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Ketotifen Inhibits Allergen-Specific T Lymphocytes' Responses by Suppressing Antigen Presentation with Concomitant Decrease of HLA-DQ Antigen on Macrophages

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SUMMARY **Allergen activates T lymphocytes responsive to interleukin 2 (IL-2) in allergic patients but not in normal individuals. This response was suppressed by anti-allergic agent, Ketotifen (4-(1-methyl-4-piperidylidene)-4H-benzo [4, 5] cyclohepta [1, 2-b] thiophen-10 (9H)-one hydrogen (fumarate). Prolonged culture of antigen-presenting adherent cells impaired the ability to present *Dermatophagoides farinae* (Df) antigen to T cells, whereas stimulation of adherent cells with recombinant interferon-γ (IFN-γ) restored the antigen-presenting capability. The maintained antigen presenting ability of adherent cells treated with IFN-γ was also suppressed by Ketotifen. Fluorescence activated cell sorter (FACS) analysis disclosed that Ketotifen selectively reduced the expression of HLA-DQ antigen, crucial restriction elements in Df antigen-related responses, on macrophages but not on B cells, even in the presence of IFN-γ. Collectively, Ketotifen prevented macrophages from inducing allergen-activated T lymphocytes' responsiveness to IL-2 at least in part by decreasing the expression of HLA-DQ antigen.**

Despite recent advances in therapeutic strategies for allergic diseases, the incidences of childhood asthma are increasing.[1] Lymphocytes from allergic patients show proliferation,[2-7] and IL-2 production,[8] on stimulation with an allergen such as the mite. Elsewhere we showed that Df antigen-specific T lymphocytes from patients were activated *in vitro* when peripheral blood mononuclear cells (PBMC) were stimulated with Df antigen in the presence of the T lymphocyte growth factor, IL-2, whereas normal lymphocytes were not responsive.[9-12] The cellular basis of this response disclosed that HLA-DQ$^+$ adherent cells were suggested to present Df antigen to T cells of CD4$^+$ subset and then to induce IL-2 responsiveness.[10]

The responses induced in allergic patients may reflect the *in vivo* immunological functions of the allergen-sensitized lymphocytes and should prove useful for identifying etiological allergens and for monitoring the clinical activity of the atopic diseases. Allergen-activated T cells responding to IL-2 may correspond to the two phenotypes of the T helper cells since human Th1 and Th2 clones are propagated efficiently with IL-2 *in vitro*.[13-15] Ketotifen, chemical entry of benzocycloheptathiophene, is one of the effective oral prophylactics for atopic diseases.[16,17] This agent was evidently reported to have a weak immunosuppressive activity as shown by the inhibition of allergen-induced lymphocytes' responsiveness to IL-2.[18] In this study, we investigated the mechanisms by

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which Ketotifen suppressed the antigen presentation thereby inhibiting the allergen specific T lymphocytes responses with special reference to major histocompatibility complex (MHC) class II antigens and the class II inducing cytokines, IFN-γ and IL-4.[19,20]

MATERIALS AND METHODS

Subjects

Peripheral blood mononuclear cells (PBMC) were obtained from 8 patients with bronchial asthma, 3-22 years of age (13.6 ± 5.9). Patients with bronchial asthma have attacks of wheezing, dyspnea, chest tightness, and coughs that are reversible spontaneously or with treatment. Diagnoses were based on such clinical features and a skin test. The serum immunoglobulin E (IgE) score against Df, determined by the radio allergosorbent test (RAST),[21] varied from 3 to 4, corresponding to more than 3.5 IU/ml, in each patient. Informed consent was obtained from each patient.

Separation of adherent cells and nonadherent cells

Mononuclear cells were separated from heparinized peripheral blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation.[22]

Monocyte-enriched adherent cells were isolated by a 2-hr adherence to plastic petridishes (Corning 100-mm tissue culture dish No. 250-20) followed by removal with EDTA, as described by Minami *et al*.[23] The nonadherent cells were recovered and were used as IL-2-responding cells. Monocyte-enriched adherent cells contain 80-90% of peroxidase+ cells, whereas monocyte-depleted nonadherent cells contain less than 5% of peroxidase+ cells. In experiments where MHC class II antigen expression was examined, non-T cells were prepared by depleting PBMC of aminoethylisothiouronium bromide (AET) treated sheep red blood cell (SRBC)-binding cells as described.[24]

Staining/quenching solution

Propidium iodide (Sigma, St. Louis MO) stock solution (0.5 mg/ml in 4.9% EDTA) in a volume of 1.5 ml was diluted with 7.5 ml of EDTA solution (4.9%, pH 7.0, Wako Pure Chemical Industries Inc, Japan) and was added to 1.5 ml of Triton X-100 (8%, v/v, Wako Pure Chemical Industries Inc, Japan) to lyse the lymphocytes. Drawing ink (0.1 ml of a ≧10 fold dilution) (LEITZ, Wetzlar) was included to quench the background fluorescence. This solution was kept at -20°C and designated the LSQ Medium (Lysing-staining-quenching (medium).

