

The Effect of Ketotifen on the *In Vitro* Immune Function of Asthmatic Children*

Kue-Hsiung Hsieh, M.D.



Ketotifen, an oral anti-asthma drug, is a benzocycloheptathiophene derivative that was synthesized by Sandoz Laboratories in January 1970. The first phase of clinical trials began in August 1971 and the drug appeared on the market in Switzerland at the beginning of 1978. Several papers have been published which demonstrate elegantly the pharmacological actions of ketotifen that account for its clinical effectiveness. Those actions include: (1) inhibiting the antigen-induced release of histamine and SRS-A from human basophils and neutrophils,^{1,4} (2) interfering with the action of SRS-A,^{4,5} (3) enhancing the tissue response to isoprenaline,⁴⁻⁶ and (4) inhibiting cellular uptake of Ca^{++} .⁷

In spite of extensive studies on the effects of ketotifen on mediator release, to the author's knowledge there is still no report dealing with the influence of ketotifen on the lymphocyte function although the antigen-induced mediator release involves the production of an IgE antibody which reflects the net result of the reaction between T and B lymphocytes after antigen stimulation.

This trial was conducted to study the influence of ketotifen on *in vitro* lymphocyte functions with particular emphasis on the changes of lymphocyte subpopulations and *in vitro* IgE biosynthesis.

SUMMARY The *in vitro* effect of ketotifen on the immune function was studied in 35 newly diagnosed asthmatic children. At a concentration of 100 μ g/ml, ketotifen was able to decrease the total number of T cells and suppress their blastogenic response to T-cell mitogens (PHA and Con A), but it did not affect the number of B cells, their blastogenic response to B-cell mitogen (PWM), *in vitro* IgE biosynthesis and expressions of complement and Fc receptors on polymorphonuclear leukocytes.

ASIAN PACIFIC J ALLERG IMMUN 1983;1:85-88.

MATERIALS AND METHODS

The study population consisted of 35 newly diagnosed asthmatic children who participated in a three-month, open clinical trial of ketotifen. The patients were 24 boys and 11 girls, ranging in age from six to fifteen years. None had received steroids in the two weeks prior to the trial and bronchodilators were withheld for at least three days prior to the collection of blood samples.

Preparations of mononuclear cells, lymphocytes and polymorphonuclear leukocytes: Peripheral blood mononuclear cells were isolated by the method of Boyum.⁸ After washing three times with Hanks' balanced salt solution (HBSS, GIBCO), the mononuclear cells were suspended at a concentration of 2×10^6 cells/ml in a complete culture medium (RPMI-1640 containing 10% heat-inactivated foetal calf serum, 100 units/ml of penicillin, 100 μ g/ml of streptomycin and

2mM/ml of L-glutamine, GIBCO). A 10-ml cell suspension was placed into a plastic petri dish (100 x 15 mm, Falcon Plastics) and incubated for one hour in a humidified incubator with a 5% CO_2 atmosphere at 37°C. At the end of incubation, the nonadherent cells were decanted, washed three times with HBSS and resuspended in HBSS at a concentration of 2×10^6 cells/ml. The final cell suspension contained < 5% esterase-positive monocytes and was referred to as containing pure lymphocytes. After removal of the mononuclear cells at the interface layer, the packed cells at the bottom of the centrifuge tube were mixed with an equal volume of HBSS and 6% dextran in normal saline (0.2 ml/ml of cell suspension) was added. After standing at room temperature for one hour, the leukocyte-rich supernatant was col-

*From the Department of Paediatrics, National Taiwan University Hospital, Taipei, Taiwan 100, Republic of China.

lected and the contaminated RBC were lysed with cold distilled water. The polymorphonuclear leukocytes (PMN) were washed three times with HBSS and then adjusted to a concentration of 2×10^6 cells/ml. The purity of PMN in the final cell suspension was more than 97 per cent and viability was over 98 per cent using the trypan blue dye exclusion test.

