

Pilot Study of Oral Tolerance to Keyhole Limpet Hemocyanin in Humans

Down-regulation of KLH-reactive Precursor-cell Frequency

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Oral tolerance has long been recognized as a method of evoking antigen-specific suppression of immune responses and has recently been used to suppress autoimmune diseases in both animals and humans (reviewed in ref. 1). To determine whether the oral administration of a protein antigen could down-regulate immune responses in humans, we conducted a two-part pilot study using KLH as an antigen. In part 1, two healthy volunteers, who had not been immunized with KLH, were fed 30 mg of KLH every other day for a minimum of six weeks, and KLH-reactive precursor cell frequency was measured. In part 2, six healthy volunteers were fed KLH or OVA for four weeks and immunized by the intradermal injection of KLH. We observed substantial numbers of KLH-reactive cells in the peripheral blood of unimmunized subjects (natural immunity) and found a decrease in the cell frequency with feeding. In addition, oral KLH decreased the frequency in KLH-reactive cells in immunized subjects fed KLH versus those fed ovalbumin (OVA).

METHODS

Preparation of Antigens

KLH and OVA (Grade V) were purchased from Calbiochem (La Jolla, CA) and Sigma Chemical (St. Louis, MO), respectively. Both antigens were dialyzed against phosphate-buffered saline, passed twice through 0.45 μ m membrane filter systems, and lyophilized in 50-mL polystyrene culture tubes (approximately 30 mg/tube).

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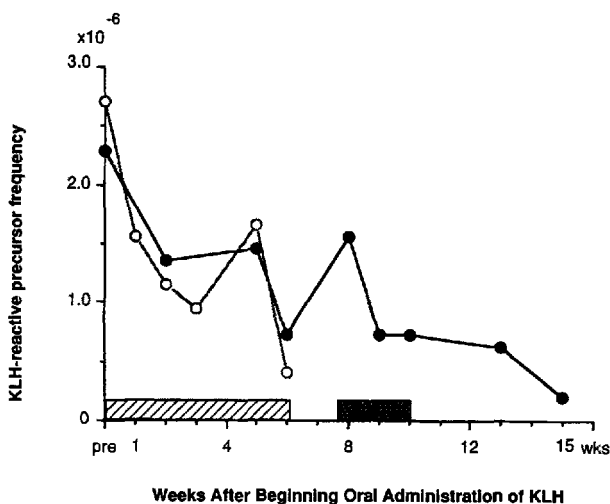


FIGURE 1. Time course of KLH-reactive precursor cell frequency. Thirty milligrams of KLH was administered orally every other day to each subject. Subject A (closed circles) took KLH for six weeks (the hatched bar) and then ceased for 10 days. Oral KLH was readministered for 18 consecutive days between the middle of the eighth week and the end of the tenth week of the study (the coarse hatched bar). Subject B (open circles) took KLH every other day for six weeks (the hatched bar).

Subjects and Administration Schedule

For part 1, two healthy male volunteers (ages 37 and 46) were fed 30 mg of KLH dissolved in water plus an equivalent amount of grapefruit juice every other day. Subject A followed this schedule for six weeks. After 10 days without receiving the antigen, that is, missing five doses, he was then given KLH for 18 consecutive days, after which he received no further LKH (10-week-course). Subject B received KLH on alternate days for the same six weeks. Both subjects were examined serially at one- or two-week intervals. For part 2 of the study, six other healthy volunteers (3 men and 3 women), whose ages ranged from 24 to 41 years, were paired into three study groups; each pair of subjects was examined concurrently. One subject in each group received 30 mg of KLH orally every other day for four weeks, and the other received 30 mg of OVA orally. At the beginning of the fifth week of the study, subjects in each group were immunized with intradermal injections of either 1 or 5 mg of KLH.

