

Lamina Propria Lymphocytes Produce Interferon- γ and Develop Suppressor Activity in Response to Lactoglobulin

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This study examines the *in vitro* response of human lamina propria lymphocytes (LPLs) to food antigens. LPLs were obtained from jejunum of healthy individuals undergoing gastric bypass operations for morbid obesity. Proliferation was assayed by [^{3} H]thymidine incorporation and cytokine production by ELISA. LPLs proliferated in response to keyhole limpet hemocyanin, ovalbumin, lactoglobulin, and phytohemagglutinin (PHA), but not to yeast. The responses to lactoglobulin and PHA were inhibited by the CTLA4/Fc chimera and by MAbs against CD2, CD58, CD80, and CD86, indicating stimulation of CD28⁺ LPLs with antigen-presenting cells through activation of the CD2 pathway. Besides producing IL-2, IL-10, and TNF- α , LPLs synthesized large amounts of IFN- γ (100 ng/ml) with lactoglobulin, a process dependent upon CD80/CD86, CD40/CD40L, and IL-12. After a three-day culture with lactoglobulin, ovalbumin, or PHA, LPLs developed suppressor activity that reduced proliferation of naive LPLs to these same stimuli. In summary, LPLs first respond to lactoglobulin by proliferation and IFN- γ production, then by development of antigen nonspecific suppression.

KEY WORDS: lamina propria; lymphocytes; interferon- γ ; lactoglobulin.

The intestine mounts immune responses against pathogens while promoting antigen-specific tolerance toward dietary proteins. Defensive responses occur when bacteria invade epithelial cells, causing the latter to secrete chemokines and inflammatory cytokines (1). Alternatively, pathogens, which pass through the epithelium, may be taken up by antigen-presenting cells (APCs), initiating pathogen-specific immune responses (2). Oral tolerance, in contrast, develops in response to food antigens and is mediated by suppression, anergy, and apoptosis (3–6). This study evaluates for the first time the responses of human intestinal T lymphocytes to food constituents.

There are multiple barriers in the gastrointestinal tract that block contact of pathogens or undigested food with the immune system, such as the glycocalyx and IgA secretion. M cells take up and transport particulate antigens from the mucosal surface to the Peyer's patches, where dendritic cells process the antigens and present them to T cells. Suppressor T cells, often CD4⁺, originating in the Peyer's patches, migrate to the lamina propria. After repeated contact with the initiating antigen, they produce TGF- β that inhibits local T-cell responses to specific and nonspecific antigens (bystander suppression) (7). These CD4⁺ T cells can transfer oral tolerance (8).

The role of IFN- γ in oral tolerance is controversial: IFN- γ knockout mice do not develop oral tolerance, yet addition of IFN- γ reduces its generation in normal mice (9, 10). Feeding the novel antigen, keyhole limpet hemocyanin (KLH), to humans reduced both

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KLH-specific T-cell proliferation *in vitro* and delayed skin test responses *in vivo*. Furthermore, this feeding augmented serum and mucosal antibody levels, showing that T-cell tolerance occurred with B-cell priming (11).

Food allergies may result from a breakdown in oral tolerance. The major food allergens are water-soluble glycoproteins that are stable to heat, acid, and proteases. The most common is cow's milk, which can be divided into casein and whey (the latter consisting mainly of lactoglobulin, LG) (12). Other allergens include peanuts, soybeans, and fish, as well as chicken egg white, composed of ovalbumin (OVA), ovomucoid, and ovotransferrin. Pasteurization does not denature all proteins, but rather may increase the immunogenicity of some, such as LG. In contrast, several of the other proteins, such as bovine serum albumin, are denatured by extensive heating, in the treatment fetal bovine serum usually receives prior to its use as a culture medium supplement (13).

In the present study, intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated from the proximal jejunum of healthy individuals lacking food allergies who underwent gastric bypass operations for morbid obesity. These lymphocytes were tested for proliferation, cytokine production, and suppressor activity in response to purified food antigens. The results reflect normal occurrences in patients who should have already developed oral tolerance to these antigens.

