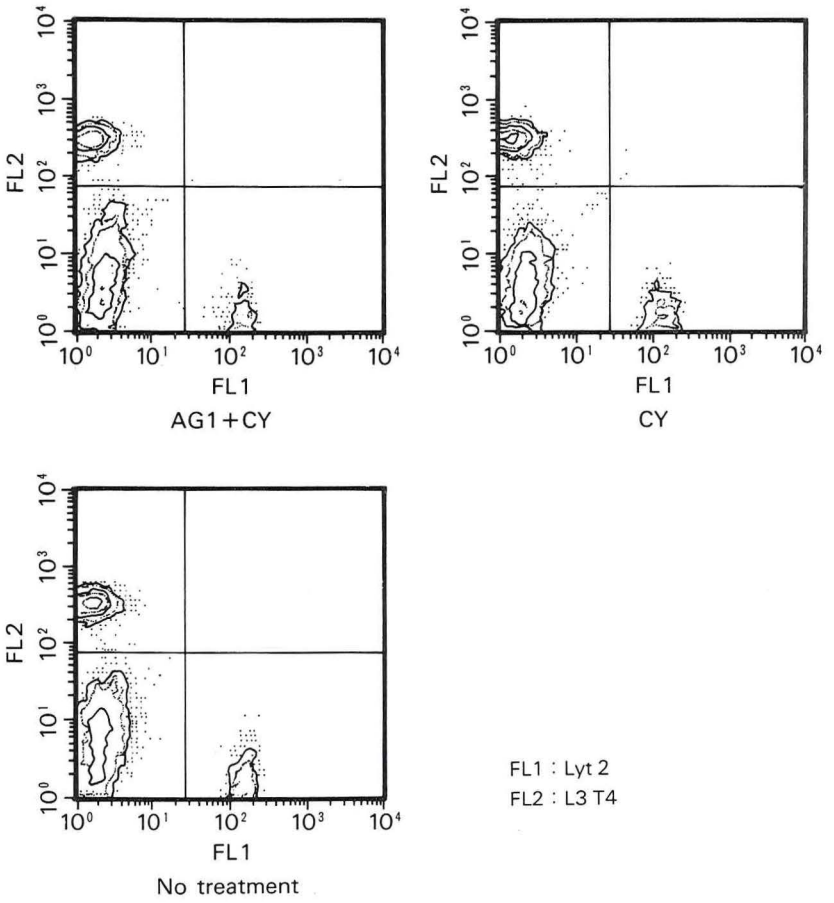


Figure 1. Flowcytometry analysis of spleen cells from NOD mice two weeks after cyclophosphamide injection. $AG1^+$ cells completely disappeared in anti-asialo GM1-treated mice.



FL1 : Lyt 2
FL2 : L3 T4

Figure 2. Flowcytometry analysis of spleen cells from NOD mice two weeks after cyclophosphamide injection. There were no significant differences between anti-asialo GM1-treated mice and those treated with normal rabbit Ig in terms of L3T4- and Lyt2-positive cell percentages.

