Studies on Thermophile Products. VII. Effect of 1,3-Di-14-methylpentadecanoyl Glycerol and Its Related Isofatty Acids on T Cell Proliferation in Vitro

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Received October 29, 1993; accepted February 2, 1994

It has been found that Bacillus steaothermophilus UK563-derived immuno-suppressant fraction (Fr. 5-B) consists of 1,3-diacylglycerols with saturated iso- and anteiso-type fatty acids (C_{14:0}-C_{18:0}) as major components. The compound, 1,3-di-14-methylpentadecanoyl glycerol (1,3-diolo C_{16:0} G), was synthesized and its effect on T cell proliferation was investigated together with its related iso fatty acids. While 1,3-diolo C_{16:0} G, iso C_{16:0}, iso C_{17:0}, iso C_{18:0}, methyl ester (OME) and anteiso C_{17:0} OMe suppressed the mixed lymphocyte reaction (MLR) of C57BL/6 against BALB/c mice, iso C_{15:0}, 1,3-acylglycerols with normal C_{16:0}, C_{16:1}, and C_{18:0} did not, suggesting that the presence of iso fatty acids with a certain chain length may be essential for the suppression of MLR. 1,3-Diolo C_{16:0} G and iso C_{16:0} strongly inhibited the autologous MLR of mesenteric lymph node cells against self-antigen presenting cells in MRL/MpJ-lpr/lpr (MRL/lpr) mice, but had no effect on concanavalin A-induced T cell proliferation.

Keywords 1,3-di-14-methylpentadecanoyl glycerol; iso fatty acid; MRL/MpJ-lpr/lpr mouse; mixed lymphocyte reaction; autologous mixed lymphocyte reaction

We previously reported the isolation of immuno-suppressant, Fr. 5-B from the debris of Bacillus steaothermophilus UK563 autolysate. Fraction 5-B inhibited several immune responses, including the mixed lymphocyte reaction (MLR), expression of class II major histocompatibility molecule (IIa) antigen on macrophages and cytolytic lymphocyte generation. These results strongly suggest that Fr. 5-B consists of a number of components, which have different site of action. To help clarify this, we determined the structure of Fr. 5-B and found it to consist of 1,3-diacylglycerols. The fatty acid moieties of the glycerols consisted of iso and anteiso types C_{15:0}, C_{16:0} and C_{17:0} as major components and C_{14:0} and C_{18:0} as minor components. In the present study, 1,3-di-14-methylpentadecanoyl glycerol (1,3-diolo C_{16:0} G), which is one of major components of Fr. 5-B, was synthesized as a putative representative of 1,3-diacylglycerols with iso fatty acid components and its effect on T cell proliferation was studied, together with that of its related iso fatty acids. The structural elucidation of Fr. 5-B will be published elsewhere.

MATERIALS AND METHODS

Mice Male MRL/MpJ-lpr/lpr (MRL/lpr, 6, 9 and 16 weeks old), C57BL/6 (6 weeks old) and BALB/c (6 weeks old) mice were purchased from Nippon SLC (Shizuoka, Japan). They were maintained under specific pathogen-free conditions until used.

Materials RPMI 1640 and Hank's balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Flow Laboratories (Mclean, VA, U.S.A.). \[^{3}H\]Thymidine (Tdr) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.) and concanavalin A (Con A) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 13-Methyltetradecanoic acid (iso C_{15:0}), 14-methylpentadecanoic acid (iso C_{16:0}), 15-methylhexadecanoic acid (iso C_{17:0}) methyl 15-methylhexadecanate (iso C_{17:0} OMe), methyl 14-methylhexadecanate (anteiso C_{17:0} OMe), 1,3-dipalmitin (1,3-di C_{16:0} G), 1,3-distearin (1,3-di C_{18:0} G) and 1,3-dipalmitin glycerol ether (1,3-di C_{16:0} G ether) were obtained Funakoshi Co. (Tokyo, Japan). Nylon wool was a generous gift from Unitika Co., Ltd. (Uji, Kyoto, Japan), cyclosporin A (CsA) from Sandoz Ltd., Biological and Material Research (Basel, Switzerland) and lobenzarit (CCA) from Chugai Pharmaceutical Co., Ltd. (Gotenba, Shizuoka, Japan).

