Neuraminidase Treatment Enhances Allogeneic Stimulation of Unprimed CD8$^+$ T Cells

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Abstract : Many cell types are known to stimulate CD8$^+$ T cells in allogeneic recognition such as mixed lymphocyte reaction (MLR). Whereas dendritic cells are most potent among them, T cells are usually considered very poor in stimulating CD8$^+$ T cells although there are some tumor cells that are weakly stimulatory. T cells, as a stimulator, cultured in the presence of concanavalin A that were otherwise nonstimulatory to CD8$^+$ T cells appeared to stimulate CD8$^+$ T cells strongly when they were pretreated with neuraminidase. The enhancement of MLR by neuraminidase could be achieved by treating either the stimulators or responders with neuraminidase. Removal of negatively-charged sialic acid moieties from the cell surface, which reduced electrostatic repulsion between responders and stimulators to give better cell-cell contact might be responsible for the enhanced MLR. In addition, neuraminidase treatment also appeared to deliver activation signal to responding T cells since it could activate CD8$^+$ T cells in synergy with phorbol myristate acetate. The maximal responses were observed when both responders and stimulators were treated with neuraminidase.
Key words : CD8$^+$ T cells, mixed lymphocyte reaction, neuraminidase

Mixed lymphocyte reaction (MLR) is a typical *in vitro* demonstration of alloreactivity of the immune system. It has been well established that CD4$^+$ T cells are the major responding T cells in MLR, which recognize the difference in class II major histocompatibility complex (MHC) antigens expressed on stimulating cells, and that primary MLR by CD8$^+$ T cells is directed predominantly to class I alloantigens (Sprent and Schaefer, 1985; Guimezanes *et al.*, 1985; Mizuochi *et al.*, 1986). However, it is not yet clear what the requirements are for the allogeneic antigen presenting cells (APC) to stimulate the T cells to proliferate. Although recognition of alloMHC via T cell receptor (TCR) is undoubtedly the most critical event in the response, some other costimulatory signals delivered by other surface molecules on APC appear to play important roles. Stimulation of T cells requires the presence of viable APC (Sprent and Webb, 1987). Whereas many different APC can deliver activation signals to sensitize T cells, stimulation of unprimed T cells to proliferate appears to be restricted to specialized cells such as dendritic cells (Steinman *et al.*, 1986).

Like P815 mastocytoma cells and transformed fibroblasts, various tumor T cell lines exhibit some APC functions for CD8$^+$ T cells (Sprent and Schaefer, 1986). Other T tumor cells exhibit only minimal APC functions, probably due to their low-level expression of class I MHC (Sprent and Schaefer, 1988). Upregulating the density of class I molecules on the cells with IFN-γ causes a marked increase in their APC function. In contrast to tumor T cells, normal T cells exhibit almost no APC function for CD8$^+$ T cells unless the cultures are supplemented with IL-2 (Sprent and Schaefer, 1989).

In this study we found that treatment of responding T cells with neuraminidase markedly enhanced their response in allogeneic stimulation when normal T cells preactivated with concanavalin A were used as stimulators. Furthermore, it appeared that neuraminidase treatment delivered some activation signals in CD8$^+$ T cells that were synergistic with phorbol ester. Other possible interpretations are also discussed.

Materials and Methods

Cell lines and animals

Hybridomas J11d.2 (anti-B), GK1.5 (anti-CD4), 28-16-8S (anti-I-A^{bd}) were purchased from American Type Culture Collection (Rockville, USA). Hybridoma 3.168 (anti-CD8) was a kind gift from Dr. Sprent (The Scripps Research Institute, La Jolla, USA). C57BL/6 and CBA/

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J mice were obtained from Korea Research Institute of Biotechnology and Bioscience (Taejon, Korea).

