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γ-Aninobutyric acid (GABA) suppresses antigen-specific immune responses in ovalbumin γ(OVA)-immunized BALB/c mice

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Abstract: γ-Aminobutruic acid (GABA) is known to be a ubiquitous inhibitory neurotransmitter in the central nervous system. Furthermore, recent studies have shown that GABA modulates cardiovascular and respiratory functions. In this study, we examined the effect of GABA on antigen (Ag)-specific immune responses and elucidated the mechanisms underlying those effects in ovalbumin (OVA)-immunized BALB/c mice. OVA-specific proliferative responses were decreased in mice that had been administered 20 mg/kg GABA compared to those in control mice without reduction in responses to anti-CD3 monoclonal (m)Ab. The levels of OVA-specific IgG, IgG1 and IgG2a were also decreased in mice that had been administered GABA. However, GABA administration did not influence the TNP-specific IgM and IgG levels. Ag-presenting activity of $CD11c^+$ dendritic cells from GABA-treated mice was less than that of cells from control mice. These results suggest that the suppressive effect of GABA on Ag-specific immune responses contributes to the reduction of antigen-presenting function on $CD11c^+$ dendritic cells.

Key words: γ-Aminobutruic acid, antigen-specific immune response, $CD11c^+$ dendritic cells

INTRODUCTION

γ-Aminobutyric acid (GABA) serves as a major inhibitory neurotransmitter within the central nervous system[1,2], and it is also found in peripheral tissues[3]. It has been shown that GABA plays an important role in the modulation of cardiovascular functions[4] by acting not only within the central nervous system but also in peripheral tissues[5, 6]. In fact, administration of GABA has been shown to reduce blood pressure in both experimental animals[7] and human subjects[8, 9].

There are at least two types of neuronal GABA receptors, $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ligand-gated ion channels that respond to GABA by opening their integral Cl^- channel[1]. A multitude of different neuronal $GABA_A$ receptors exist due to the combinatorial assembly of its various subunits. Nineteen neuronal $GABA_A$ receptor subunits have so far been identified (α1-6, β1-3, γ1-3, δ, ε, θ or ρ1-3). Most $GABA_A$ receptors are comprised of two identical α subunits, two identical β subunits, and a single γ subunit. In some cases, the γ subunit is replaced by α δ, ε, θ, π subunit. The different receptor subtypes vary in their affinities for GABA and pharmacological properties[9, 10]. In contrast, $GABA_B$ receptors are coupled to Ca^{2+} or K^+ channels via GTP-binding proteins[11].

Many of the cells of the immune system express receptors for neuroactive molecules, which modulate immune system function, creating a link between the nervous and immune systems. It has shown that T cells express $GABA_A$ receptors and that GABA inhibits T cell responses *in vitro*[12]. Furthermore, GABA administration has been shown to inhibit delayed-type hypersensitivity (DTH) responses to a foreign antigen (Ag)[12]. These studies suggest that functional $GABA_A$ receptors are expressed by T cells and might mediate immunosuppression. However, the mechanisms by which GABA exert their immunosuppressive effects have not been fully elucidated. In this study, we examined the effects of GABA on Ag-specific immune responses in Ag-immunized BALB/c mice.

MATERIALS AND METHODS

Mice and GABA treatment: Female BALB/c mice (Japan SLC, Shizuoka, Japan) and DO11.10 mice on BALB/c background (The Jackson Lab., Bar Harbor, ME) were maintained under specific pathogen-free conditions with a 12-hr light:dark cycle at 25±2°C and 55±10% relative humidity. The mice were administered 200 μl GABA solutions containing 0, 2 or 20 mg GABA/kg body weight by gavage from 3 days before immunization and until the time of sacrifice.