MATERIALS AND METHODS

Isolation of IELs, LPLs, PBMCs, and T-Cell Subsets. Human jejunal mucosa was treated for 30 min with 1 mM dithiothreitol, then with 0.75 mM ethylenediamine tetraacetic acid (EDTA; Sigma Chemical Company, St Louis, Missouri, USA) in calcium- and magnesium-free Hanks' buffered salt solution (Biowhittaker, Biggs Ford, Maryland, USA) for three 45-min incubations at 37°C. IELs were purified by discontinuous Percoll (Pharmacia, Piscataway, New Jersey, USA) density gradient centrifugation, as described previously (14). The remaining tissue received three more 45-min EDTA treatments to fully strip the epithelium and IELs. It was then digested with collagenase (20 units/ml) for 3 hr at 37°C and pressed through a wire mesh sieve to disperse LPLs, which were then purified by a Percoll gradient. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density gradient

centrifugation. Purified IELs were $94 \pm 5\%$ CD2⁺, $89 \pm 2\%$ CD8⁺, $1 \pm 1\%$ CD14⁺, and $0 \pm 0\%$ CD20⁺. LPLs were $65 \pm 10\%$ CD4⁺, $30 \pm 11\%$ CD8⁺, $1 \pm 1\%$ CD14⁺, and $1 \pm 0\%$ CD20⁺. PBMCs were $82 \pm 4\%$ CD2⁺, $10 \pm 2\%$ CD14⁺, and $8 \pm 1\%$ CD20⁺.

The T lymphocytes were depleted of monocytes and B cells by immunomagnetic sorting using monoclonal antibodies (MAbs) against CD14 and CD20 followed by goat anti-mouse IgG-conjugated magnetic particles (Biomag Polysciences, Warrington, Pennsylvania, USA) resulting in <1% of the lymphocytes positive for the depleted phenotype. CD4⁺ and CD8⁺ T cell subsets were also obtained by immunomagnetic separation (15).

Measurement of Proliferation. Lymphocytes (2×10^5 /0.2 ml) were cultured with KLH (Sigma), yeast extract (*Saccharomyces cerevisiae*) (YST) (Difco Laboratories, Detroit, Michigan, USA), chicken OVA, purified bovine β -LG (approx 90% purified by Sigma, each at 100 μ g/ml) or phytohemagglutinin (PHA) (0.5 μ g/ml, Murex Diagnostics, Norcross, Georgia, USA). MAbs against CD2, CD40, CD40L, CD58, CD80, and CD86 (Coulter-Immunotech Inc., Miami, Florida, USA) and the CTLA4/Fc chimera (Pharmingen, San Diego, California, USA) were added (all at 1 μ g/ml) to block the reactions. Proliferation was measured by [³H]thymidine incorporation (17). In some experiments, lymphocytes were cultured on top of monolayers of KD cells (human lip fibroblasts, American Type Culture Collection, Rockville, Maryland, USA), which augment PHA-induced proliferation of intestinal lymphocytes (16).

Measurement of Responder Frequency. The frequency of responding cells was determined by limiting dilution analysis (17). CD4⁺T lymphocytes from LPLs and PBMCs were cultured in serial dilutions with LG or PHA and with irradiated (5000 rads) autologous PBMCs (1×10^4 /0.1 ml) as feeder cells in round-bottomed culture wells. Proliferation was measured after 10 days. Sixteen replicate wells were scored as positive or negative, with negative values defined as being within 3 SD of the mean proliferation of wells containing PBMCs alone. A semi-log plot of the percentage of negative wells versus the number of cells added per well allowed a linear regression from, which was interpolated as the cell number resulting in 37% negative wells. The reciprocal of this value was an estimation of the frequency of cells responding to the stimulus (17).

Measurement of Suppression. Suppressor cells were generated by culturing LPLs (2×10^6 /ml) for three days with LG or OVA (each at 100 μ g/ml), or staphylococcal enterotoxin B (SEB, 100 ng/ml). Suppressor cells were washed, irradiated, and continued in secondary cultures with naive autologous LPLs (indicator cells) at a 1:1 ratio with each stimulus. Proliferation three days later was determined by [³H]thymidine incorporation, and percent suppression calculated as

$$1 - \left[\frac{\text{naive LPLs} + \text{stimulus} + (\text{irradiated LPLs previously stimulated})}{\text{naive LPLs} + \text{stimulus} + (\text{irradiated LPLs previously unstimulated})} \right] \times 100\%$$

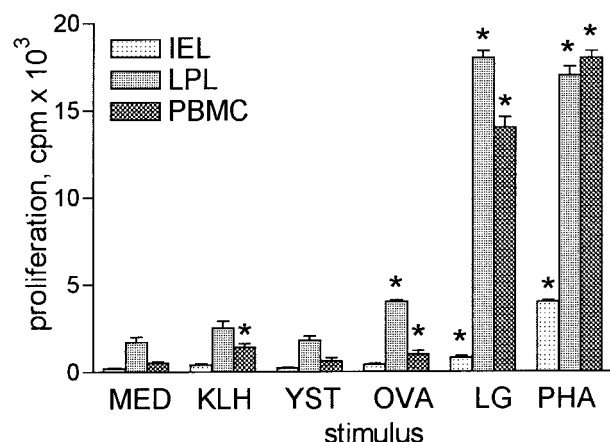


Fig 1. Lymphocyte proliferation toward food antigens. Lymphocytes were cultured for three days with each stimulus, and proliferation, determined by [³H]thymidine incorporation, was expressed as mean $\pm$ SE. Those values significantly greater than medium control were marked with asterisks ($P < 0.05$, $N = 6$).

