Studies on Thermophile Products. V.[1] Immunosuppressive Profile *in Vitro* of *Bacillus stearothermophilus* Component, Fr.5-B

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The immunosuppressive profile of *Bacillus stearothermophilus* UK563 component, Fr.5-B, is presented in *in vitro* studies. Fr.5-B (0.1—1000 ng/ml), provided it was added at the initiation of mixed leukocyte reaction (MLR), inhibited dose-dependently the incorporation of tritiated thymidine ([^{3}H]TdR) into mouse spleen cells and human peripheral blood lymphocytes. Even the addition of Fr.5-B 48 h after the onset of culture suppressed mouse MLR, unlike cyclosporin A (CYA). Fr.5-B significantly inhibited cytotoxic T lymphocyte generation determined by [^{3}H]TdR-release micro-cytotoxicity assay by using mouse mastocytoma P815 as targets. Moreover, this component decreased dose-dependently the expression of class II major histocompatibility molecules (Ia) on mouse peritoneal macrophages induced by concanavalin A supernatant. The present results revealed the unique immunosuppressive property of Fr.5-B which was different from that of CYA.

Keywords *Bacillus stearothermophilus*; immunosuppressant; mixed leukocyte reaction; cytotoxic T lymphocyte; Ia antigen; macrophage

The immunomodulating substances which attack specific target sites of cells are expected to be potent tools for the study of cellular and biochemical events of immune responses and also provide a useful prototype of drugs for immunotherapy.[2] Several groups have tried to search immunomodulating substances among microbial metabolites using an immunological assay system, and some new immunoactive low molecular weight substances have been discovered.[3] In connection with our studies on products of thermophile with potentially useful biological activity, we previously reported the isolation of *Bacillus stearothermophilus* UK563 component, Fr.5-B, that suppresses mouse mixed leukocyte reaction (MLR), but not proliferation stimulated by mitogens.[4]

In this paper, the immunosuppressive profile of Fr.5-B is presented in *in vitro* studies compared with an immunosuppressant, cyclosporin A (CYA).

Experimental

Mice Female C3H/HeJ, male C57BL/6 and BALB/c mice (6 to 8 weeks old) were purchased from Shimizu Jikken Zairyou, Ltd. (Kyoto, Japan). They were maintained under specific-pathogen-free conditions until used.

Materials RPMI 1640, Dulbecco's Modified eagle medium (DMEM) and Hank's balance salt solution (HBSS) were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Gibco Laboratories (Grand Island, NY, U.S.A.), and monoclonal anti-I-A^k antibody was obtained from Cedarlane Laboratories Ltd. (Ontario, Canada). [^{3}H]Tymidine (TdR) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.), concanavalin A (Con A) from Sigma Co. (St. Louis, MO) and lipopolysaccharide (*Escherichia coli* 055: B5, LPS) from Difco Laboratories (Detroit, MI, U.S.A.). Fr.5-B used was the preparation previously isolated from the debris of *B. stearothermophilus* UK563 autolysate.[4] Fr.5-B was the form of white powder purified by successive silica gel column chromatographies from the ethanol extract and appeared to be a lipid-like compound. CYA was a generous gift from Sandoz Ltd., Biological and Medical Research (Basel, Switzerland). Fr.5-B and CYA were dissolved in ethanol and further diluted in medium to the proper concentration in culture.

Tumor Cells AKR/J thymoma BW5147, DBA/2 mastocytoma P815, human T cell leukemia Molt 4 and human T cell acute lymphoblastic leukemia Tall 1 were supplied by the Japanese Cancer Resources Bank (JCRB).

MLR Spleen Cells: The spleen obtained from BALB/c and C57BL/6 mice was teased into single cell suspensions and filtered through nylon mesh. The cell suspension was freed of erythrocytes by treatment with ammonium chloride buffer (ACK buffer) and washed three times. Cell preparation was finally resuspended in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol (2ME), 12 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, 100 U/ml benzylpenicillin and 100 μg/ml streptomycin (RPMI 1640 complete medium).

Peripheral Blood Lymphocytes (PBL): Human blood was obtained from healthy adult donors. The PBL were isolated by Lymphoprep™ (Nycomed Pharma As, Oslo, Norway) density centrifugation. The collected cells were washed three times and filtered through nylon mesh. Cell preparation was finally resuspended in RPMI 1640 complete medium.

