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Suppressive Effect of Antibiotic Siomycin on Antibody Production

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The antibiotic thiazole compound siomycin, which we have found from the culture broth of *Actinomycetes* (strain No.806097) in search of antibody production inhibitor, showed the *in vitro* immunosuppressive property against B-cells stimulated with T-cell independent antigen DNP-LPS (dinitrophenyl-lipopolysaccharide) while it also showed inhibitory effect against T-cell proliferation. Its inhibitory mechanism was considered to be different from that of FK506, the representative of T-cell immunosuppressant. Moreover, siomycin showed inhibitory effect in both T-cell dependent and independent murine antibody production models and decreased the severity in murine collagen arthritis model. Therefore, siomycin is a unique immunosuppressant which has potential for the treatment of some antibody-mediated diseases.

The very high success rate of allotransplantation by using T-cell immunosuppressants such as cyclosporine A and FK506 has led to the establishment of this procedure as a standard therapy for the treatment of acute allograft rejection[1,2]. However, some problems remain to be solved because such a drug does not exert a satisfactory effect toward other immune syndrome[3].

Some immune diseases including rheumatoid arthritis, xenograft rejection or chronic allograft rejection involve the presence of antibody[4~6]. In xenotranplantation which is the transplantation of organs derived from different species, the loss of xenograft is induced by several causes. One of them is that excessive antibody production, resulting in acute vascular rejection to which current T-cell immunosuppressants show ineffectiveness, is triggered by the transplanted xenograft[7]. Chronic allograft rejection is a leading to organ loss owing to the obstruction of arterial vessels of the transplanted allograft. Among the numerous contributing factors, antibody plays a critical role in this disease[8,9].

Therefore, the sufficient control of antibody production may play a pivotal role in treating the above-mentioned immune diseases. A B-cell immunosuppressant, which is different from typical T-cell immunosuppressants in mode of action, would be expected to have substantial therapeutic potential in andibody-mediated diseases.

We have searched for a potent inhibitor of T-cell independent antibody production by murine B-cells, from the culture broth of *Actinomycetes*. Siomycin produced by *Actinomycetes* strain No. 806097, known as thiazole antibiotic[10], was found to inhibit antibody production by murine B-cells stimulated by T-cell independent antigen, DNP-LPS (dinitrophenyl-lipopolysaccharide).

In this paper, we describe the *in vitro* and *in vivo* immunological properties of siomycin, and show the possibility of this compound to be a potent therapeutic drug against andibody-mediated diseases.

Materials and Methods

Media Used for Seed Culture and Production

The seed medium consisted of glucose 0.5%, sucrose 0.5%, yeast extract 0.2%, peptone 0.5%, oatmeal 0.5%, tween80 0.1%, peanuts powder 0.5%, fumic acid 0.01% and $CaCO_3$ 0.2%. The production medium (pH=6.5) consisted of glucose 0.5%, MS3600 2.5%, soybean flour 1%, dried yeast 0.5%, L-asparagine 0.2%, K_2HPO_4 0.05%, NaCl 0.05% and $CaCO_3$ 0.2%.

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HPLC Analysis

Siomycin was detected by the reverse phase HPLC method using YMC-ODS-AM column (AM302, 150×4.6 mm i.d., YMC Co., Ltd.). The mobile phase was acetonitrile - water (45 : 55), (v/v). The flow rate was 1.0 ml/minute. The detection wavelength was set at 210 nm.

Drugs

FK506 was prepared in our Research Laboratories. Thiostrepton and kirromycin was purchased from Sigma.

In Vitro Suppressive Activity against Antibody Production by B-cells Stimulated with DNP-LPS

Spleen cells from female BALB/c mice (Charles River Japan Inc.), which were stimulated with DNP-LPS (Biosearch Technologies, Inc.), were cultured in flat bottomed microtiter plates at 37°C in a humidified CO_2 incubator for 6 days (spleen cells; 5.0×10^6 cells/ml, DNP-LPS; 5 μg/ml). The culture medium was RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Moregate, Bulimba, Australia), 50 mM 2-mercaptoethanol (Nakarai Chemical, Kyoto, Japan), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Rockville, MD). To assess the inhibitory activity of the compounds, serial dilutions of the drugs were performed in the culture medium before adding stimulated spleen cells. The supernatants from the culture were assayed for the presence of IgG by ELISA. To be brief about the ELISA, immobilized DNP-BSA (Lsl Co., Ltd.) was incubated with the supernatants prior to addition of HRP (horse radish peroxidase)-conjugated rabbit anti-mouse IgG (Zymed) in 96-well plate. Then, *o*-phenylene diamine and H_2O_2 were added to each well, and absorbance at 490 nm was measured to calculate the amount of DNP-specific IgG.