Assay for IL-2 responsiveness

Mononuclear cells (1×10^6) were cultured in the presence of the Df antigen (TORII & Co. Ltd., Tokyo, Japan) and then suspended in 1 ml of RPMI 1640 medium containing 40 μg/ml gentamycin and 10% heat-inactivated pooled human serum in a culture tube (Falcon Plastic 2054). After 5 days of culture (37°C, 10% CO_2), the medium was replaced with fresh medium, after which the cells were readjusted to approximately 2×10^5/150 μl of medium or were divided into 6 portions and plated in 96 well flat bottom microplates (Nunc, Roskiled, Denmark). More than 90% of 5 day-cultured lymphocytes were viable as determined by trypan blue dye exclusion. These cells were cultured for three days in the presence of 0.1 U/ml of recombinant IL-2 (Takeda Co. Ltd., Tokyo., Japan). At the end of this culture, 50 μl of LSQ medium was added to the cell suspension, after which the mixture was kept for 2 hours at 37°C. Propidium iodide (2,7-diamino-9-phenyl-10 dietylaminopropyl phenanthridinium iodide methiodide) which then was added to the lysed lymphocytes stained the dsDNA but not the ssDNA. The medium's fluorescence was quenched by the addition of drawing ink. The fluorescence intensity (long-pass filter, 580 nm) for each well was determined with Leitz PATIMED equipment (Leica). IL-2 responsiveness was evaluated as follows :

$$\text{IL-2 responsiveness (stimulation index or S.I.)} = \frac{\text{Fluorescences intensity with IL-2 – background}}{\text{Fluorescence intensity without IL-2 – background}}$$

Background means the fluorescence intensity with LSQ medium alone. Control studies done on non-lysed cells (without Triton X-100) with PI for the evaluation of dead cells showed no significant fluorescence intensity (data not shown).

To clarify the role of adherent cells to present antigens to autologous nonadherent cells, $1x10^5$ adherent cells were pulsed with 10 μg/ml of Df antigen at 37°C for 12 hours then washed three times with the medium. Thereafter they were recombined with mononuclear cells depleted of adherent cells ($1x10^6$) and were cultured for 5 days. IL-2 responsiveness was evaluated following an additional three days of coculture with rIL-2.

Treatment of cell with Ketotifen

Ketotifen was adjusted to 500 ng/ml in RPMI 1640 medium at pH 7.4, which was found to be optimal for suppressing antigen-induced IL-2 responsivenss in our previous study.[18] Mononuclear cells ($1x10^6$/ml) were treated with Ketotifen at 37°C for 12 hours, washed three times and then stimulated with Df antigen to be tested for their IL-2 responsiveness. To address its effect on adherent cells in the induction of IL-2 responsiveness, the adherent cells were pretreated with Ketotifen as mentioned above and then washed three times with the medium, followed by stimulation with Df antigen for 12 hours at 37°C. Thereafter they were washed again to remove Df antigen thoroughly and were reconstituted with autologous nonadherent cells to evaluate the intensity of IL-2 responsiveness. In contrast, to examine the direct action of Ketotifen on the nonadherent cells, nonadherent cells were pretreated with Ketotifen for 12 hours at 37°C before recombination with adherent cells.

Pretreatment of adherent cells with IFN-γ

Adherent cells were cultured with or without 500 U/ml of rIFN-γ for 2 days in RPMI 1640 medium containing 10 % human serum and then they were washed three times with the media before recombination with autologous nonadherent cells in the presence of Df antigen as described previously.[19]

FACS analysis of MHC class II expression on cultured non-T cells

PBMC depleted of SRBC-binding cells ($1x10^6$) consisting mainly of macrophages and B cells, were cultured for three days with 500 U/ml of recombinant IFN-γ (Shionogi, Osaka, Japan), or with 100 U/ml of recombinant IL-4 (Genzyme, Cambridge, MA) in the presence or absence of Ketotifen for three days. The doses of the lymphokines used in this study had already been confirmed to be optimal for the induction of MHC class II antigens on macrophages.[19,20] Thus cultured non-T cells were extensively washed and were then stained with monoclonal antibodies which define macrophage, B cells, and MHC class II antigens and they were analyzed on a fluorescence activated cell sorter (FACS) 440 (Becton-Dickinson Electronics Lab., Mountain View, CA) The monoclonal antibodies used in this study were phycoerythrin (PE)-conjugated Leu M3 (CD14), PE-conjugated Leu 16 (CD20), fluorescence isothiocynate (FITC) conjugated HLA-DR, FITC-conjugated Leu 10 (HLA -DQ) and HLA-DP antibodies purchased from Becton-Dickinson.