Purification of $CD8^+$ T cells

Lymph nodes were removed from C57BL/6 mice, and were ground to be suspended in RPMI 1640 medium. The cells were washed and resuspended in a medium containing 10% fetal bovine serum (FBS; Gibco, Detroit, USA), glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (30 μg/ml) and 5×10^{-5} M 2-mercaptoethanol. The cells were incubated at 37℃ for 45 min in the presence of culture supernatants of J11d.2 and GK1.5 along with guinea pig complements (Cedarlane, Hornby, Canada). The resulting cells were washed throughly with Hank's balanced salt solution (HBSS), resuspended in cold HBSS containing 2.5% newborn bovine serum (NBS) and poured into petri dishes precoated with 3.168 antibody. After incubating in a cold room for 3 h, the petri plates were stirred and washed with cold HBSS containing 2.5% NBS to remove unbound cells and the bound cells were collected by vigorous pipetting with warm HBSS. The resulting cells were analyzed for their purity by flow cytometry before being used, and were usually higher than 95%.

Purification of B cells

Spleen cells obtained from F1 (C57BL/6 X CBA/J) mice were incubated with J1j.10 antibody in the presence of guinea pig complement at 37℃ for 1 h. The resulting cells were washed, resuspended in HBSS and poured into tissue culture plates. The plates were incubated for 1 h at 37℃. The plates were stirred and rocked vigorously and the cells that came off the plates were collected and used as B cells. The purity of the B cells was usually higher than 95% when analyzed by flow cytometry. If necessary, the cells were irradiated with γ-ray (1,500 rad).

Preparation of T cell blasts

Lymph nodes removed from F1 (C57BL/6 X CBA/J) mice were ground on a steel mesh into single cell suspension. The cells were washed and cultured in RPMI 1640 medium containing 10% FBS, glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (30 μg/ml), 5×10^{-5} M 2-mercaptoethanol and 10 μg/ml of concanavalin A (Con A) for 3 days. The cultured cells were further incubated with J11d.2, 28-16-8S in the presence of guinea pig complement for 1 h at 37℃. The resulting cells were washed and used as T cell blasts. The purity of the T cells was usually higher than 95% when analyzed by flow cytometry. If necessary, the cells were irradiated with γ-ray (1,500 rad).

Mixed lymphocyte reaction (MLR)

$CD8^+$ lymph node cells (1×10^5/well) suspended in RPMI 1640 medium containing 10% FBS, glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (30 μg/ml), 5×10^{-5} M 2-mercaptoethanol were mixed with stimulator cells, e.g., F1 (C57BL/6 X CBA/J) spleen cells (5×10^5/well) irradiated with γ-ray (1,500 rad) or T blast cells (2×10^5/well) irradiated with γ-ray (1,500 rad). The mixture of the cells was incubated at 37℃ for 3 days before being pulsed with ^{3}H-thymidine for 16 h. The cells were harvested and the ^{3}H-thymidine incorporations was measured. All experiments were carried out in triplicate and arithmetic mean values were presented. Standard deviations were usually less than 5% of the mean values unless otherwise specified.

Neuraminidase treatment of cells

Cells (5×10^6) were incubated with 1 unit of neuraminidase (Type V; Sigma Chem. Co., St. Louis, USA) in 0.5 ml of RPMI 1640 without FBS for 2 h at 37℃. Resulting cells were washed extensively, and if necessary, they were irradiated with γ-ray (1,500 rad).

Results and Discussion

There are several types of cells that are stimulatory in allogeneic T cell responses. Dendritic cells and macrophages are usually considered as potent allogeneic stimulators (Steinman *et al.* 1986). T cells are usually not good stimulators to $CD8^+$ T cells in MLR, nor are even the T cells activated by culturing with Con A (Sprent and Schaefer, 1989). However, Con A-activated T cells designated as T blasts delivered a strong signal to $CD8^+$ T cells to proliferate markedly in MLR when they were pretreated with neuraminidase, as shown in Table 1. The nonstimulating T blasts appeared to succeed in stimulating the $CD8^+$ T cells in synergy with recombinant interleukin-2 (rIL-2) added from the beginning of the culture (Sprent and Schaefer, 1989). Spleen cells prepared from C57BL/6 (B6) and F1 (C57BL/6 X CBA/J) were used as negative and positive controls, repectively in these experiments. Enhancement of MLR by treating stimulators with neuraminidase did not seem applicable to other potential stimulators. Resting T cells or a tumor cell e.g., P815 that carry alloantigens on their surfaces did not stimulate $CD8^+$ T cells even after neuraminidase treatment (data not shown).