In Vitro Immunosuppressive Activity

The two assays, in both of which the decline of metabolic activity was an index of the immunosuppressive property, were performed. One of them was mixed lymphocyte reaction (MLR) assay. In this assay spleen cells from female C57/B6 mice (Charles River Japan Inc.) were irradiated and mixed with the same number of spleen cells from female BALB/c mice (Charles River Japan Inc.) in 96-well U-bottomed plate. Both cells were suspended in the same medium as that used in antibody production assay. (1.5×10^6 cells/ml). These cells were cultured at 37°C in a humidified CO_2 incubator for 3 days. The metabolic activity of the cells was measured by an MTT assay[11]. Technically another method is also the same as MLR assay with exception that stimulus is murine anti-CD3 mAb (1 μg/ml) in place of C57/B6 spleen cells. As well as the case of antibody production assay, serial dilutions were performed in the culture medium before incubating with stimulus to address the immunosuppressive property of the drugs.

In Vivo Antibody Production Model Immunized with DNP-LPS

Female BALB/c mice (Charles River Japan Inc.) were immunized intravenously with DNP-LPS (10 μg/mice) on day 0. Mice were bled on day 4 after immunization (7 mice/group), and anti-DNP antibody (IgG) levels in each serum were determined by ELISA. The method is same as the case of *in vitro* antibody production assay. 0.3, 3 or 30 mg/kg/day of siomycin were intraperitoneally administrated (day 0～3).

In Vivo Antibody Production Model Stimulated with Alloantigen

Female C3H mice, 6 weeks old, (Charles River Japan Inc.) were intraperitoneally administrated with spleen cells (1.0×10^7 cells/mice) derived from female BALB/c mice (Charles River Japan Inc.) on day 0. Mice were bled on day 6 after immunization (7 mice/group), and anti-allo IgG1 levels in each serum were determined by flowcytometry. Briefly, Each serum was incubated with spleen cells (1×10^5 cells/well) from BALB/c mice for 30 minutes prior to addition of FITC-conjugated anti-mouse IgG1. The number of FITC-labeled spleen cells, whose fluorescent intensity is more than five, per total cells were calculated in order to determine the quantity of IgG1. 0.3, 3 or 30 mg/kg/day of siomycin was intraperitoneally administrated (day 0～5).