In experiments in which HLA-DP antigens were stained, the cell, first incubated with HLA-DP antibody were washed to remove free antibody and then the cell-bound antibody were reacted with $F(ab')_2$ of FITC conjugated goat anti-mouse Ig antibody (Tago, Burlingame, CA) after extensive washes to remove unbound secondary antibody, the cells were subsequently added with PE-Leu M3 or PE-Leu 16 antibody and then they were analyzed on a FACS 440.

Statistical analysis

Statistical analyses were performed both with students' two tailed *t* test and with Wilcoxon matched pair signed rank test. The limit of significance was $p<0.05$.

RESULTS

Suppression of allergen-induced IL-2 responsiveness by Ketotifen

As previously presented, Ketotifen expressed marked suppression for allergen-induced IL-2 responsiveness in a dose dependent manner (Table 1). The suppressive effect of Ketotifen on the response was evident when it was applied to adherent cells (macrophages), whereas treatment of nonadherent T cell-rich populations with Ketotifen showed weak suppression (Fig.1).

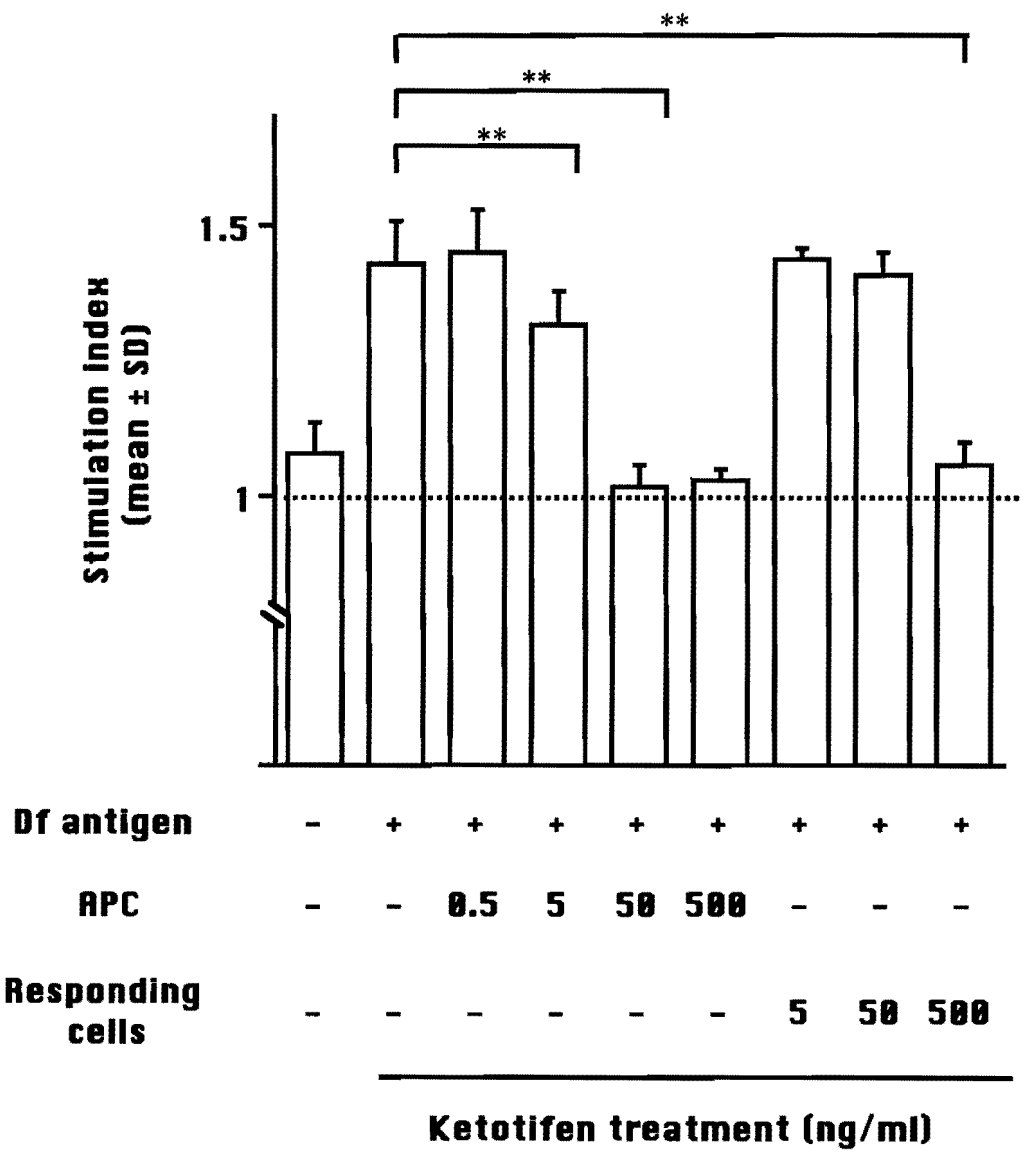

Fig. 1. Comparisons of the susceptibility of adherent cells and nonadherent T cell-rich population to Ketotifen in the induction of Df-specific IL-2 responsiveness. Either adherent cells or T cell-rich population were individually treated with Ketotifen, and then both cell fractions were recombined to evaluate the response to IL-2. Responding T cell-rich population are inhibited by as high as 500 ng/ml of Ketotifen, while the antigen