Synthesis of 1,3-Diolo C_{16:0} G 1,3-Diolo C_{16:0} G was synthesized according to the procedure described by Bentley et al., and its purity was checked by means of NMR and MS.

MLR As previously described, the MLR test against alloantigen was carried out using 5 x 10^5 C57BL/6 spleen cells (responder) and 5 x 10^5 mitomycin C (MMC)-treated BALB/c spleen cells (stimulator) in 0.2 ml RPMI 1640 medium supplemented with 10% FCS, 50 \mu M 2-mercaptoethanol, 12 \mu M N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 100 \mu M benzylpenicillin and 100 \mu g/ml streptomycin (RPMI 1640 complete medium). The cells were incubated with test samples for 4 days at 37 \degree C in a humidified atmosphere of 5% CO_2; 95% air. The cultures were pulsed with 0.5 \mu Ci of \[^{3}H\]Tdr for a period of 16 h before the end of culture and harvested onto glass fiber filter paper using an automatic harvester LM 101 (Medi-Con Corp., Osaka, Japan). The filter papers were dried and processed for liquid scintillation counting to determine the incorporated \[^{3}H\]Tdr. Test samples were dissolved in ethanol and further diluted

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in medium to the required concentration in culture.

AMLR of MRL/pr Mice Mesenteric lymph nodes (LN) from MRL/pr mice were teased to give a single cell suspension and filtered through nylon mesh. The cell suspension was freed of erythrocytes by treatment with ammonium chloride buffer and washed. LN cells (T cell-rich fraction) passed over a nylon wool column according to the method of Julius et al. were used as responder cells. Whole spleen adherent cells (SAC, stimulator cells) were prepared by allowing spleen cells to adhere for 1 h to a 100-mm Plastic Tissue Culture Dish (No. 3003, Falcon Labware, Div. of Becton & Dickinson Co., Oxnard, CA, U.S.A.). The adherent cells were then treated with MMC (incubated with 25 μg/ml MMC at 37°C for 30 min and washed three times with HBSS). AMLR was performed in flat-bottomed microtiter plates (Corning®, Corning, NY, U.S.A.), with each well containing 1 x 10^6 responder cells and 3 x 10^6 stimulator cells, with or without test samples in 0.2 ml RPMI 1640 complete medium. The cells were incubated for 5 days at 37°C in a humidified atmosphere of 5% CO₂: 95% air. The cultures were pulsed with [³H]Tdr for the last 16 h, and the incorporated [³H]Tdr was determined as described for MLR.

Con A Response The Con A response was determined in flat-bottomed microtiter plates with each well containing 5 x 10^4 spleen cells of MRL/pr mice, with or without 1.25 μg/ml Con A, and test samples in 0.2 ml RPMI 1640 complete medium. The cells were incubated for 2 days. The cultures were pulsed with [³H]Tdr for the last 16 h, and the incorporated [³H]Tdr was determined as described for MLR.

RESULTS

MLR The effect of 1,3-diiso C16:0 G and its related isofatty acids on T cell responses in the alloreactive MLR of C57BL/6 mice against spleen cells of BALB/c mice is shown in Fig. 1. 1,3-Diiso C16:0 G, iso C16:0, iso C17:0, iso C17:0 OMe, and antiso C17:0 OMe suppressed the MLR response by approximately 20% at a concentration of 1 μg/ml, while iso C15:0 did not. 1,3-Diacylglycerols with C16:0, C18:0 and C16:1 fatty acids, and 1,3-di C16:0 G either were completely ineffective in inhibiting MLR (data not shown). The immunomodulating drug, CCA, also suppressed MLR-stimulated T cell proliferation by 20% at 10 μg, and the immunosuppressant CsA produced an inhibition greater than 90%.

AMLR of MRL/pr Mice 1,3-Diiso C16:0 G and iso C16:0 which suppressed the MLR response against alloantigen, were tested at concentrations of 0.01 and 1 μg/ml. As shown in Fig. 2, 1 μg/ml of the lipids inhibited by more than 40% the incorporation of [³H]Tdr into LN cells when added at the start of culture, and even 0.01 μg/ml produced an inhibition of approximately 25%. CCA produced an inhibition of about 30% at 10 μg/ml, and CsA an inhibition of 90% at 0.12 μg/ml.