Enumeration of lymphocyte subpopulations: the total number of T cells (E rosettes), active T cells and B cells (EAC rosettes) was determined using the method of Kerman *et al*⁹ with slight modification.¹⁰ T-cell subsets defined by monoclonal antibodies, anti-OKT3 for total T cells, anti-OKT4 for helper T cells and anti-OKT8 for suppressor T cells, were determined by the method described previously.¹¹ For studying the effect of ketotifen on the distribution of lymphocyte subpopulations, lymphocytes were incubated in HBSS containing 100 μ g/ml of ketotifen (pure powder from Sandoz Laboratories) for one hour in a 37°C water bath. After washing, the lymphocyte markers were determined as previously described. There was no difference in the viability between treated and untreated cells (97% vs 96%, mean).

Enumeration of Fc and complement receptors on PMN: The Fc and complement receptors on the PMN were determined according to the method described previously.¹² Briefly, anti-SRBC antibodies were raised in rabbits by injecting weekly 0.5 millilitre of 50% SRBC suspension in HBSS until a reasonable complement fixation titre was reached. The unfractionated antibody was diluted optimally and was then used to prepare erythrocyte-antibody (EA) and erythrocyte-antibody-complement (EAC) complexes as described by Kerman *et al*⁹ and the present author.¹² A PMN either binding or phagocytosing three or more EA or EAC was considered to be a cell with an Fc or complement receptor. To study the effect of ketotifen on the ex-

pressions of Fc and complement receptors on PMN, the PMN were incubated in HBSS containing 100 μ g/ml of ketotifen in a 37°C water bath for one hour; they were washed and their surface receptors were determined as described previously. The rates of viability for treated and untreated cells were 95 per cent and 96 per cent, respectively (mean).

Performance of lymphoproliferative response to phytomitogens: Blastogenic responses to concanavalin A (Con A), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) were measured using the Bradley's method.¹³ One tenth millilitre of mononuclear cells in a concentration of 1.5×10^6 cells/ml was added simultaneously to 0.1 ml solutions of RPMI-diluted PHA-M (final dilution 50x), Con A (final concentration 20 μ g/ml) or PWM (final dilution 50x) to round-bottom multiple-well microtitre plates (Flow Laboratories). Ketotifen was added to the test wells to attain a final concentration of 100 μ g/ml. The cultures were incubated for three days in a humidified incubator with a 5% CO₂ atmosphere at 37°C. At the end of the incubation, 20 μ l of RPMI-1640 containing 1 μ Ci of ³H-thymidine were added. The cells were harvested the next morning and their radioactivity was counted. Both the controls and stimulated cultures were set up in triplicate. The stimulation index was calculated by dividing the cpm of the stimulated cultures by those of their unstimulated counterparts. The viability rates for treated and untreated cells after three-day's incubation were 79 per cent and 75 per cent, respectively.

In vitro IgE biosynthesis was done using the method reported previously.¹⁴ Two millilitres of 4×10^6 cells/ml of mononuclear cells in a complete culture medium were cultured in 160 x 10 mm plastic culture tubes (Falcon Plastics) in the presence of 10 μ l/ml of PWM and incubated for one week in a humidified incubator with a 5%

CO₂ atmosphere at 37°C. Ketotifen was added to the tubes to attain a final concentration of 100 μ g/ml. At the end of incubation, the culture tubes were vortexed and centrifuged at 1,200x g for 15 minutes and the supernatants were aspirated and stored at -70°C until the IgE concentrations were determined. There was no difference in the viability rates of treated and untreated cells after seven day's culture (69% vs 71%).

Determination of IgE concentrations: IgE concentrations in the culture media were determined in duplicate by using Phadebas IgE PRIST kits (Pharmacia, Sweden). For convenience of calculation, the IgE concentrations were changed from IU/ml to pg/ml by a multiplying factor of 2420 (1 IU=2,420 pg).

Statistics: The student's t test was used for statistical analysis throughout the study.