presenting adherent cells are suppressed by 50 to 500 ng/ml of Ketotifen. Data from independent experiments performed on 4 patients were pooled and represented as mean ± SD. APC: antigen-presenting cells; Signifi cant differences compared to values for the corresponding cultures without Ketotifen treatment: ** $p<0.01$.

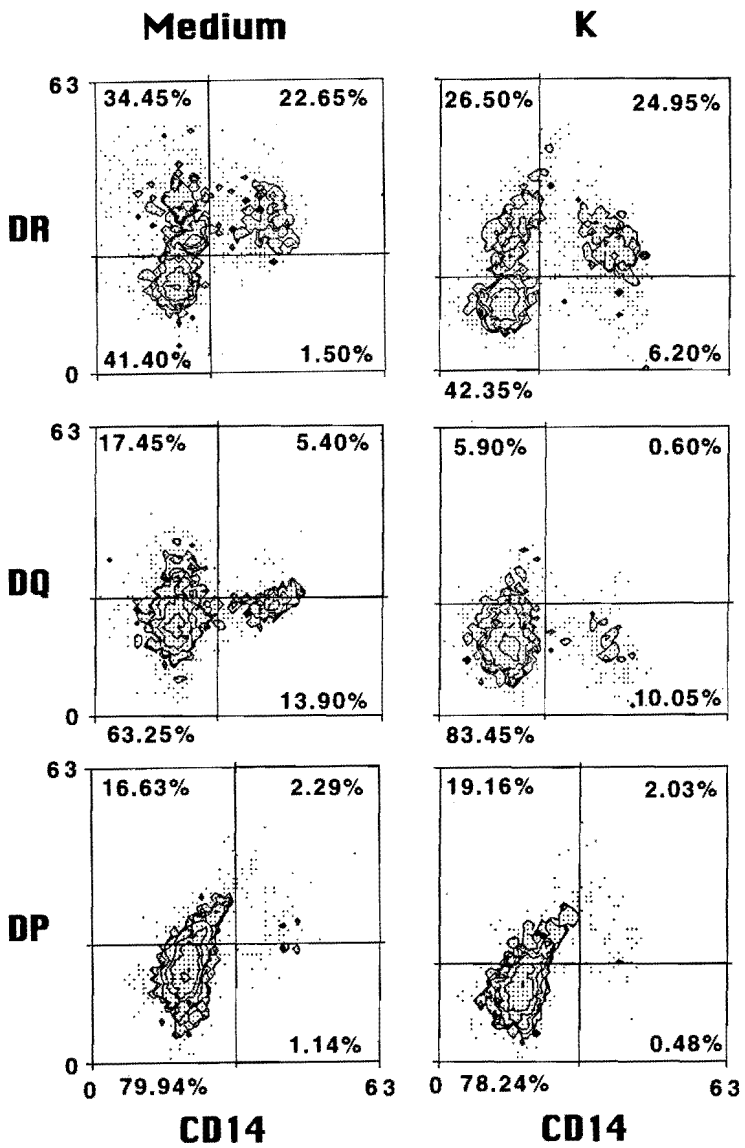

Fig. 2a.

Fig. 2. A representative data of the two color analysis of the expression of HLA class II antigens on macrophages and B cells. Non-T cells, unstimulated or stimulated with IFN-γ or IL-4 were cultured for three days in the absence or presence of 500 ng/ml of Ketotifen (K), and then the expression of HLA-DR, -DQ and -DP antigens on macrophages were evaluated using flow cytometry. Two color fluorescence data are displayed as log red fluorescence on the X axis and log green fluorescence on the Y axis. The scattergram is divided into four quadrants by setting the X and Y cursors. The cursors were set to exclude 95-98% of the red or green fluorescence of cells incubated with isotypic control monoclonal antibodies. The expression of HLA-DQ antigens on macrophages was significantly suppressed by the Ketotifen with out marked change in HLA-DR and -DP antigens. The suppressive effect on the HLA-DQ by macrophages was still observed when the cells were stimulated with IFN-γ (a) HLA class II expression on macrophages cultured without cytokines. (b) HLA class II expression on macrophages cultured with IFN-γ (c) HLA class II expression on macrophages cultured with IL-4.