The mouse MLR was performed in flat-bottomed microtiter plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), with each well containing 5×10^5 C57BL/6 spleen cells (responder cells, H-2^b) and 5×10^5 mitomycin C-treated (incubated with 25 μg/ml mitomycin C at 37 °C for 30 min and washed three times with HBSS) BALB/c spleen cells (stimulator cells H-2^d) in 0.2 ml RPMI 1640 complete medium. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2: 95% air. The human MLR test was established by using normal human PBL. Responder lymphocytes (2.5×10^5 cells) were co-cultured with an equal number of mitomycin C-treated stimulator lymphocytes in 0.2 ml RPMI 1640 complete medium. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2: 95% air. The cultures were pulsed with 0.5 μCi of [^{3}H]TdR during 16 h before the end of culture and harvested onto glass fiber filter paper. The papers were dried and processed for a liquid scintillation counting.

Cytotoxic T Lymphocyte (CTL) Generation Cytotoxic activity of splenocytes was determined as described by Nakamura *et al.*,[5] with only minor modifications.

Effector Cells: Single cell suspensions of effector cells were obtained from the mouse bulk MLR (96 h). The collected cells were washed 3 times and cellular debris was removed by passing through nylon mesh. They were finally resuspended in RPMI 1640 complete medium.

Target Cells: Suspension culture of P-815 mastocytoma (H-2^d) cells maintained in RPMI 1640 complete medium was washed 3 times and resuspended in RPMI 1640 complete medium. Tritium-labeled P-815 cells were prepared by incubating the cells for 24 h in the presence of [^{3}H]TdR (1 μCi/ml).

^{3}H-Release cytotoxic assay was performed in round-bottomed microtiter plates. To each well with 1×10^5 target cells in 0.1 ml of complete medium 4×10^5 effector cells in 0.1 ml of RPMI 1640 complete medium were added and incubated at 37 °C in a CO_2-incubator for 4 h. At the end of the incubation period, 100 μl of the supernatant was removed and the released radioactivity was counted by a liquid scintillation counter. Supernatants from wells containing target cells alone in 0.2 ml medium served as control for the baseline release of [^{3}H]TdR and 1×10^5 of [^{3}H]TdR-labeled target cells were also counted to determine total [^{3}H]TdR incorporated into the target cells. The percentage of specific

cytotoxicity was calculated as follows:

$$\text{cytotoxicity (\%)} = \frac{\text{cpm of experimental group} - \text{cpm of medium control}}{\text{total cpm} - \text{cpm of medium control}} \times 100$$

Expression of Class II Major Histocompatibility Molecules (Ia) Antigen of Peritoneal Macrophages Peritoneal exudate cells (PEC): C3H/HeJ mice were injected i.p. with 2.5 ml of 2% casein. Four days after the injection, PEC were harvested by peritoneal lavage with 3 ml of ice cold DMEM containing 20 units of heparin/ml. They were centrifuged at 1000 rpm for 5 min and resuspended at 5×10^5 cells/ml in RPMI 1640 complete medium.

Interferon-γ (IFN-γ): The splenocytes obtained from ddY mice were cultured in RPMI 1640 complete medium at a concentration of 2×10^6 cells/ml with Con A (2 μg/ml) in 25 cm^2 tissue culture flask (Corning®, NY). Cells were cultured for 3 d at 37 °C in a humidified atmosphere of 5% CO_2: 95% air. After incubation, the cells at 1×10^7 cells/ml were incubated again with 10 μg/ml of Con A as a stimuli to produce IFN-γ at 37 °C. After 24 h of incubation, the supernatant was collected by centrifugation at 2000 rpm for 5 min. To remove residual Con A, the supernatant was mixed with Sephadex® G-50 (Pharmacia, Uppsala, Sweden) at 20 °C for 1 h and centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant termed Con A sup was filtrated by a 0.45 μm membrane filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and stored at −20 °C.