RESULTS

Changes in the distribution of lymphocyte subpopulations after *in vitro* incubation with ketotifen are shown in Table 1. There was no difference in the mean percentage of active T, B, OKT4 and OKT8 cells either before or after incubation with ketotifen, but the mean percentage of total T cells detected either by the E-rosette technique or monoclonal antibody (OKT3 cells) after incubation was much lower than the percentage of T cells before incubation ($p < 0.001$).

Figure 1 shows the dose-dependent suppressive effect of ketotifen on the lymphoproliferative response of lymphocytes to PHA. At a concentration of 1 μ g/ml, ketotifen was able to suppress only 7-30 per cent of the response, but when the concentration was increased to 10 μ g/ml, the suppression reached a magnitude greater than 90 per cent.

The effect of ketotifen on the blastogenic responses to Con A, PHA and PWM is shown in Table 2. It is clear that ketotifen only suppressed lymphoproliferative re-

sponses to T-cell mitogens, e.g. Con A and PHA, but not to PWM which is a B-cell mitogen. The mean baseline cpm for the cultures in the presence of ketotifen was similar to that for the cultures without ketotifen, indicating that the drug had no effect on the cell viability during the three-day culture.

Table 3 shows that the ketotifen had no influence on Fc and com-

plement receptor expressions on PMN.

Figure 2 demonstrates that ketotifen did not affect *in vitro* IgE biosynthesis. The amount of IgE produced by B cells in the presence of ketotifen was not different from that produced by B cells in the absence of ketotifen (1453±867 pg/ml for the former and 1430±849 pg/ml for the latter).

DISCUSSION

The inhibition of antigen-induced mediator release has been established as being one of the mechanisms accounting for the effectiveness of ketotifen in the treatment of asthma.¹⁴ Antigen-induced mediator release from human leukocytes involves the binding of an antigen with a cell-bound specific IgE antibody. Also, because IgE production is a highly T-cell dependent process,¹⁵ it would be interesting to know whether ketotifen exerts its anti-asthmatic effect through its influence on the T-cell functions and IgE production in addition to the pharmacological actions mentioned previously.¹⁻⁷

Under experimental conditions involving a concentration of ketotifen (100 µg/ml incubated for one hour), the drug was found to be capable of decreasing the total T-cell number detected using either the E-rosette technique or the monoclonal antibody like anti-LKT3. Moreover, ketotifen also was found to suppress lymphoproliferative responses to T-cell mitogens, such as Con A and PHA, but not to the B-cell mitogen (PWM). Thus, it was determined that ketotifen is able to suppress T-cell functions in terms of decreasing the total T-cell number and the level of blastogenesis to mitogens.

Intracellular nucleotides have been reported to regulate the E-rosette-forming capability of T cells. While an increase of cAMP inhibits the formation of E-rosettes,¹⁶ an increase of cGMP enhances such a biological function.¹⁷ Martin *et al*¹ reported that ketotifen was able to inhibit the activity of cAMP phosphodiesterase which would indirectly increase the intracellular concentration of cAMP. Such an effect may be used to explain the suppressive activity of ketotifen on the T-cell function.

One may argue that, as the average peak serum level of ketotifen is only around 1 µg/ml,¹⁸ after taking

Table 1. *In vitro* effect of ketotifen on the distribution (%) of lymphocyte subpopulations

| Ketotifen | Active T | Total T | B (EAC rosettes) | OKT3 | OKT4 | OKT8 |
|-----------|-----------|----------|------------------|----------|-----------|----------|
| With | 26.1±8.2* | 63.5±8.0 | 19.9±6.3 | 68.4±7.2 | 41.7±11.2 | 29.0±9.5 |
| Without | 24.8±7.3 | 56.0±9.4 | 21.2±5.7 | 59.9±7.9 | 38.2±11.1 | 27.5±7.3 |
| P | > 0.05 | < 0.001 | > 0.05 | < 0.001 | > 0.1 | > 0.3 |

*Mean ± SD (n=35)

Lymphocytes were cultured in the presence (100 µg/ml) or absence of ketotifen for one hour before lymphocyte subpopulations were determined.