Measurement of Cytokine Production. Supernates were taken from cultures of lymphocytes (2×10^6 /ml) stimulated by KLH, YST, OVA, LG, or PHA for one or three days (depending upon the time of maximal secretion for each cytokine). Levels of IL-2, IL-4, IL-10, IL-12, TNF- α , and IFN- γ were determined by ELISA (R&D Systems, Minn, Minnesota, USA). In some cases, MAb against IL-12, CD80, CD86, CD40, or CD40L (each at $1 \mu\text{g/ml}$) were added at the beginning of the cultures with the stimulus.

Statistical Analysis. For each data set, an arithmetic mean and SEM were calculated. Pairs of data sets were compared with Student's *t* test for paired or independent variables.

RESULTS

LPLs and PBMCs Proliferate Briskly in Response to LG and PHA. IELs, LPLs, and PBMCs proliferated best in response to LG and PHA, with blastogenesis by LPLs and PBMCs being equivalent and peaking on day 3 (Figure 1). When T-cell subsets from LPLs and PBMCs were tested separately, proliferation occurred almost exclusively in the CD4⁺ T cell subset (not shown). Smaller responses of LPLs and PBMCs occurred with KLH and OVA, while no response was induced by YST, used as a food protein control. Adding a monolayer of KD fibroblasts to the antigen-stimulated lymphocyte cultures, which augments IEL and LPL responsiveness to PHA (16), had no effect on the proliferation of any lymphocyte type to the food antigens.

The roles of several cell surface receptors were determined by MAb inhibition studies (Figure 2). LG- and PHA-stimulated blastogenesis of LPLs was partially reduced by MAb ($1 \mu\text{g/ml}$) against CD2 or

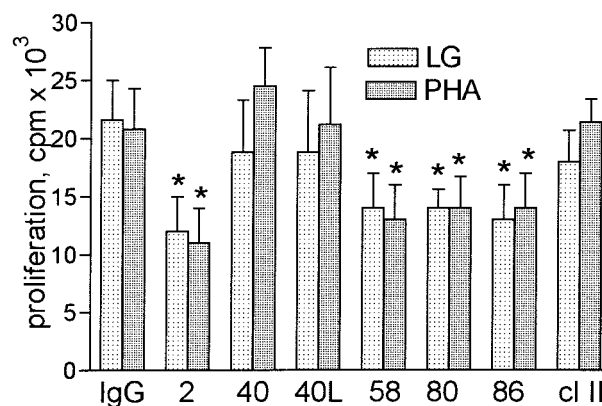


Fig 2. Surface markers involved in proliferation of LPLs to LG and PHA. Proliferative responses to LPLs to LG or PHA were measured in the presence of IgG control or MAb against various surface markers. Those values marked with an asterisk indicated significant reductions in proliferation ($P < 0.05$, $N = 5$).

CD58, suggesting a role for the CD2 pathway, and against CD80 or CD86, as well as the CTLA4/Fc chimera ($1 \mu\text{g/ml}$), suggesting involvement of APCs. LPL proliferation to LG or PHA did not change with the addition of MAb against CD40, CD40L, or MHC class II (DR); neutralizing MAb against IL-4, IL-12, TNF- α , IFN- γ , or TGF- β ; or IL-1ra.

The frequencies of proliferating CD4⁺T cells from LPLs and PBMCs were determined by limiting dilution analysis (Figure 3). LPLs contained more precursors for LG than for PHA ($1/4500$ vs $1/12000$). Both were less than the precursor frequencies in PBMCs for LG or PHA ($1/500$ vs $1/300$) in this representative of three experiments, showing that