The expression of Ia antigen of PEC was determined as follows. The sample was prepared in 24-well flat bottomed culture plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) in a volume of 250 μl which was seeded with 500 μl of PEC (2.5×10^5 cells/well), with or without Con A sup 250 μl, that was used as a INF-γ-like stimulator. The plates were then incubated for 3 d at 37 °C in a humidified CO_2 incubator. The adherent PEC (macrophages) were collected with the aid of a soft, wide-tipped rubber policeman. Cells were washed 3 times with HBSS supplemented with 2% FCS and 20 mM NaN_3 (HBSS buffer) and suspended in HBSS buffer (2×10^7 cells/ml). The suspension (50 μl) was incubated with 50 μl of FITC-conjugated anti-mouse I-A^k monoclonal antibody diluted in HBSS buffer at 4 °C for 1 h and washed 3 times with HBSS. The amount of Ia molecule expressed on macrophages was detected with FITC-conjugated anti-mouse I-A^k monoclonal antibody by fluorescence spectrophotometer model F-3000 (Hitachi, Ltd., Tokyo, Japan).

Cytotoxic Assay Viability of splenocytes used in the MLR was counted by a flowcytometer (Epics® Profile II, Coulter Corporation, Hialeah, Florida, U.S.A.) after staining with fluorescein diacetate–propidium iodide (FDA–PI, Sigma Co.).[6] The assay using BW5147 thymoma, Tall 1 leukemia and Molt 4 leukemia was performed in flat-bottomed microtiter plates, with each well containing 10^4 cells in 0.2 ml of RPMI 1640 complete medium. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2: 95% air for 2 d. The cultures were pulsed with 0.5 μCi of [^{3}H]TdR during 16 h before the end of culture and counted.

Results

MLR The time dependency of the effect of Fr.5-B and CYA on mouse MLR has been studied. Fr.5-B and CYA were added at the initiation of mouse MLR or 48 h after the onset of culture. The results are illustrated in Fig. 1. Fr.5-B and CYA inhibited dose-dependently the incorporation of [^{3}H]TdR into mouse spleen cells when added at the initiation of culture. The concentration causing 50% inhibition (IC_{50}) values of Fr.5-B was 1 ng/ml, as determined from Fig. 1. The IC_{50} value of CYA was 11 nM (13 ng/ml) which was deduced in the other experiment based on four arbitrary doses. Even the addition of Fr.5-B 48 h after the onset of culture, inhibited mouse MLR in a dose-dependent manner (IC_{50} values, 3 ng/ml). However, when CYA was added at 10 or 100 nM 48 h after the start of alloantigen stimulation, no inhibition was seen and the increase in cell division closely mirrored that of the control culture.

Viability of splenocytes used in the MLR was counted by a flowcytometer after staining with FDA–PI. As far as the cytotoxicity tests are concerned, Fr.5-B was non-toxic at less than 1 μg/ml against splenocytes used in the MLR (data not shown). However, when CYA was added at a highly suppressive concentration of 100 nM at the initiation of mouse MLR, viability of splenocytes decreased slightly (76% of control).

Fr.5-B and CYA were tested for its ability to suppress the *in vitro* proliferative response of human lymphocytes to alloantigen stimulation. The results of representative experiments in which various quantities of Fr.5-B and CYA were added to the human MLR at the initiation of culture are illustrated in Fig. 2. The IC_{50} values of Fr.5-B and CYA were 25 ng/ml and 9 nM (11 ng/ml), respectively.

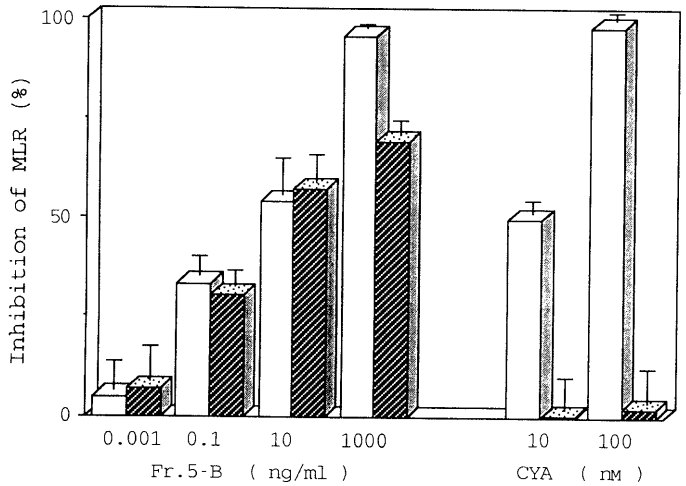

Fig. 1. Time Dependency of Effect of Fr. 5-B and Cyclosporin A (CYA) on Mouse Mixed Leukocyte Reaction (MLR)