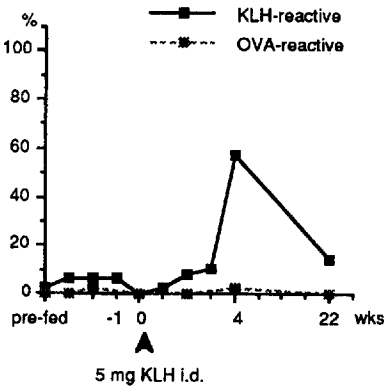
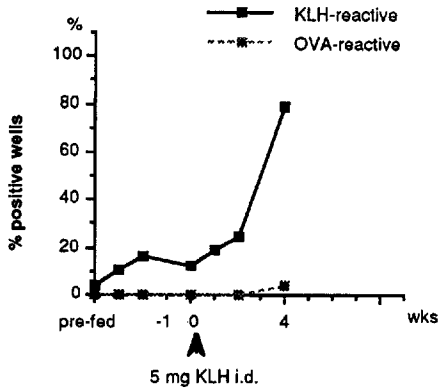
Preparation of Antigen-presenting Cells

Peripheral blood mononuclear cells, separated by Ficoll-Paque density gradient centrifugation before the administration of KLH was begun, served as antigen-presenting cells (APC) in all experiments. The APC in aliquots of 10^7 cells per tube were kept frozen at -70°C until use.

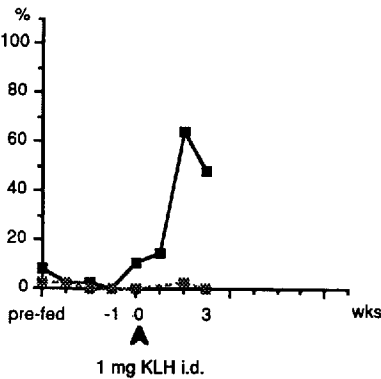
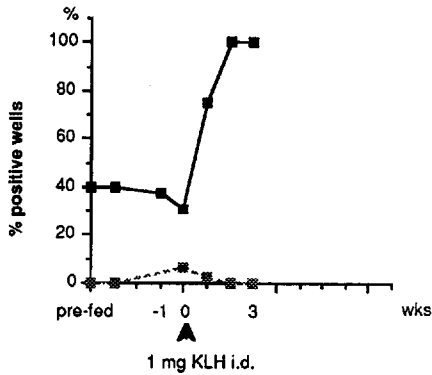
OVA-fed subjects

KLH-fed subjects

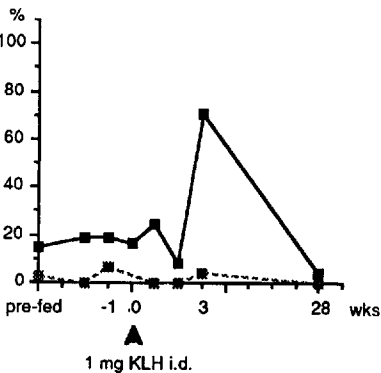
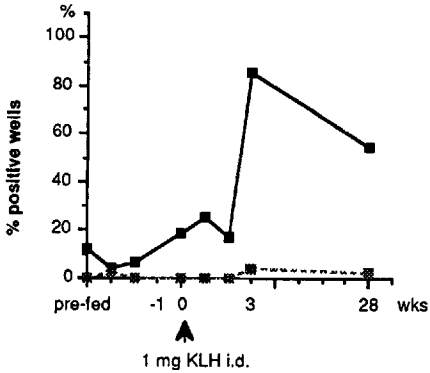
Experiment 1



Experiment 2



Experiment 3



Weeks After Immunization

Weeks After Immunization

Immunological Parameters

KLH- and OVA-reactive precursor cell frequencies were measured according to a previously reported method.² Preliminary experiments showed that 2.5 and 5.0 $\mu\text{g/mL}$ of KLH and between 10 and 50 $\mu\text{g/mL}$ of OVA had the highest efficiency in generating antigen-reactive cell lines. Culture media were supplemented with 10% pooled human AB sera. The cell culture was maintained for 13 days with 5% IL-2 (ABI, Columbia, MD) and 2 U/mL of recombinant IL-4 (Genzyme, Boston, MA), which were added every 3 to 4 days. An aliquot of the cell line from each well was then checked for reactivity to the relevant antigen, that is, either KLH or OVA, by plating, in duplicate (1 to 2×10^4 line cells together with 1×10^4 APC). The APC had been preincubated with or without 100 $\mu\text{g/mL}$ of the antigen for 1 hour and then irradiated (4500 rad) and washed to remove the antigen. The cells were incubated for three days, and [^3H]thymidine (0.63 $\mu\text{Ci/well}$) was added 18 hours prior to cell harvesting. Cell lines that had a stimulation index (mean Δ cpm of wells containing antigen-pulsed APC divided by mean Δ cpm of wells containing antigen-nonpulsed APC) exceeding 3.0 and whose Δ cpm in positive wells was more than 500 were considered as being reactive to the antigen. Previous experiments performed by this method for generating cell lines have shown that cells in a positive well were derived from a single antigen-reactive cell.² Thus, either of the higher percentages of positive wells obtained in a total of 48 wells set up for each concentration of KLH (2.5 and 5.0 $\mu\text{g/mL}$) and OVA (10 and 50 $\mu\text{g/mL}$) was used as representative KLH- and OVA-reactive precursor cell frequencies at the time of blood sampling. For example, 10% positive wells indicated that the precursor cell frequency was $10/100/[2 \times 10^5] = 0.5 \times 10^{-6}$. Preliminary studies showed a difference between the lots of pooled human AB sera in terms of efficiency in generating KLH-reactive cell lines. The lot that gave less than 20% positive wells (cell frequency, 1.0×10^{-6}), rather than one that gave high percentages used in part 1 of the study, was selected for part 2 of the study.