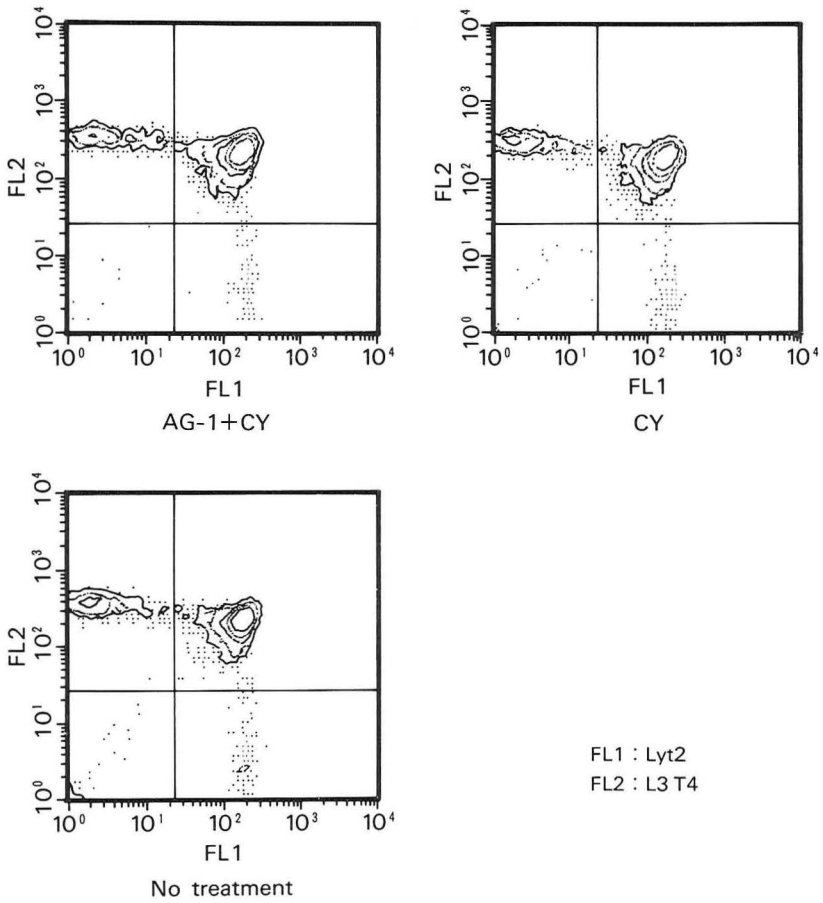


Figure 3. Flowcytometry analysis of thymus cells from NOD mice two weeks after cyclophosphamide injection. There were no significant differences between anti-asialo GM1-treated mice and those treated with normal rabbit Ig in terms of L3T4- and Lyt2-positive cell percentages.

activity of spleen cells is significantly reduced in mice treated with anti-asialo GM1 antibody. Furthermore, flowcytometry demonstrated the complete disappearance of anti-asialo GM1-positive cells. However, we could not find significant reduction in the relative percentages of T-lymphocyte subsets. These results suggest that NK cells play a crucial role in the development of diabetes in the NOD mouse model. The present results are consistent with our previous report in that the NK cell activity increased just before and after the onset of diabetes in cyclophosphamide-treated NOD mice (10).

It has been reported that anti-asialo GM1 may also react with non-NK cells, such as cytotoxic T cells, including LAK cells and certain macrophages. However, the majority of asialo GM1 positive spleen cells are supposed to be responsible for NK activity (11–14). Recently, asialo GM1 positive spleen cells have been demonstrated to contain cytoplasmic perforine and a small proportion of these cells coexpressed CD8 (I.I). In addition, all NK1.1$^-$ positive cells were stained with anti-asialo GM1 except for a small number of asialo GM1$^+$ NK1.1$^-$ cells. These asialo GM1$^+$ NK$^-$ cells are assumed to express CD8 (Okumura and Nishimura, personal communication). In the present study, *in vivo* injection of anti-asialo GM1 did not produce a significant reduction in the proportion of CD8$^+$ spleen cells. This is probably attributable to the asialo GM1$^+$ CD8$^+$ cell population being too small for a comparison of cell numbers to be made between individual mice with and without anti-asialo GM1 treatment. Taken together, it may be concluded that NK cells are one of the effecter cells attacking B cells in the islets, though the possibility remains that the small subset of asialo GM1$^+$ NK1.1$^-$ CD8$^+$ cells, as well as NK cells, function as effecters in the development of diabetes in NOD mice. Research aimed at elucidating the characteristics of these asialo GM1$^+$ Lyt-2$^+$ cells is presently being done.

It has been reported that the majority of the infiltrating lymphocytes in the islets are CD4$^+$ T cells. These CD4$^+$ cells have been proposed to react to self MHC class II expressed on pancreatic B cells and may thereby activate effecter cells such as asialo GM1$^+$ cells by producing lymphokines.