The data are presented as the percentage of inhibition based on response in the control diluent and show the mean +S.E. of triplicate determinations. Mean cpm of [^{3}H]TdR uptake for MLR (4 d) in which the sample was added at the initiation of MLR (□) was 28832 ± 1646, and unstimulated lymphocytes average 2726 cpm. Mean cpm of control that the sample was added 48 h after onset of culture (▨) was 32785 ± 1751, and unstimulated lymphocytes average 2511 cpm.

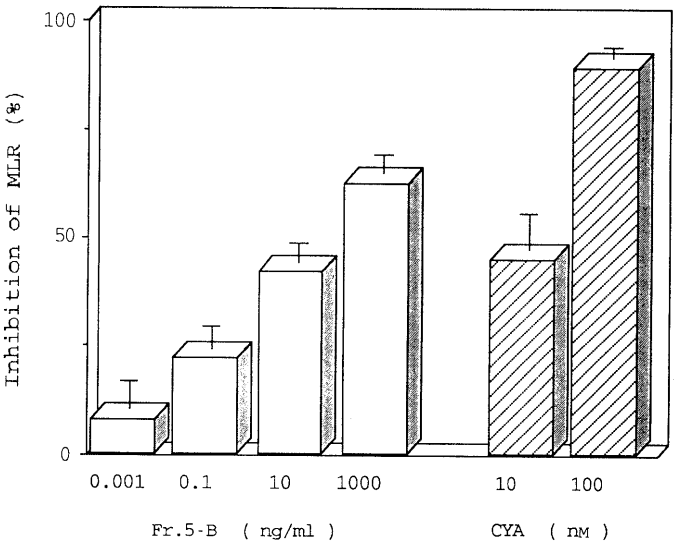

Fig. 2. Effect of Fr. 5-B and CYA on Human MLR

The data are presented as the percentage of inhibition based on response in the control diluent and show the mean +S.E. of 4 determinations. Mean cpm of [^{3}H]TdR uptake for human MLR was 21302 ± 1524. Unstimulated lymphocytes average 697 cpm of [^{3}H]TdR.

Both Fr.5-B and CYA did not affect the viability of lymphocytes in the human MLR system (data not shown).

CTL Alloreactive CTL was generated from bulk MLR by mixing 5×10^6 C57BL/6 mouse lymphocytes with 5×10^6 mitomycin C-treated BALB/c mouse lymphocytes. Various dilutions of Fr.5-B and CYA were added to mouse bulk MLR at the initiation of the culture. After 4 d of incubation, the cells were washed and the cytotoxicity of the CTL was determined by a short-term [^{3}H]TdR release micro-cytotoxicity assay by using P-815 cells as targets. As demonstrated in Table I, Fr.5-B and CYA significantly inhibited the generation of CTL in a dose-dependent manner. The IC_{50} value of Fr.5-B was 0.9 ng/ml at the short term. At a 16 h incubation period in which the spontaneous release of [^{3}H]TdR from P-815 may occur in addition to the specific generation of CTL, Fr.5-B and CYA were also inhibited.

Ia Expression We examined the effect of Fr.5-B on the expression of macrophage Ia expression *in vitro*. The amount of Ia molecule expressed on macrophage was detected with FITC-conjugated anti-mouse I-A^k monoclonal antibody. As can be seen in Fig. 3, Con A sup which was used as an INF-γ-like stimulator increased approximately 4-fold basal Ia antigen expression on macrophages 3 d after initiating the culture. Fr.5-B slightly decreased basal Ia antigen expression, and clearly decreased the stimulated Ia antigen expression in a dose-dependent manner, like positive control prostaglandin E_2.