Statistical Analysis

To analyze the decrease in KLH-reactive precursor cell frequency for the two subjects in part 1 of the study, the two-way analysis of variance (two-way ANOVA) was used. The two-way ANOVA with repeated measures (repeated-measure ANOVA) was performed for analyzing the differences of immunological parameters in the KLH-fed and OVA-fed subjects in part 2 of the study.

RESULTS

Part 1

As shown in FIGURE 1, in subject A (closed circles), KLH-reactive precursor cell frequency was 2.3×10^{-6} before the oral administration of KLH; the cell frequency

FIGURE 2. Time course of the percentage of positive wells for KLH- and OVA-reactive cell lines. The percentages of positive wells for KLH and OVA are shown in solid and broken lines, respectively. KLH or OVA was administered orally for four weeks prior to the intradermal immunization with KLH. The immunization is denoted by large arrowheads. The results obtained in the subjects who received oral KLH are shown on the right side of the FIGURE, and those from the oral OVA subjects are shown on the left side.

decreased to about half the original level in two weeks and decreased further to one third, until the end of the first part of the feeding schedule (the hatched bar in FIG. 1). The frequency of KLH-reactive cells increased when oral KLH was discontinued and then decreased once again to one tenth of the original level after 18 days' consecutive intake of KLH (the coarse hatched bar). At four months and nine months after the discontinuation of KLH, KLH-reactive precursor cell frequency was found to be approximately 1.0×10^{-6} , less than one half of the original level (data not shown). Subject B (open circles) showed a rapid decrease in the cell frequency: 2.7×10^{-6} before starting KLH, and less than 0.5×10^{-6} after six weeks' oral administration of KLH (the hatched bar). The decrease in KLH-reactive precursor cell frequency during the first six weeks of KLH feeding in both subjects was statistically significant ($p = 0.0157$), when analyzed by the two-way ANOVA. The decrease in precursor cell frequency was not due to the impaired function of frozen APC, because there was no difference in the percentage of positive wells in the assay plates containing frozen APC or fresh APC.

Part 2

The intradermal injection of KLH, at concentrations of both 1 and 5 mg, elicited strong cellular immune responses to the antigen in all six subjects, as shown in FIGURE 2. KLH-reactive precursor cell frequency (solid lines) reached a peak two to four weeks after immunization, whereas OVA-reactive precursor-cell frequency (broken lines) remained at low levels. Throughout the postimmunization period, all three subjects who received KLH orally had lower numbers of KLH-reactive cells than the three paired subjects who received OVA orally. This down-regulation observed in the three KLH-fed subjects showed a positive trend but did not achieve statistical significance ($p = 0.071$) when the results prior to immunization (zero week) were compared with those obtained at the peak of KLH-reactive cell frequency by means of a repeated-measure ANOVA (FIG. 3).