Murine Collagen Arthritis Models

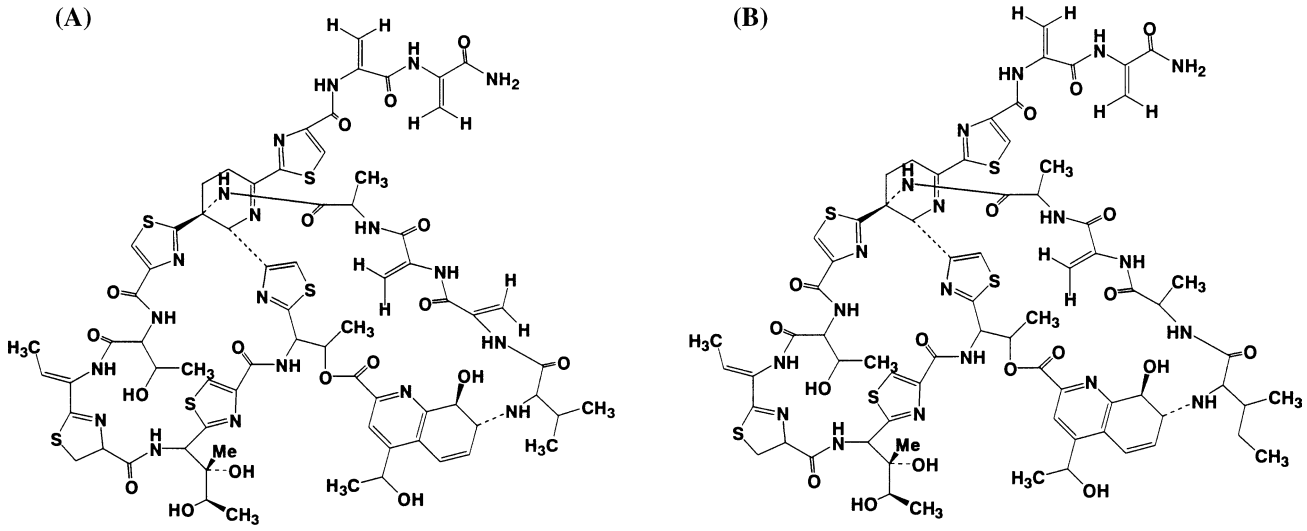
Male DBA/1 mice, 7 weeks old, (Charles River Japan Inc.) were sensitized with bovine type II collagen and adjuvant complete freund (Wako Jyunnyaku, Japan), and 3 weeks later secondary immunization was performed (10 mice/group). Simultaneously administration of siomycin (0.3, 3 or 30 mg/kg/day i.p.) was started (day 0) and proceeded until day 10. Mice were bled on day 11. Total antibody (IgG, IgM and IgA) levels in each serum were determined by ELISA. The degree of arthritis was defined as score of edema of each 4 feet (The conditions were classified into 4 grades; score 0: normal condition, score 1: partial inflammation, score 2: overall inflammation and score 3: inability of bending of articulation). We have observed its severity on day 11. Total scores regarding each 4 feet were expressed as a dot in Fig. 4B.

Table 1. IC_{50} values of siomycin, thiostrepton and FK506 against murine splenic proliferation and T-cell independent antibody production.

	murine splenic proliferation		T-cell independent antibody production
stimulus	allogeneic splenocyte	anti-CD3 mAb	DNP-LPS
siomycin	0.21	0.22	0.03
thiostrepton	0.48	1.17	0.08
FK506	0.0002	0.0002	16.1

(μg / ml)

Fig. 1. Structure of siomycin (A) and thiostrepton (B).



Results

Inhibitory Effect of Siomycin and Thiostrepton on Activation of B and T Cells *in Vitro*

We have searched for B-cell immunosuppressants from the culture broth of *Actionomycetes*. The broth extract of the *Actinomycetes* strain No.806097 showed suppressive activity against antibody production by B cells stimulated with DNP-LPS. The antibiotic thiazole siomycin (Fig. 1A) was found to suppress antibody production while this substance also possessed inhibitory action against T-cell proliferation stimulated with alloantigen or soluble anti-CD3 mAb (Table 1). Moreover, we have investigated whether the related thiazole substance thiostrepton (Fig. 1B) affect on B-cells and T cells, and we have discovered it exerted the similar effect as siomycin (Table 1).

On the other hand, T-cell immunosuppressant FK506, which strongly suppresses the activation of T-cells followed by inhibition of B-cell activation and differentiation, showed only a weak suppressive activity against T-cell independent antibody production (Table 1). These results indicate that siomycin and thiostrepton directly work on B-cells whereas FK506 indirectly work on B-cells *via* T-cell

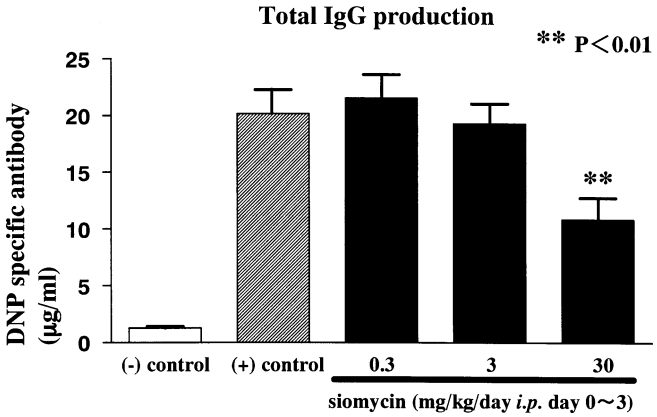
inactivation. From this point of view, these antibiotics may compensate for the conventional therapy in antibody-mediated diseases.