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Immunization: For ovalbumin (OVA) immunization, OVA (2 mg/ml) emulsified in an equal volume of complete Freunds adjuvant (Sigma Chemical Co., MO) was injected in the footpads of mice with a volume of 50 μl on day 3 after the start of GABA administration. For TNP-Ficoll immunization, mice were intraperitoneally immunized with 25 μg of TNP-Ficoll (Sigma) absorbed in 2 mg of Aluminium Hydroxide Gel Adjuvant (HCI Biosector, Denmark) on days 10 and 17 after the start of GABA administration.

Proliferation assay: To prepare single cell suspensions, lymph nodes were squeezed with two slide glasses in RPMI-1640 medium (Sigma) and filtered through mesh. Then cells were washed two times with RPMI-1640 medium and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 100 μg/ml streptomycin and 100 U/ml penicillin. Five x 10^5 splenocytes were stimulated with 200 μg/ml OVA or with plate-bound anti-CD3 mAb (coated overnight at 2 □g/ml) in a 96-well flat-bottom plate at 37°C under 5% CO_2 for 72 hr. For the last 20 hr of culture, 0.5 μCi of [^{3}H] thymidine deoxyribose (TdR) was added to the wells, and the amount of [^{3}H]TdR incorporated was measured by a Matrix beta counter (Packard Instrument B.V. Chemical Operations, Netherlands).

Determination of Ag-specific Ab levels: On day 13 after the start of GABA administration for mice immunized with OVA and on day 17 for mice immunized with TNP-Ficoll, blood was collected from mice and centrifuged, and sera were stored at –20°C until used. Serum levels of OVA-specific Abs were measured by ELISA. Ninety-six-well ELISA plates were coated with 10 μg/ml OVA in 50 mM carbonate buffer (pH 9.6) and left overnight at 4°C. The plates were washed with 0.05% Tween 20-PBS (PBS-T) and blocked with 1% bovine serum albumin (BSA)-PBS-T. After washing, various dilutions of the samples were added and incubated for 2 hr at room temperature. Then the plates were washed and incubated with alkaline phosphatase (AP)-conjugated anti-mouse IgG Ab, AP-conjugated anti-mouse IgG1 Ab or AP-conjugated anti-mouse IgG2a Ab (Southern Biotechnology Associates, Inc., AL) for 2 hr at room temperature. After the plates had been washed, 1 mg/ml *p*-nitrophenyl phosphate (Sigma) in 10% diethanolamine buffer was added to the wells. The reaction was terminated by adding 3N NaOH, and the OD was determined at 405 nm.

Serum levels of TNP-Ficoll-specific Abs were also measured by ELISA. The plates were each coated with 10 μg/ml TNP-BSA in 50 mM carbonate buffer (pH 9.6) and left overnight at 4°C. The following steps were the same as those used for OVA-specific Ab measurement except for the use of AP-conjugated anti-mouse IgG Ab (Southern Biotechnology Associates, Inc.) and AP-conjugated anti-mouse IgM Ab (Stressgen Biotechnologies, CA).

Antigen-presenting cell function: CD11c$^+$ cells were purified from 3 to 4 mouse spleens that had treated with a vehicle or with 2 mg/kg or 20 mg/kg GABA using CD11c microbeads and a MACS LD column (Miltenyi Biotec, Inc., CA) according to the manufacturer's instructions. T cells were purified from DO11.10 transgenic mice, which express OVA-specific T cell receptors, using a nylon wool column. T cells (2.5 x 10^5 cells) from the DO11.10 mouse spleen were cultured in a 96-well plate with CD11$^+$ cells (3 x 10^3 cells) in the presence of 200 μg/ml OVA for 48 hr at 37□/5% CO_2. Cultures were pulsed with 0.5 μCi of [^{3}H]TdR for the last 12 hr of culture. Incorporated [^{3}H]TdR was determined by a Matrix beta counter (Packard).

Statistical analysis: Data were analyzed using one-way analysis of variance followed by Scheffe's post hoc test for multiple comparisons. Data are expressed as means±SD. Differences were considered significant at $P<0.05$.