Cytotoxicity Two dilutions of Fr.5-B and CYA were added to the cultures of mouse thymoma BW5147, human T-leukemia Tall 1 and Molt 4 cell lines. After 2 d of incubation, the amount of incorporated [^{3}H]TdR was measured. The results are presented in Table II. The percent inhibitions of proliferation by Fr.5-B at 1000 ng/ml against these three cells was 0, 3 and 5%, respectively. On the other hand, the inhibitions of CYA at 100 nM were 8, 76 and 15%, respectively. This result suggests that Fr.5-B was non-toxic at less than 1 μg/ml against constitutive mouse and human T cell proliferation such as BW5147, Tall 1 and Molt 4 cell lines.

TABLE I. Effect of Fr. 5-B on Cytotoxic T Lymphocyte (CTL) Generation

Sample	Concentration (ng/ml)	Specific lysis (%, mean ± S.E., $n=5$) 4 h		16 h	
Control	—	27.4 ± 1.6		77.6 ± 4.1	
Fr. 5-B	0.001	28.4 ± 2.2	(0)[a]	57.8 ± 2.4[b]	(26)
	0.1	22.0 ± 2.5	(20)	39.2 ± 4.8[c]	(50)
	10	7.5 ± 1.9[c]	(73)	40.0 ± 4.4[c]	(49)
	1000	4.2 ± 2.0[d]	(85)	24.7 ± 2.7[d]	(68)
CYA	10[e]	31.4 ± 1.5	(0)	75.5 ± 2.6	(3)
	100	4.7 ± 2.3[c]	(83)	33.8 ± 5.1[c]	(56)

a) % inhibition. *b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$, *versus* control. *e*) nM.

Discussion

The immunological network is in a dynamic equilibrium regulated by various kinds of cells. To control the balance of the network, low molecular weight agents that have specificity to the function of each group of cells are of use.[2] CYA has been used successfully as the primary drug to suppress the rejection of transplants.[2b,3a,7] However, its specific immunosuppressive effect on the one hand and clinical nephrotoxicity on the other, have led us to search for more specific and less toxic new immunosuppressants. Fr.5-B was identified in 1991 in our laboratories during a

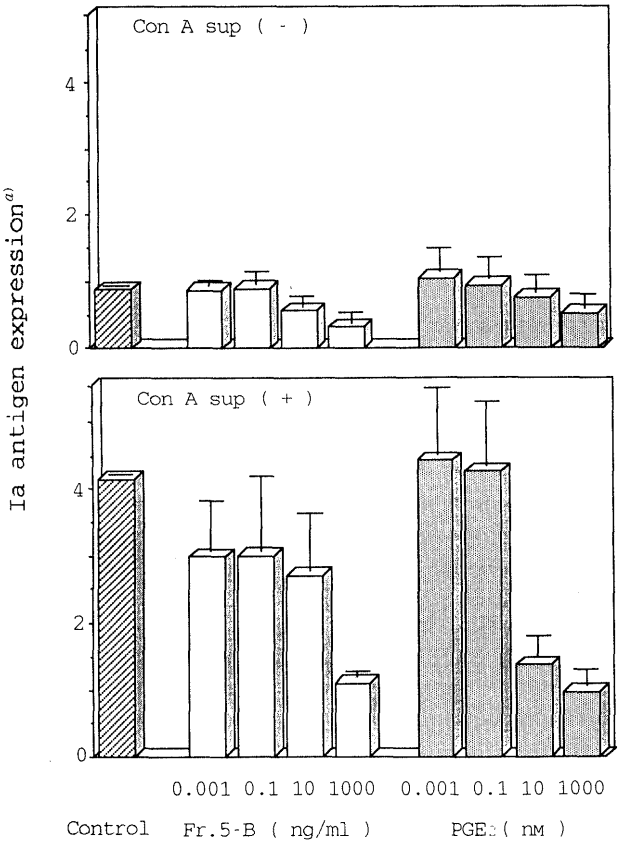

Fig. 3. Effect of Fr. 5-B on Ia Antigen Expression on Macrophages

a) The basal [Con A sup (−)] and stimulated [Con A sup (+)] amount of Ia antigen expressed on macrophages was detected with FITC-conjugated anti-mouse I-A^k monoclonal antibody and expressed as relative fluorescence intensity. The data show the mean ± S.E. of 4 determinations.