Inhibitory Effect of Siomycin on Antibody Production *in Vivo*

We have shown the immunosuppressive activities against B-cells of siomycin *in vitro*. Then, we evaluated its inhibitory effect on antibody production *in vivo* on the assumption that this compound should show a therapeutic value against various antibody-mediated diseases.

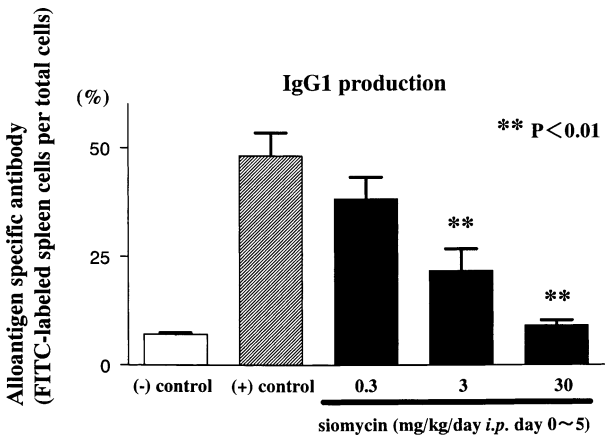
The effects of siomycin on *in vivo* T-cell independent antibody production were assessed after immunization of female BALB/c mice with DNP-LPS. Administration of 30 mg/kg/day (i.p., day 0～3) of siomycin showed suppressive (about 50% inhibition) effect on the production of anti-DNP antibody in this model (Fig. 2). Subsequently, siomycin was evaluated through *in vivo* T-cell dependent antibody production model in which female C3H mice were

Fig. 2. Suppressive effect of siomycin on antibody production in DNP-specific antibody production model.



The results are shown as means±S.E.M. (n=7). P<0.01 versus (+) control: Statistical analysis was performed by Dunnett's multiple comparison test.

Fig. 3. Suppressive effect of siomycin on antibody production in alloantigen specific antibody production model.



The results are shown as means±S.E.M. (n=7). P<0.01 versus (+) control: Statistical analysis was performed by Dunnett's multiple comparison test.

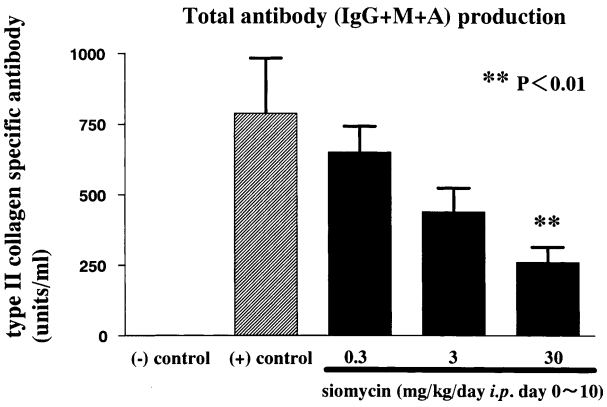
immunized with splenocytes from female BALB/c mice as alloantigen. Consequently, it inhibited anti-alloantibody production dose-dependently from 30 mg/kg/day down to 0.3 mg/kg/day (i.p., day 0～5) (Fig. 3). These results indicate that siomycin inhibits both T-cell dependent and independent antibody production *in vivo*, and that it may have potential against some antibody-mediated diseases presently untreatable with available drugs.

Effect of Siomycin in Murine Collagen Arthritis Model

Murine collagen arthritis model in which score of edema is an indicator of the pathology is known as one of rheumatoid arthritis models. We subsequently investigated whether siomycin improves this score.