DISCUSSION

In this pilot study, we found that oral administration of a protein antigen can down-regulate cell-mediated immunity to the fed antigen in humans as measured by cell frequency. The substantial numbers of KLH-reactive cells in the peripheral blood of the two nonimmunized subjects in part 1 of the study decreased with the oral administration of KLH, indicating that oral KLH suppressed preexisting or natural cellular immunity to this antigen. The presence of KLH-reactive cells in the subjects who had not been immunized with KLH is consistent with the reported detection of low levels of anti-KLH antibodies in the sera of nonimmunized human subjects.³ Part 2 of the study showed that prior four-week oral administration of KLH showed a trend in decreasing the number of antigen-reactive cells triggered after intradermal immunization with KLH. Of note is that all three subjects fed OVA experienced moderate pruritus at the site of the KLH injection, whereas none of the three subjects who received KLH orally did.

Other investigators have studied immune responses to KLH in human subjects immunized with KLH following oral⁴ or nasal⁵ administration of KLH. In fed subjects, a decrease in T-cell but not B-cell responses was observed following immunization; 50 mg of KLH was fed daily for a total of 10 days. In nasally treated subjects, 100 mg KLH was administered weekly over 3 to 4 weeks. Nasal administration, alone, primed for antibody responses but suppressed both antibody and DTH responses following subcutaneous immunization with KLH. In our studies, we immunized with a 10- to 50-times higher amount

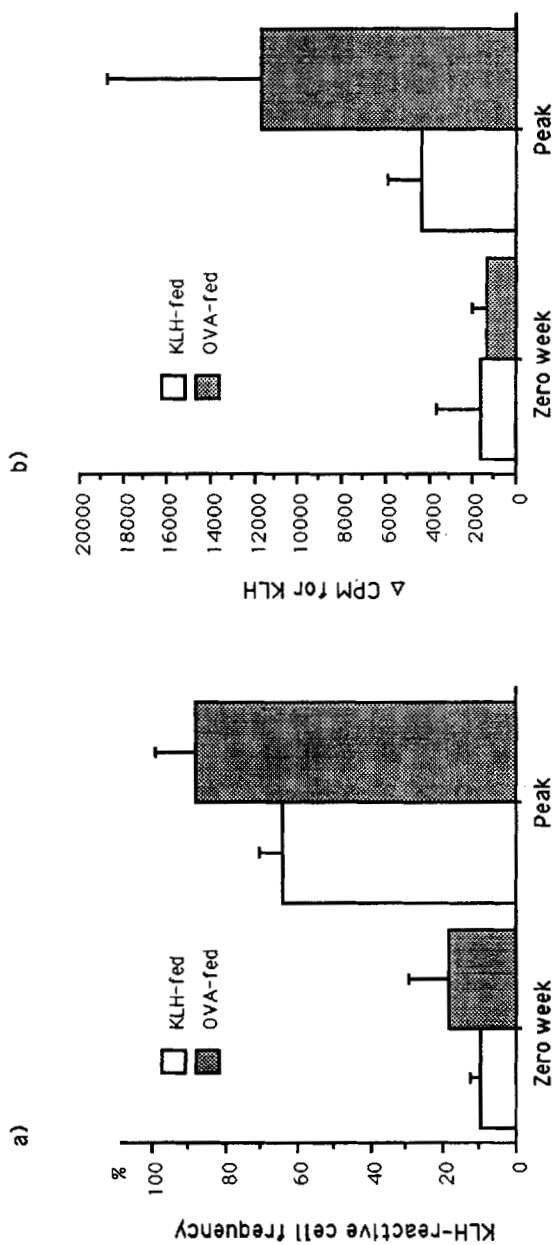


FIGURE 3. Comparison of the results of (a) KLH-reactive precursor cell frequency and of (b) Δ cpm for KLH between the week of immunization (zero week) and the week of the peak results (peak).

(1 mg or 5 mg) of KLH than the aforementioned studies. This most probably explains why we observed only a trend in the suppression of cellular immune responses as measured by reactive cell lines following immunization.

Oral tolerance is now recognized to involve multiple mechanisms, including active suppression, anergy, and deletion, depending on the dose fed.⁶ Subsequent to this pilot study with KLH, we have observed the induction of antigen-specific TGF- β -secreting cells following oral administration of myelin antigens.⁷ In terms of KLH, our results in this pilot study and the results of others demonstrate that mucosal administration of KLH has a clear effect in reducing cell mediated-type responses to KLH. Given our better understanding of the dose-dependent mechanisms of oral tolerance and the increase in human studies involving oral administration of autoantigens for the treatment of autoimmune diseases, further studies will help delineate the immune responses to oral antigens in humans.

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