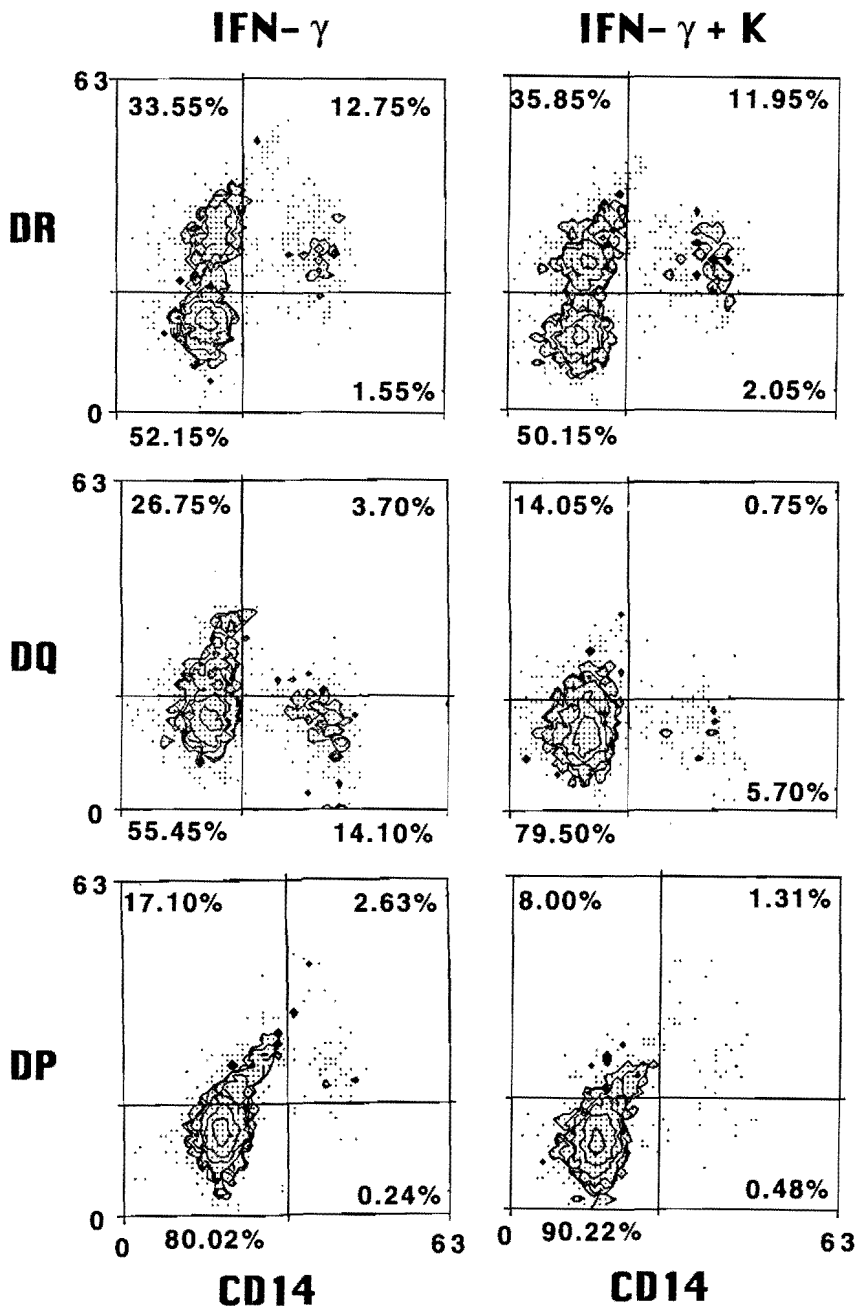
IFN-γ
IFN-γ + K
DR
DQ
DP
63
0
33.55%
12.75%
1.55%
52.15%
35.85%
11.95%
2.05%
50.15%
26.75%
3.70%
55.45%
14.10%
14.05%
0.75%
5.70%
79.50%
17.10%
2.63%
0.24%
80.02%
8.00%
1.31%
0.48%
90.22%
CD14
CD14

Fig. 2b.