REFERENCES

1. Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K. and Tochino, Y. (1980). Breeding of a non-obese, diabetic strain of mice. *Exp. Anim.*, **29**, 1–13.
2. Takei, I., Maruyama, T., Taniyama, M., and Kataoka, K. (1986). Humoral immunity in the NOD mouse. In *Insulitis and type I diabetes: Lessons from NOD mouse* (edited by S. Tarui, Y. Tochino, and K. Nonaka), pp. 101–110. Academic Press Japan, Tokyo.

3. Maruyama, T., Takei, I., Taniyama, M., Kataoka, K. and Matsuki, S. (1984). Immunological aspect of non-obese diabetic mice: immune islet cell-killing mechanisms and cell-mediated immunity. *Diabetologia*, **27**, 121–123.
4. Maruyama, T., Takei, I., Asaba, Y., Yanagawa, T., Takahashi, T., Itoh, H., Suzuki, Y., Kataoka, K., Saruta, T. and Ishii. T. (1989). Insulin-autoantibodies in mouse models of insulin-dependent diabetes. *Diabetes Research*, **11**, 61–65.
5. Hattori, M., Buse, J. B., Jackson, R. A., Glimsher, L., Dorf, M. E., Minami, M., Makino, S., Moriwaki, K., Kuzuya, H., Imura, H., Strauss, W. M., Seidmann, J. G. and Eisenbarth, G. S. (1986). The NOD mouse: Recessive gene in the major histocompatibility complex. *Science*, **231**, 733–735.
6. Miyazaki, A., Hanafusa, T., Yamada, K., Miyagawa, J., Fujino-Kurihara, H., Nakajima, H., Nonaka, K. and Tarui, S. (1985). Predominance of T-lymphocytes in pancreatic islets and spleen of pre-diabetic non-obese diabetic (NOD) mice: a longitudinal study. *Clin. Exp. Immunol.*, **60**, 622–630.
7. Koike, T., Itoh, Y., Ishii, T., Itoh, I., Takabayashi, K., Maruyama, N., Tomioka, H. and Yoshida, S. (1987). Preventive effect of monoclonal anti L3T4 antibody on development of diabetes in NOD mice. *Diabetes*, **36**, 539–541.
8. Nagata, M., Yokono, K., Hayakawa, M., Kawase, Y., Hatamori, N., Ogawa, W., Yonezawa, W., Shii, K. and Baba, S. (1989). Destruction of pancreatic islet cells by cytotoxic T-lymphocytes in non-obese diabetic mice. *J. Immunol.*, **143**, 1155–1162.
9. Lee, K. U., Amano, K. and Yoon, J. W. (1988). Evidence for initial involvement of macrophages on development of insulitis in NOD mice. *Diabetes*, **37**, 989–991.
10. Maruyama, T., Yanagawa, T., Takei, I., Asaba, Y., Takahashi, T., Kataoka, K., Saruta, T. and Ishii, T. (1988). Increased cytotoxicity to islet cells and antibody-dependent cell-mediated cytotoxicity in a cyclophosphamide-injected non-obese diabetic (NOD) mouse. (Abstract) *Diabetes*, **37**, No. 5, 16A.
11. Kasai, M., Iwamori, M., Nagai, Y., Okumura, K. and Tada T. (1980). A glycolipid on surface of mouse natural killer cells. *Eur. J. Immunol.*, **10**, 175–180.
12. Habu, S., Fukui, H., Shimamura, K., Kasai, M., Nagai, Y., Okumura, K. and Tamaoki, N. (1981). Reduction of NK-activity and enhancement of transplanted tumor growth in nude mice. *J. Immunol.*, **127**, 34–38.
13. Suttles, J., Schwarting, G. A. and Stout, R. D. (1986). Flow cytometric analysis reveals the presence of asialo GM1 on the surface membrane of alloimmune cytotoxic T lymphocytes. *J. Immunol.*, **136**, 191–197.
14. Mercurio, A. M., Schwarting, G. A. and Robbins, P. W. (1984). Glycolipids of the mouse peritoneal macrophage. *J. Exp. Med.*, **160**, 1114–1125.

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