Neuraminidase treatment of stimulators (T blasts) were not the only way to enhance stimulation of the responder $CD8^+$ T cells; treatment of the responding $CD8^+$ T cells with neuraminidase also enhanced the MLR. As shown in Table 2, T blasts, without treatment with neu-

Table 1. Neuraminidase treatment of stimulators markedly enhanced proliferation of $CD8^+$ T cells in MLR

	Stimulator (irradiated)	Proliferation ($cpm \times 10^{-3}$)
Exp. 1.	T blasts (F1)	0.5
	T blasts (F1)+rIL-2	29.2
	rIL-2	3.6
	B6 spleen cells	0.6
	F1 Spleen cells	36.3
Exp. 2.	T blasts (F1)	0.9
	T blasts (F1)-N'dasea	131.4
	T blasts (B6)-N'dase	0.2
	F1 spleen cells	85.2

$CD8^+$ T cells were cultured with stimulator cells as indicated for 72 hrs followed by pulsing with ^{3}H-thymidine before harvest. Stimulator cells were spleen cells from either C57BL/6 (B 6) or F1 (C57/Bl/6 x CBA/J), or F1 T cells stimulated with Con A as described in Materials and Methods. Neuraminidase treatment was carried out as described in Materials and Methods.
[a]Neuraminidase-treated stimulator

Table 2. Responses of neuraminidase-treated $CD8^+$ T cells in MLR

Stimulator (Irradiated)	Neuraminidase treatment of stimulators	Proliferation ($cpm \times 10^{-3}$)
Resting T cells (F1)	-	0.7
Resting T cells (F1)	+	6.0
T blasts (F1)	-	56.0
T blasts (F1)	+	65.0
T blasts (B6)	-	0.4
T blasts (B6)	+	0.5
B6 spleen cells	-	0.7
F1 Spleen cells	-	63.8

$CD8^+$ T cells were treated with neuraminidase as described in Materials and Methods and cultured with stimulators treated with neuraminidase or with nontreated stimulators.

Table 3. Neuraminidase carried over to neighboring cells to exert its enzymatic action

	Stimulator (irradiated)	Proliferation ($cpm \times 10^{-3}$)
Exp. 1.	Resting T (F1)+T blasts (F1)	3.9
	Resting T (F1)-N'dase[a]+T blasts (F1)	16.7
	Resting T (F1)-N'dase	0.9
	Resting T (F1)	0.6
Exp. 2.	T blasts (F1)	5.9
	B cells (B6)+T blasts (F1)	6.9
	B cells (B6)-N'dase+T blasts (F1)	16.5
	B cells (B6)-N'dase+Resting T (F1)	0.5
	Spleen cells (B6)	0.9

[a]Neuraminidase-treated stimulators

Table 4. The effect of neuraminidase inhibitor on MLR where responders and/or stimulators were treated with neuraminidase

Cell combination	Neuraminidase inhibitor	Proliferation ($cpm \times 10^{-3}$)
R+S	-	0.6
R+SN	-	9.3
R+SN	+	0.7
RN+S	-	16.4
RN+S	+	1.1
RN+SN	-	21.4
RN+SN	+	19.6

Neuraminidase-treated responder cells ($CD8^+$ T cells; RN) or nontreated responders cells (R) were mixed with stimulator cells (T blasts) treated with neuraminidase (SN) or not (S), and they were cultured for MLR in the presence or in the absence of neuraminidase inhibitor(N-acrtylneuraminic acid, 200 g/ml).

raminidase, could stimulate the $CD8^+$ T cells that were pretreated with neuraminidase as strong as the T blasts that were pretreated with neuraminidase did to nontreated $CD8^+$ T cells. Moreover, resting T cells which were very poor stimulators whether treated with neuraminidase or not seemed to stimulate the neuraminidase-treated $CD8^+$ T cells although the intensity of the response was very limited.