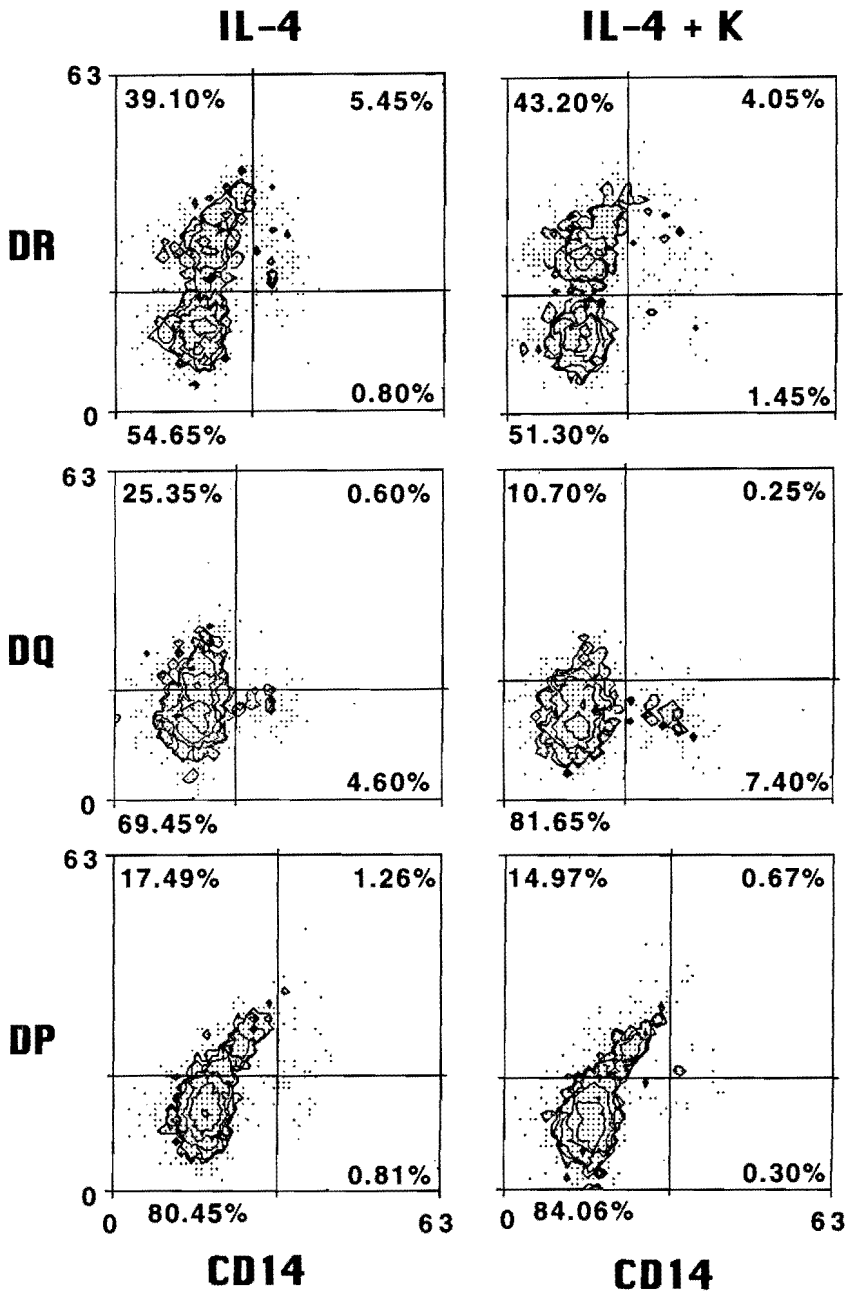
IL-4
IL-4 + K
63
0
39.10%
5.45%
0.80%
54.65%
43.20%
4.05%
1.45%
51.30%
DR
25.35%
0.60%
4.60%
69.45%
10.70%
0.25%
7.40%
81.65%
DQ
17.49%
1.26%
0.81%
80.45%
14.97%
0.67%
0.30%
84.06%
DP
CD14
CD14

Fig. 2c.

Table 1. Relationahip between the dose of Ketotifen and its suppressive effect.

Antigen	Ketotifen (ng/ml)	IL-2 responsiveness (mean ± SD)
-	-	1.06 ± 0.13
Df	-	1.56 + 0.06
Df	5	1.16 ± 0.11**
Df	50	1.09 ± 0.14**
Df	500	1.03 ± 0.10***

Stimulation of lymphocytes with Df antigen significantly induced IL-2 responsiveness in lymphocytes from patients with bronchial asthma (line 2) ($p<0.01$), compared with cultures without antigen (line 1). Treatment with Ketotifen reduced the Df-induced IL-2 responsiveness in a dose dependent manner (line 3, 4, and 5). Data from three individuals were pooled. Significant differences compared to values for the corresponding cultures without Ketotifen-treatment (line 2): **$p<0.01$; *** $p<0.001$.