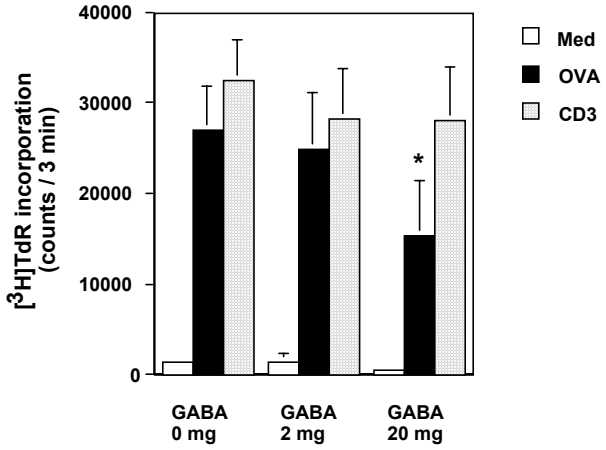

Fig. 1: Effect of GABA on proliferative responses. BALB/c mice were administered genistein for 13 days and immunized with OVA. Splenocytes from GABA-treated mice were cultured with OVA or anti-CD3 mAb for 72 hr. Proliferative responses were determined by measurement of the amount of incorporated [^{3}H]TdR. Each value is the mean±SD of seven mice per group. * $P<0.05$.

RESULTS

After 13 days of GABA administration, we examined the *in vitro* proliferative responses of splenocytes by co-culture with OVA or anti-CD3 mAb. The proliferative responses to OVA were significantly suppressed in 20 mg/kg GABA-treated mice compared to those in control mice, while the proliferative

responses to anti-CD3 mAb were not suppressed (Fig. 1). These results suggest that GABA inhibits the induction of Ag-specific T cells but does not suppress T cell functions directly.

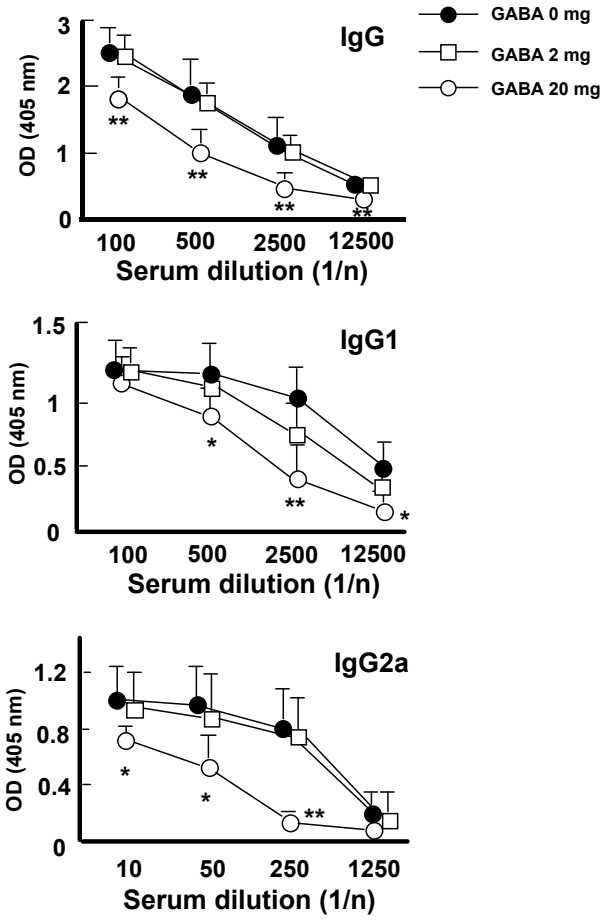

Fig. 2: Effect of GABA on OVA-specific Ab production. BALB/c mice were administered GABA for 13 days and immunized with OVA. Collected sera were serially diluted, and OVA-specific IgG, IgG1 and IgG2a levels were measured by ELISA. Each value is the mean±SD of seven mice per group. * $P<0.05$, ** $P<0.01$ as compared to controls.