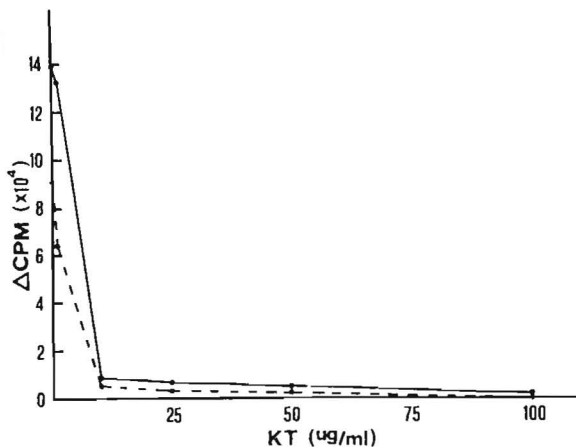


Fig. 1 Regarding the dose-dependent suppressive effect of ketotifen (KT) on the lymphoproliferative response to phytohaemagglutinin, each curve represents one experiment. The concentrations of KT ranged from 0 to 100 µg/ml.

Table 2. The effect of ketotifen on lymphoproliferative responses to Con A, PHA and PWM[@]

| Mitogen | - Ketotifen | + Ketotifen | P |
|---------|-------------|-------------|--------|
| No | 1493±252* | 1632±409 | |
| Con A | 42.1± 4.5# | 13.3± 1.3 | < 0.01 |
| PHA | 215.4±17.8 | 75.6±10.9 | < 0.01 |
| PWM | 31.8± 3.2 | 26.8± 4.2 | > 0.05 |

[@]n=35, *Counts per minute (Mean ± SEM), #Stimulation index (Mean ± SEM) Ketotifen was added to the test cultures as a final concentration of 100 µg/ml.

Table 3. Effect of ketotifen on the expressions of complement and Fc receptors on PMN*

| Ketotifen | % of PMN with complement receptor | % of PMN with Fc receptor |
|-----------|-----------------------------------|---------------------------|
| With | 42.5±20.6 [#] | 40.1±16.2 |
| Without | 44.6±17.4 | 40.8±13.8 |
| P | > 0.05 | > 0.05 |

*PMN: Polymorphonuclear leukocytes

[#]Mean ± SD (n=35)

PMN were cultured in the presence (100 µg/ml) or absence of ketotifen for one hour before surface receptors were determined.

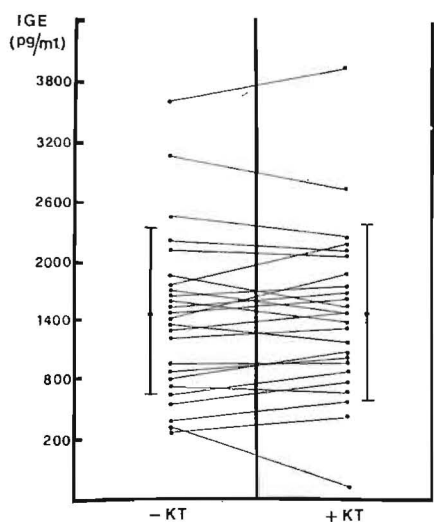


Fig. 2 The effect of ketotifen on the *in vitro* IgE biosynthesis: 4×10^6 mononuclear cells/ml were cultured in the presence (+KT, 100 µg/ml) and absence (-KT) of ketotifen for one week, after which time the concentration of IgE in the supernatant was determined.

a single recommended dose (i.e. 1 mg), the *in vitro* effect of ketotifen on the T-cell functions observed in this study, which involved the use of concentrations as high as 100 µg/ml, may not occur *in vivo*. As a matter of fact, Figure 1 shows that ketotifen at a concentration of 1 µg/ml was able to suppress the level of blastogenesis by only 7-30 per cent, whereas a magnitude of suppression greater than 90 per cent could be achieved by using a concentration of 10 µg/ml. Because a concentration of 50-100 µg/ml of ketotifen was required to obtain 50 per cent inhibition of antigen-induced release of both histamine and SRS-A,²⁻⁴ 100 µg/ml was used in this study for convenience of comparison.