Table 2. Effect of Ketotifen on the antigen-presenting ability of IFN-γ stimulated macrophages.

	Treatment of adherent cells		IL-2 responsiveness (mean ± SD)		
Antigen	IFN-γ	Ketotifen	Case 1	Case 2	Case 3
-	-	-	1.00 ± 0.00	1.01 ± 0.31	1.02 ± 0.01
-	+	-	1.08 ± 0.22	0.96 ± 0.01	0.93 ± 0.00
Df	-	-	1.04 ± 0.01	1.05 ± 0.00	1 01 ± 0.02
Df	+	-	1.38 ± 0.25**	1.46 ± 0.01**	1.59 ± 0.01**
Df	+	+	0.95 ± 0.01	0.96 ± 0.01	0.94 ± 0.03

Adherent cells, cultured for two days in the absence of IFN-γ lost the capability to present Df antigen to autologous nonadherent cells (line 3). Stimulation of adherent cells with 500 IU of IFN-γ restored the antigen presenting ability to responder T calls (line 4). The upregulated responses by IFN-γ were clearly suppressed by treatment with 500 ng/ml of Ketotifen (line 5.) Significant differences compared to values for the corresponding cultures without any treatment: ** $p<0.01$.

Inhibitory effect of Ketotifen on the antigen-presenting capability of IFN-γ-stimulated macrophages

As the previous results indicated that IFN-γ stimulated antigen-presenting ability of macrophages both in human,[25] and murine[26] system and in Df antigen-stimulated T lymphocytes responses in particular,[19] we were interested in the stimulus of IFN-γ that might overcome the inhibitory effect by Ketotifen. The results were clearly shown in Table 2, in which two days' culture without stimuli lost the antigen presenting ability of macrophages. Thus diminished antigen presentation was upregulated by the presence of IFN-γ to induce IL-2 responsiveness efficiently. Addition of Ketotifen, however, blocked the antigen presentation by IFN-γ treated macrophages.

Modulation of MHC class II expression by Ketotifen

To elucidate the mechanism whereby Ketotifen suppresses antigen presentation by macrophages, the expression of HLA class II antigen, which has been shown to be essential in the macrophage-T cell interactions, was investigated. As Table 3 presented Ketotifen profoundly inhibited the expression of HLA-DQ antigen but not of HLA-DR or-DP antigen on $CD14^+$ macrophages, while this anti-allergic agent did not affect the HLA-DR,-DQ and -DP molecules on $CD20^+$ B cells. The suppressive effect on the expression of HLA-DQ antigen on macrophages was still observed when the cells were treated with IFN-γ a well-known stimulus for the expres-

Table 3. Effect of Ketotifen on the expression of HLA class II antigen on B cells and macrophages.

Surface phenotype	Medium	K	IFN-γ	IFN-γ +K	IL-4	IL-4 +K
CD20+DR+/CD20+	79.5 ± 5.8	90.3 ± 0.2	88.7 ± 3.1	80.1 ± 11.7	81.7 ± 2.2	84.9 ± 3.4
CD20+DQ+/CD20+	55.3 ± 7.6	52.1 ± 14.8	47.1 ± 9.6	43.9 ± 10.0	47.6 ± 13.3	61.4 ± 2.0
CD20+DP+/CD20+	71.6 ± 1.8	78.9 ± 1.9	82.4 ± 2.3	66.9 ± 13.2	85.5 ± 13.4	60.8 ± 13.5
CD14+DR+/CD14+	83.7 ± 10.2	83.6 ± 3.5	89.7 ± 0.5	85.8 ± 0.4	90.6 ± 0.5	79.9 ± 6.3
CD14+DQ+/CD14+ -	33.7 ± 5.7	23.1 ± 10.2*	40.4 ± 19.6	21.2 ± 14.6*	19.7 ± 14.6	17.3 ± 13.0
CD14+DP+/CD14+ -	11.3 ± 4.5	5.8 ± 3.8	12.7 ± 6.2	14.1 ± 8.4	40.2 ± 7.9	52.8 ± 13.2

Non-T cells cultured for 3 days with cytokines in the presence or absence of Ketotifen were analyzed with respect to the expression of MHC class II antigen. Mean±SD (% expression) of three independent experiments were shown. K:Ketotifen. Significant differences compared to values for the corresponding cultures without Ketotifen-treatment: *$p < 0.05$.

sion of MHC class II antigens. Another MHC class II-inducing factor , IL-4 has the property to reduce the HLA-DQ antigen expression on macrophages by itself, which masked the suppressive effect by the Ketotifen. A representative data illustrating the two dimensional profiles of MHC class II expression was presented in Fig. 2.