We next investigated OVA-specific IgG, IgG1 and IgG2a production. The levels of OVA-specific IgG, IgG1 and IgG2a were significantly decreased in 20 mg/kg genistein-treated mice compared to those in control mice (Fig. 2). To examine the possibility that GABA directly stimulates B cells and results in decreased Ab production, we further analyzed the effects of genistein on B cell response by using a thymus-independent Ag, TNP-Ficoll. The levels of TNP-specific IgM and IgG were increased on day 7 after immunization, and the increase levels were sustained until day 14. However, GABA administration did not influence the level of either TNP-specific IgM or IgG in experimental period (Fig. 3). These results suggest that the suppressive effect of GABA on Ab responses is partly derived from the suppressed induction of Ag-specific T cells but not from a direct inhibitory effect on B cells.

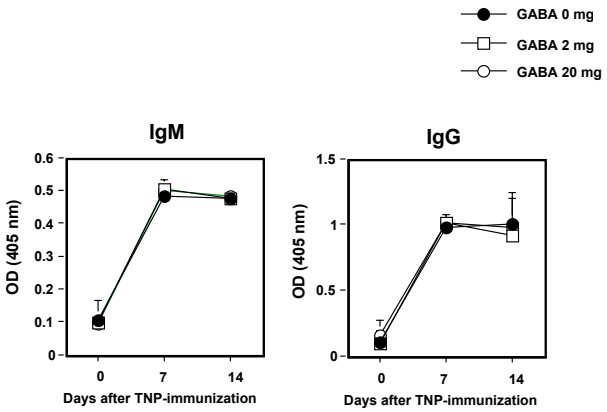

Fig. 3: Effect of GABA on TNP-specific Ab production. BALB/c mice were administered genistein for 17 days and immunized with TNP-Ficoll twice. Sera were collected on days 3, 10 and 17 after the start of genistein administration, and TNP-specific IgM and IgG levels were measured by ELISA. Each value is the mean±SD of six mice per group.

Finally, to address the target cell populations that suppress the induction of Ag-specific T cells, we determined the function of dendritic cells, which are major Ag-presenting cells[13]. When purified DO11.10 mouse T cells were cultured with OVA, the level of their proliferation response was 9,748 ± 19 cpm. Addition of $CD11c^+$ dendritic cells from control mice to the culture increased their response to 15,707 ±2,919 cpm. Notably, $CD11c^+$ dendritic cells from mice that had been treated with 20 mg/kg GABA did not increase the proliferation response of DO11.10 mouse T cells (Fig. 4).

DISCUSSION

It has been postulated that there is a mechanism for cross talk between the nervous and immune systems. Indeed, many of immune cells express receptors for neuroactive molecules[14]. In this study, we focused on GABA, which is known to be a ubiquitous inhibitory neurotransmitter in the central nervous system, and examined the effect of GABA on Ag-specific immune responses. An *in vitro* study has shown that GABA inhibits Ag-primed and anti-CD3 mAb-induced T cell proliferation responses[12]. When T cells recognize Ag presented on MHC molecules, a series of signal cascades is induced, resulting in activation of T cells. In

this process, an increase in intracellular Ca^{2+} concentration has been shown to be important because Ca^{2+}-dependent calcineurin activation is crucial for regulation of transcription factors, NFAT and AP-4[15]. The inhibitory action of GABA on T cell activation is related to the change in intracellular Ca^{2+} concentration. The activation of $GABA_A$ receptors induces a Cl^- current, the direction of which depends on the ion's equilibrium potential and the cell's resting membrane potential. Quiescent T cells have membrane potentials between –50 and –70 mV, and their Cl^- equilibrium potential is approximately –35 mV[16, 17]. The activation of $GABA_A$ receptors might cause depolarization. Depolarization in T cells interferes with the Ca^{2+} influx and causes inhibition of T cell activation[18]. A direct inhibitory effect of GABA on T cell proliferation response was not observed when spleen cells were stimulated with anti-CD3 mAb (Fig. 1). This indicates that functions of $CD11c^+$ dendritic cells are more susceptible to GABA than are function of T cells (Figs. 1 and 4).