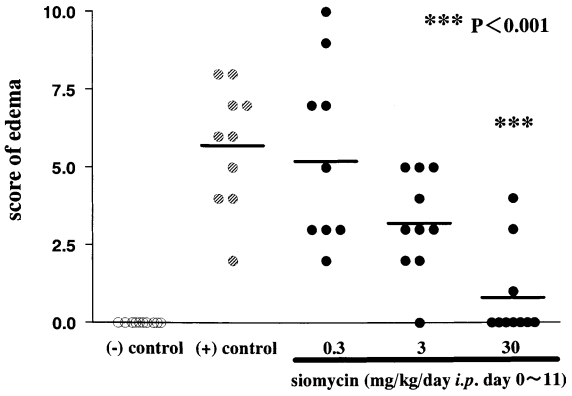
Siomycin also inhibited total antibody (IgG, IgM and IgA) production dose-dependently from 30 mg/kg/day down to 0.3 mg/kg/day (i.p., day 0～10) in this model (Fig. 4A), and led to the observation on day 11 that it significantly improved the score of edema at a dose of

Fig. 4A. Suppressive effect of siomycin on antibody production in murine collagen arthritis model.



The results are shown as means±S.E.M. (n=10). P<0.01 versus (+) control: Statistical analysis was performed by Dunnett's multiple comparison test.

Fig. 4B. Improvement of pathogenesis (score of edema) in administration of siomycin in murine collagen arthritis model.



A dot means the total score of edema in one mouse. Horizontal line shows the average of score of edema in one group. P<0.001 versus (+) control. Statistical analysis was performed by Dunn's multiple comparison test.

30 mg/kg/day (i.p., day 0～10) (Fig. 4B). The definition of edema is described at methods section.

Discussions

During our screening for B-cell immunosuppressants, we found antibacterial thiazole compound siomycin from the culture broth of the *Actinomycetes* strain No.806097. To the best of our knowledge, this is the first time siomycin and its related thiazole antibiotic thiostrepton have been reported to inhibit antibody production. Their inhibitory mechanism against *in vitro* antibody production was considered to be different from that of T-cell immunosuppressant FK506 because these two thiazole antibiotics suppressed T-cell independent antibody production which FK506 doesn't inhibit to the same degree.

Then, we studied the inhibitory effect of siomycin in two murine antibody production models to investigate whether *in vitro* profile of siomycin reflect *in vivo* properties. One model is T-cell independent antibody production using DNP-LPS as antigen, and another model is T-cell dependent using allo-splenocytes as antigen. In the DNP-LPS model it showed moderate suppressive (about 50% inhibition) activity at a dose of 30 mg/kg/day (i.p., day 0～3). In the allo-splenocytes model it showed almost complete suppressive property at a dose of 30 mg/kg/day (i.p., day 0～5). Consequently, siomycin showed suppressive effect in both of the antibody production models.

Moreover, in murine collagen arthritis model which is a model for autoimmune disease, siomycin significantly improved the score of edema after administration of 30 mg/kg/day. These results suggest that siomycin might exert a therapeutic effectiveness against various autoimmune diseases concerning with autoantibodies through its immunomodulating activity. Moreover, it might bear potential for the treatment of xenograft rejection and chronic allograft rejection.

On the other hand, siomycin and thiostrepton also blocked the alloantigen-induced mixed lymphocyte reaction or anti-CD3 mAb induced T-cell activation suggesting that they might be available for the treatment of acute rejection in allo-transplantation as well.

Cyclic thiazole peptide siomycin and thiostrepton have been known as antibacterial compounds which bind to 50S ribosome resulting in inhibition of synthesis of protein in bacteria[12]. Interestingly, antibiotic kirromycin, inhibitor of protein synthesis by inactivating EF-Tu[13], showed no suppressive activity on B-cells (data not shown).

FRORENZ SASSE *et al.* reported that cyclic thiazole peptide argyrin B consisting of 8 amino acid residues was a potent inhibitor of T-cell independent antibody production and had slight antibiotic activity[14]. Combining this report with our data about these antibiotics, there might be some correlation among structural property, antibacterial mechanism and inhibitory effect against T-cell independent antibody production.

We have concluded that antibiotic thiazole compound siomycin is unique immunosuppressant which might have potential for the treatment of some autoimmune diseases, mainly, xenograft rejection, and progression of chronic allograft rejection.

Moreover, there might be a necessity of further investigation of its mechanism as to intracellular responses to identify the related target for the discovery of novel drugs toward antibody-mediated diseases.

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