DISCUSSION

Previous data showed that allergen-induced Il-2 responsiveness in peripheral blood lymphocytes, which is specifically induced in allergic individuals was markedly suppressed by the anti-allergic agent, Ketotifen.[18] In that study, Ketotifen was obviously found to impair preferentially antigen-presenting capability when the allergen such as Df and ovalbumin were used. In as much as the mechanism of inibition has been unclear, the present study was undertaken to focus the antigen presenting ability of macrophages stimulated with IFN-γ We also examined the expression of MHC class II antigen, which is closely related to the antigen presentation. The results obtained by the experiments with 2 day-cultured macrophages were consistent with our previous data using the fresh macrophages as antigen-presenting cells (APC). Namely, although the antigen presentation by macrophages was lost by prolonged culture, it was restored by the incubation of macrophages with IFN-γ. However, restoration of antigen-presenting ability by IFN-γ was clearly antagonized with Ketotifen (Table 2), although IFN-γ restored the function which was abrogated by the prolonged culture. Therefore, Ketotifen seemed to provide rather strong suppression toward macrophages, in that it also reduced the antigen presentation by IFN-γ activated macrophages.

The suppressive effect of Ketotifen on antigen presentation was correlated with the reduction of HLA-DQ antigen expression as demonstrated in Table 3. As is clearly shown, Ketotifen substantially decreased the expression of HLA-DQ antigen without any modifications of HLA-DR and-DP antigens. Interestingly, the agent did not change HLA-DR, -DQ or-DP on B cells. This finding suggests the suppressive effect by Ketotifen is selective for macrophages and is concordant both with the widely-accepted notion that macrophages act as major antigen presenting cells except for some restricted cases,[28] and with our own previous data in this response to Df antigen.[10]

In murine system, Th1 clones have been clearly shown to proliferate in response to macrophages but not to B cells whereas Th2 clones preferentially respond to B cells.[29] However human Th1 and Th2 cells could respond

to either macrophages or B cells once they had been activated and fully differentiated into memory cells.[30] Therefore, data implicating the inhibitory actions preferential to macrophages may not predispose to the amplification of Th2 cells, since Th1 and Th2 cells in atopic patients may already be developed *in vivo* after repeated exposure to allergens.

The suppressive effect on HLA-DQ antigen was further substantiated by the co-culture experiments with Ketotifen and MHC class II-inducing cytokines. IFN-γ, a potent factor to enhance antigen-presenting capability by increasing Ia molecules,[25,26,31] has been shown to upregulate the expression of HLA-DR,-DQ and -DP antigens of macrophages but not of B cells.[32] As Table 3 demonstrated, IFN-γ enhanced the expression of HLA-DQ antigen on macrophages, which was substantially suppressed by the simultaneous application of Ketotifen.

Similar to IFN-γ, MHC class II-inducing properties have been implicated in one of the pleiotropic activities of IL-4.[20,27] Using this function, effect of Ketotifen on MHC class II expression in IL-4 treated cells was investigated. IL-4 apparently acted on macrophages to enhance HLA-DR and -DP antigen expression and to conversely reduce the expression of HLA-DQ antigen (Table 3). With respect to the expression of HLA-DQ antigen, te Velde reported that IL-4 increased both HLA-DR and -DQ on macrophages, counteracting the data of Gerrard *et al.* and ours wherein IL-4 expressed upregulation of HLA-DR and -DP and downregulation of HLA-DQ. This discrepancy may result from the different culture conditions: in the former report, the cells were cultured in a modified Iscove's medium with human serum albumin and autologous serum, whereas RPMI 1640 medium with 10 % FCS was used in the latter studies. In this study, IL-4 sharply increased the expression of HLA-DR and-DP antigen on macrophages, which was not suppressed by Ketotifen. The effect of Ketotifen on HLA-DQ expression by IL-4 treated macrophages was less evident since IL-4 itself decreased HLA-DQ expression on macrophages.

Collectively, HLA-DQ antigen on macrophages seemed to be selectively suppressed by Ketotifen, which could account for the inhibition of antigen-presenting capability of macrophages, in the light of HLA-DQ as restriction molecules in the induction of IL-2 responsiveness as reported previously.[10] In addition to the effect on the nonadherent cells, Ketotifen inhibits the nonadherent cells less effectively. This direct action on nonadherent cells may be mediated by co-stimulation pathway via CD28 molecules.[33] Also Ketotifen may modify protein tyrosin kinases triggered by T cell receptor-stimulation,[34,35] which needs further investigation. Although Ketotifen has been used as a therapeutic measure for the patients with bronchial asthma since 1982,[16,17] no severe side effects have been reported. This study may disclose the mechanisms whereby this drug effects on patients with bronchial asthma.

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