It seems very clear that neuraminidase enhances MLR response when the responders or stimulators were treated with the enzyme. These results might result from the possibility that neuraminidase used to treat the stimulators were carried over to the responders, or vice versa. If so, it would not be clear which part was necessary to be treated with neuraminidase for higher MLR response. In order to examine the possibility of carry-over of neuraminidase, $CD8^+$ T cells were subjected to respond with T blasts in the presence of nonstimulators, e.g., resting T cells or B cells that were pretreated with neuraminidase. T cell blasts could stimulate $CD8^+$ T cells when neuraminidase-treated resting T cells or B cells were added to the culture, while untreated resting T cells or B cells resulted in no effects (Table 3). These results strongly suggest that the enzyme was carried over from stimulator to responder or the other way around.

The next question would be which part, stimulator or responder, is susceptible to neuraminidase treatment in MLR. Thus, stimulators and/or responders were treated with neuraminidase before they were cultured for MLR. As shown in Table 4, neuraminidase treatment of either responders or stimulators enhanced the MLR, as expected, although it appeared that neuraminidase treatment of the responders ($CD8^+$) resulted in a higher response than treatment of the stimulators (T blasts). However, the maximal response could be observed when the both were treated with neuraminidase. Note that the effect of neuraminidase could be abolished in the presence of a neuraminidase inhibitor, N-acetylneuraminic acid when either of the two was treated with neu-

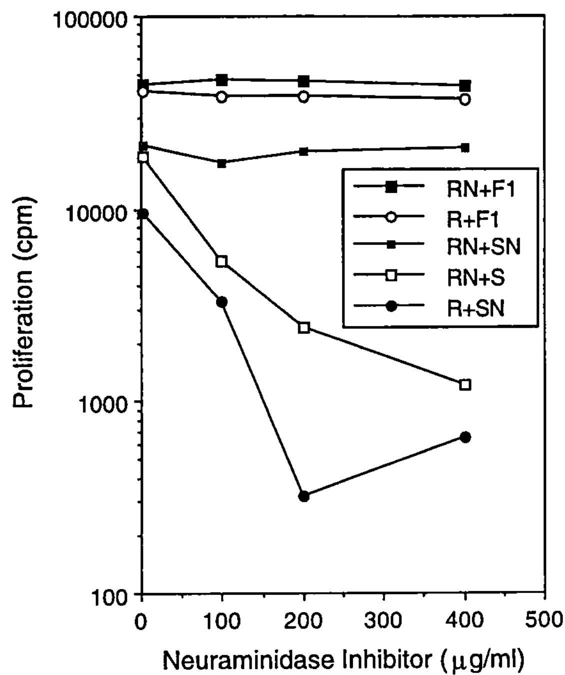

Fig. 1. Enhancement of MLR by neuraminidase could be inhibited by neuraminidase inhibitor. Neuraminidase-treated responder cells ($CD8^+$ T cells; RN) or nontreated responders cells (R) were mixed with stimulator cells (T blasts) treated with neuraminidase (SN) or not (S), or splenocytes (F1) prepared from F1 (C57BL/6 X CB A/J) mice, and were cultured for MLR in the presence of neuraminidase inhibitor in the concentration indicated. The proliferation of (R+S) was 650 cpm. Spleen cells from F1 mice were used as positive control for stimulating $CD8^+$ responders.