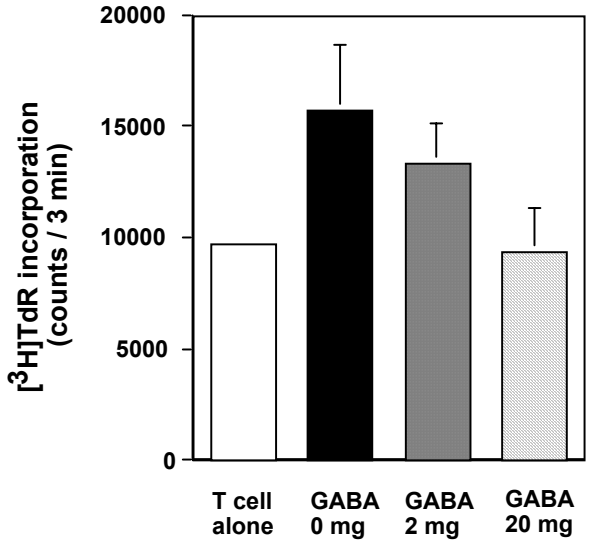

Fig. 4: Effect of GABA on function of $CD11c^+$ dendritic cells. $CD11c^+$ dendritic cells were purified from control mice and from 2 mg/kg GABA- and 20 mg/kg GABA-treated mice as described in Materials and Methods. $CD11c^+$ cells were co-cultured with DO11.10 mouse T cells in the presence of OVA. Proliferation responses of DO11.10 mouse T cells were determined by measuring [^{3}H]TdR activity. Level of proliferation response to OVA in $CD11c^+$ cells alone was less than 200 counts/ 3 min. Results are expressed as means±SD of quadruplicate cultures.

A direct inhibitory effect of GABA was observed in $CD11c^+$ dendritic cells (Fig. 4) but not in T and B cells (Figs. 1 and 3). The $GABA_A$ receptor is composed of α, β and γ subunits, and the composition of each subunit defines affinity for GABA. It has been shown that murine T cells express α1, α2, β1, β2, γ3, and δ but not α5, β3 and γ1 $GABA_A$ receptor subunits [19]. In human PBMC, clear positive signals of α1, α3, α4, γ and ε GABA receptors were detected by RT-PCR analysis. Real-time PCR amplication of the $GABA_A$ receptor revealed that the α1 subunit is expressed in $CD8^+$ and irradiated B cells and is strongly expressed in $CD4^+$ cells. Furthermore, the $GABA_A$ receptor α3 subunit is expressed in $CD4^+$ and irradiated B cells but not in $CD8^+$ cells[20]. Therefore, the distribution of $GABA_A$ receptor subunits is different among immune cells. The $GABA_A$ receptor subunit expressed in dendritic cells and its physiological relevance are not clear at present. Further study is needed to elucidate the immunosuppressive effect of GABA.

GABA inhibits disease progression in prediabetic NOD mice and NOD/scid mice adoptively transferred with diabetogenic T1D cells. It was shown that inhibition of T cell function by GABA is responsible for inhibition of disease progression[21]. Studies on the effects of GABA on immune cells have mainly focused on T cells and their $GABA_A$ subunits[12, 18, 21]. However, a recent study has shown that the $GABA_B$ receptor is functionally expressed in neutrophils and acts as a chemoattracant receptor via a seine/threonine kinase Akt-dependent pathway [22].

In summary, we showed in this study that GABA suppresses Ag-specific immune responses but not T and B cell functions directly. This suppressive effect of GABA partly contributes to the reduction of Ag-presenting function of $CD11c^+$ dendritic cells.

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