TABLE II. Effect of Fr. 5-B and CYA on Proliferation of Mouse and Human T Leukemia

Sample	Concentration (ng/ml)	[^{3}H]TdR uptake (cpm, mean ± S.E., $n=4$) BW5147		Tall 1		Molt 4	
Control	—	38090 ± 652		18371 ± 1295		35295 ± 1094	
Fr. 5-B	10	37917 ± 2109	(96)[a]	17943 ± 348	(98)	36137 ± 2141	(102)
	1000	38850 ± 1217	(102)	17836 ± 424	(97)	33543 ± 1416	(95)
CYA	10[b]	35319 ± 1278	(93)	17406 ± 511	(95)	32417 ± 945	(92)
	100	35223 ± 1428	(92)	4470 ± 334[c]	(24)	29888 ± 723[d]	(85)

a) % of control. *b*) nM. *c*) $p<0.001$, *d*) $p<0.01$, *versus* control.

search for immunosuppressive substances among thermophile products and constituents. This has been isolated from the debris of *B. stearothermophilus* autolysate,[4] and its chemical structure is under study.

The data obtained from the present experiments show that the new immunosuppressant, Fr.5-B, strongly inhibited the proliferative response of alloantigen-stimulated lymphocytes and the cytotoxic T cell generation at its non-toxic concentrations. Furthermore, it was shown that Fr.5-B added 48 h after the onset of culture, suppressed mouse MLR in a dose–dependent manner, unlike CYA. As already reported in one preliminary study, CYA has to be added within the first 6 h of stimulation in order to suppress mouse lymphocyte proliferation by at least 50%.[8]

The mode of action of Fr.5-B was unique and different from that of CYA. Fr.5-B seems to be a more specific suppressor on the proliferative response of lymphocytes and the cytotoxic T cell generation to alloantigen stimulation because it did not affect mitogen-induced proliferation of lymphocytes.[4] By many investigator's results obtained from *in vitro* studies and animal models, at least two well established mechanisms have been advanced to explain the effects of CYA; first, CYA inhibits the release of lymphokines *in vitro*, especially interleukin 2 (IL-2)[9]; secondly, CYA therapy prevents clonal expansion of helper and cytotoxic T cells[10] and semi-selectively spares suppressor cells.[11] Newly developed and highly potent immunosuppressant, FK506 also suppresses the production of lymphokines at about a hundred times lower concentration than CYA.[12] Although the cascade and the action of lymphokines are still unclear, FK506 may prevent the clonal expansion of T cells and the cytotoxic T cell generation with the inhibition on the production of lymphokines by T lymphocytes, especially IL-2.[13]

The T lymphocyte response to antigen requires initial antigen processing by an Ia (immune response associated antigen)-bearing macrophage-like cell, followed by the secretion of IL-1.[14] IL-1 triggers the production of IL-2 by helper T cells and thus amplifies ongoing T cell responses.[15] T helper cells recognize exogenous antigen in association with Ia antigen on the surface of antigen presenting cells.[2a,16] Fr.5-B suppressed already ongoing mouse MLR in a dose–dependent manner (Fig. 1). Further, Fr.5-B clearly suppressed expression of the Ia antigen on macrophages in doses which were effective in inhibiting MLR and CTL (Fig. 3). These results suggest that Fr.5-B mainly affect the expression of the Ia antigen that has an important function in primary immune response. As a result, Fr.5-B specifically inhibits the proliferative response of lymphocytes to alloantigen stimulation at non-toxic concentrations.

The MLR and the generation of CTL used here are generally regarded as *in vitro* correlates of allograft rejection.[7] Accordingly, it is interesting to study further the immunosuppressive mechanism of Fr.5-B that may prove useful in organ transplantation.

It is widely accepted that activated T cells cause much of the tissue damage in graft rejection and autoimmune disease, and T cells activation is triggered by means of interactions among the Ia antigen, antigen peptide and T cell receptor.[17] Activities which can modify the level of Ia antigen expression may regulate T cell activity. Though the molecular mechanism of action of Fr.5-B remains unclear, it may provide a valuable tool for investigating the immunoregulation system *via* Ia antigen expression.

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