Con A Response The effect of the lipids on the Con A response of MRL/pr mouse lymphocytes was studied using readily available spleen cells. When added to the cultures of spleen cells with 1.25 μg/ml Con A for 2 days, 1,3-diiso C16:0 G and iso C16:0, which suppressed MLR and AMLR, had no effect on lymphocyte proliferation.
CCA also was ineffective, but CsA was able to inhibit Con A-induced T cell proliferation as well as MLR and AMLR (data not shown).

DISCUSSION

1,3-Diiso C_{16:0} G, as well as iso C_{16:0}, iso C_{17:0}, iso C_{17:0} OMe and anteciso C_{17:0} OMe, inhibited the mouse MLR (4d) against alloantigen, but did not inhibit the proliferation (2d) stimulated by Con A. Although the inhibitory effects on MLR were lesser than that of Fr. 5B,\textsuperscript{21} it is probable that these lipid components are responsible for at least a part of the inhibitory activity of Fr. 5-B.

1,3-Diacylglycerols with normal fatty acids and 1,3-di C_{16:0} G ether had no effect on the MLR and Con A responses. Previously, we reported that iso fatty acids (C_{15:0} and C_{16:0}) exhibited inhibitory effects on \textit{in vitro} erythrocyte hemolysis and albumin denaturation, and \textit{in vivo} carrageenin-induced edema formation.\textsuperscript{5} The difference in activities between diacylglycerols with iso fatty and normal fatty acids may be partly explained by the fact that mammalian serum added to the lymphocyte culture medium contains an abundance of lipid components (approximately 360 \mu g/ml free fatty acids and unesterified fatty acids, 90 \mu g/ml triglycerols and 200 \mu g/ml phospholipids in DMEM-10\% FCS) consisting of normal fatty acids.\textsuperscript{6} Since 1,3-diiso C_{16:0} G and iso fatty acids are not naturally-occurring lipids in mammals, they might be expected to have an effect on lymphocyte proliferation.

Recently, it has been reported that addition of polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and arachidonic acid, to lymphocyte culture medium results in the inhibition of lymphocyte proliferation,\textsuperscript{7} interleukin-2 production\textsuperscript{8} and protein kinase C activity.\textsuperscript{9} In addition, the emulsion of EPA exhibits an inhibitory effect on the interaction between antigen-presenting cells (APC) and T cells by suppressing APC functions.\textsuperscript{10} In the experiments \textit{in vitro}, the final inhibitory concentrations of polyunsaturated fatty acids in the culture medium were 50–500 \mu M, while those of the lipids containing iso fatty acids were less than one-tenth of that. This suggests that the lipids containing iso fatty acids act on target cells in a different way from polyunsaturated fatty acids.

AMLR is the well known phenomenon where the co-culture of T cells with MMC-treated autologous APC results in an increased DNA synthesis in the responding cells. The MRL/\textit{pr} mouse strain spontaneously develops a systemic autoimmune disease with histopathological features of human systemic lupus erythematosus and rheumatoid arthritis.\textsuperscript{11} In the present study, in our search for immunomodulating activity by these unusual lipids from thermophiles in autoimmune diseases, we used the AMLR in MRL/\textit{pr} mice as an assay system involving the T cell response. The results showed that 1,3-diiso C_{16:0} G and iso C_{16:0} inhibited the AMLR response of MRL/\textit{pr} mice more than the MLR response against alloantigen, and that the nature of the effect was a similar to that of the immunomodulating drug, CCA, but not the immunsuppressant, CsA. In AMLR, it is suggested that self-reactive T cells in responder cells proliferate by recognizing self Ia-expressing T cells, and then suppressor T cells proliferate.\textsuperscript{12} It is probable that 1,3-diiso C_{16:0} G does not affect the lymphocyte proliferative function of helper T cells because they did not inhibit the Con A response of lymphocytes. Our interpretation is that the inhibitory effect of these lipids on AMLR is probably due to increased induction of suppressor T cells, suppression of self-reactive T cells and/or suppression of non-specific T cells. However, this requires confirmation.

This report is the first description of the suppression of lymphocyte proliferation by 1,3-diacylglycerols containing iso fatty acids. As for the biological action of these lipids, further studies are needed to examine this in greater detail.

Acknowledgement This work was supported by a Grant-in-Aid for Scientific Research (No. 04671350) from the Ministry of Education; Science and Culture, Japan.

REFERENCES