Table 5. Synergistic effects of neuraminidase treatment with PMA to stimulate $CD8^+$ T cells

Responder	Neuraminidase treatment	Costimulation	Proliferation (cpm × 10^{-3})
B6 $CD8^+$ T	-	PMA (10 ng/ml)	2.2
B6 $CD8^+$ T	-	Ionomycin (100 ng/ml)	0.4
B6 $CD8^+$ T	+	-	0.1
B6 $CD8^+$ T	+	PMA (10 ng/ml)	12.9
B6 $CD8^+$ T	+	Ionomycin (100 ng/ml)	0.4

Fresh or neuraminidase-treated $CD8^+$ T cells were cultured in the presence of PMA or ionomycin for 3 days, and were pulsed for 16 h with ^{3}H-thymidine before harvested.

raminidase. The neuraminidase inhibitor would block the enzyme activity that could be carried over from one side to the other, if it occurred. However, when the both were treated with neuraminidase, the effect of neuraminidase decreased little by the neuraminidase inhibitor. This was more clearly shown in Fig. 1, where increasing doses of the neuraminidase inhibitor were used. These results also indicate that once the cells are treated with neuraminidase before culture, no further action of the enzyme is required to obtain a maximal response.

These results strongly imply that neuraminidase treatment elicited a profound change on both part of the cells in MLR. The possible explanation for the neuraminidase action would be that elimination of negatively-charged sialic acids from the cell surface resulted in enhanced responder-stimulator contacts, which would otherwise be responsible for repulsion of each other, to achieve higher responses in MLR. Alternatively, removal of sialic acids from the responder might have directly delivered some activation signals to the cells for proliferation. Indeed, neuraminidase treatment could stimulate $CD8^+$ T cells in synergy with phorbol 12-myristate 13-acetate (PMA) (Table 5). Since PMA, a potent protein kinase C activator (PKC), usually constitutes a part of strong activation signal, this result suggests strongly that neuraminidase treatment delivers a certain activation signal into the $CD8^+$ T cells. This signal delivered by neuraminidase treatment seems different from that delivered by PKC since the signal did not work in synergy with ionomycin; as well established, PKC and ionomycin usually constitute strong activation signals, as observed also in this experiment (data not shown).

Requirements for T cell stimulation in alloresponses remain to be clarified. Recognition of MHC molecules on stimulator cells by responding T cells would be undoubtedly a critical event in the response. However, expression level of MHC molecules on the cell surface does not seem to be the only parameter that might affect (Hopkins *et al*., 1993). In fact, it is not clear whether alloreactive T cells recognize alloMHC directly or recognize foreign MHC in the context of self MHC. Minimal changes in self MHC molecules caused change to alloantigen (Grandea *et al*., 1993), and it has been also reported that T cells recognize MHC independently of the presence of peptides (Elliot *et al*., 1990; Chattopadhyay *et al*., 1994; Killion *et al*., 1995). However, involvement of peptides has been documented in some examples of T cell alloreactivity (Marrack and Kappler, 1988; Heath *et al*., 1989; Rötzschke *et al*., 1990; Alexander-Miller *et al*., 1993).

Recognition of alloMHC, regardless of the involvement of peptides, is not the sole requirement for T cells to be stimulated. Costimulatory signals delivered by accessory molecules on the cell surface including several adhesion molecules seem to play an important role in the T cell responses (Zehnder *et al*., 1995). It has been reported that soluble factors might be also involved in the alloreactivity (Schiott *et al*., 1996), and even psychological stresses might change the magnitude of the T cell responses in MLR (Fleshner *et al*., 1995). Neuraminidase treatment of either responders or stimulators that resulted in enhanced MLR might be caused by elimination of sialic acids from the cell surfaces. Elimination of sialic acid

moieties from glycoproteins on the cell surfaces is believed to reduce electrostatic repulsion between the responders and stimulators to give better cell-cell contact, and thus allow the cell surface membrane proteins more access to other ligands such as TCR, LFAs, CD8, CD2, etc. In fact, it was observed that cells tended to cluster after neuraminidase treatment (data not shown). Another possible explanation is that removal of sialic acids form the surface of the responders triggered an activation signal within the responders that worked in synergy with stimulations delivered by other agents such as PMA. The activation signaling elicited by neuraminidase treatment might also occur in stimulators and cause production of cytokines (Chen *et al.*, 1997) that in turn stimulated the responders.

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