Ketotifen had no effect on *in vitro* IgE biosynthesis. This result can be explained by the findings

that ketotifen did not decrease the number of B cells or the level of lymphoproliferative response to B-cell mitogen on the one hand nor did it tilt the balance between the number of helper (OKT4) and suppressor (OKT8) T cells on the other hand.

The effect of long-term administration of ketotifen on the immune response of asthmatic children is currently under investigation.

ACKNOWLEDGMENT

The author expresses his great gratitude to Ms. Chuen-Horng Chen, Li-Hwa Lee and Chiou-Chyn Lin for their excellent technical help.

REFERENCES

1. Martin U, Romer D. The pharmacological properties of a new, orally active anti-an-

aphylactic compound: ketotifen, a benzocycloheptathiophene. *Drug Res* 1978; 28: 770-82.

2. Tomioka H, Yoshida S, Tanaka M, Kumagai A. Inhibition of chemical mediator release from human leukocytes by a new anti-asthma drug, HC20-511 (ketotifen). *Monogr Allergy* 1979; 14:313-7.
3. Ross WJ, Harrison RG, Jolley MRJ, et al. Anti-anaphylactic agents: 1, 2 (acylamino) oxazoles. *J Med Chem* 1979; 22:412-27.
4. Ney UM, Bretz U, Martin U. Pharmacology of ketotifen. *Res Clin Forum* 1982; 4:9-16.
5. Bretz U, Martin U, Ney U, Gradwohl P. Pharmacology of ketotifen. Symposium: A new approach to the treatment of bronchial asthma. Proceedings and Discussions, Cairo, April 10th, Wander Ltd., Berne, Switzerland, 1981:65-70.
6. Greenwood C. The pharmacology of ketotifen. *Chest* 1982; 82:45s-8s.
7. Lowe DA, Richardson BP. The effects of cyproheptadine, ketotifen and sodium nitroprusside on mechanical activity and calcium uptake in guinea-pig *Taenia coli* in vitro. *Prog Resp Res* 1980; 14:134-40.
8. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; 21(suppl 97):77-89.
9. Kerman R, Smith R, Ezdinli E, Stefani S. Unification and technical aspects of total T, active T and B lymphocyte rosette assay. *Immunol Communication* 1976; 5: 685-94.
10. Hsieh KH. Different responses of T-cell subpopulations to allergen challenge in asthmatic children and normals. *Ann Allergy* 1980; 45:177-80.
11. Hsieh KH. Study of T-cell subpopulations defined by monoclonal antibodies in asthmatic children with or without atopic eczema and normals. *Ann Allergy* 1982; 48:345-8.
12. Hsieh KH. Decreased Fc and complement receptors on the phagocytes of asthmatic children. *Ann Allergy* 1980; 44:313-6.
13. Bradley CM. Cell proliferation, In: Mishell MM, Shiigi SM, eds, *Selected Methods in Cellular Immunology*. San Francisco: W.H. Freedman and Co, 1980:158-61.
14. Hsieh KH. *In vitro* IgE biosynthesis in asthmatic children. *Ann Allergy* 1979; 42: 187-91.
15. Tada T. Regulation of reaginic antibody formation in animals. *Prog Allergy* 1975; 19:122-94.
16. Galant SP, Rome RA. Beta-adrenergic inhibition of human T-lymphocyte rosettes. *J Immunol* 1975; 114:512-3.
17. Galant SP, Lundak RL, Eaton L. Enhancement of early human E-rosette formation by cholinergic stimuli. *J Immunol* 1976; 117:48-51.
18. Kennedy GR. Metabolism and pharmacokinetics of ketotifen in children. *Res Clin Forums* 1982; 4:17-20.