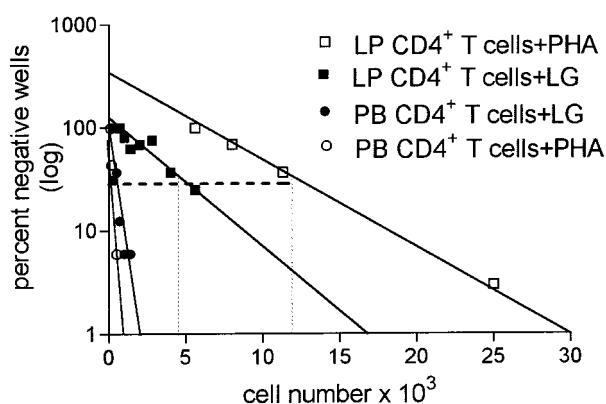


Fig 3. Limiting dilution analysis of CD4⁺ subset in LPLs and PBMCs to LG or PHA. Each line represents the linear regression for each combination of cell type and stimulus. The *x* intercept at 37% negative wells (shown by dropped lines) gives the cell number whose reciprocal is the frequency of responsive cells: $1/4500$ for CD4⁺ LPL+LG; $1/12,000$ for CD4⁺ LPL+PHA; $1/500$ for PB CD4⁺ T cells + LG; and $1/300$ for PB CD4⁺ T cells + PHA. This is a representative of three experiments.

LPLs have fewer memory cells for either stimulus than do circulating lymphocytes.

High Levels of IFN- γ are Synthesized by LPLs in Response to LG. Cytokine production was measured in cultures of IELs, LPLs, and PBMCs stimulated with KLH, YST, OVA, LG, or PHA (Figure 4). Kinetics studies of cytokine production by lymphocytes stimulated with LG or PHA were first performed to identify the day of maximal synthesis as being day 3 for IFN- γ and day 1 for the other cytokines (not shown). For the most part, IELs secreted only small amounts of cytokines, except for IL-4 and IL-12, which were produced in greater abundance by PHA-stimulated IELs than by LPLs or PBMCs (18). LPLs produced more IL-2 in response to PHA than LG, and more than the amounts secreted by PBMCs. IL-10 and TNF- α levels were similar for LPLs and PBMC (19). Extremely high levels of IFN- γ (100 ng/ml) were produced by LPLs (with LG or PHA) and by PBMCs (with PHA).

Several surface molecules or cytokines may mediate LG-stimulated IFN- γ synthesis by LPLs (Table 1). MAb blocking of IL-12, CD80/CD86, and CD40/CD40L all significantly disrupted IFN- γ production by LPLs, while only the blocking of CD80/CD86 reduced IFN- γ levels by PBMCs. IL-2 production by LG-stimulated LPLs was somewhat reduced by MAbs against IL-12 and the CD80/CD86 combination, while TNF- α production was partially blocked by MAbs to CD80 and CD86, although none of these inhibitory events reached significance.

Bystander Suppression by Antigen-Activated LPLs Reduces Proliferation of Naive Autologous LPLs. To determine whether suppressor cells developed in response to stimulation, LPLs were initially cultured with medium or LG for three days, then irradiated to prevent their own proliferation, and recultured at a 1:1 ratio with unstimulated autologous LPLs. This cell mixture was stimulated with LG for three more days and proliferation measured. LPLs that had been activated with LG in the initial culture subsequently reduced the responsiveness of naive autologous LPLs to LG by 51% (Figure 5), while unstimulated LPLs had no effect on the naive response. These findings were not due to changes in kinetics (not shown). To demonstrate specificity, LG-induced suppressor LPLs were added to naive LPLs stimulated with OVA, SEB, and PHA; proliferative responses to each in the secondary culture were reduced by about 50% (Figure 5). OVA, PHA, and SEB stimulation also induced suppressor activity in LPLs. YST stimulation, however, did not.

Although IFN- γ is produced in abundance during the initial culture of LPLs with LG, adding neutralizing MAb against this cytokine did not alter the development of LG-specific suppressor cells (not shown). To determine whether inhibitory cytokines were responsible for suppression, neutralizing MAbs to IL-4, IL-10, or TGF β were added to the secondary cultures ($N = 5$). Suppression by LPLs remained the same, indicating that the inhibition was not due primarily to these cytokines.

DISCUSSION

The response mounted by intestinal lymphocytes to food antigens may be critical in maintaining intestinal immunological tolerance or, in its absence, to development of food allergies. Studies using rodents have examined tolerance to human foods such as LG and OVA. These, however, may be novel antigens for rodents, triggering primary events, but common ones for humans, inducing secondary events. When antigens (particularly soluble, monomeric proteins) are presented in the absence of adjuvant (bacteria or lipopolysaccharide) or APCs, oral tolerance ensues. Particulate antigens with adjuvant and APCs lead to a protective immune response with secretion of proinflammatory cytokines. The numbers of APCs in jejunal mucosa are hard to quantify since there is a mixture of macrophages and dendritic cells without absolute identifying markers. Because the goals of this study were to evaluate, for the first time, human intestinal T-cell responses to commonly ingested antigens, intestinal lymphocytes were derived from normal jejunum, where most food is digested and absorbed.

Of the common food antigens tested, LG, then OVA, were the most effective inducers of cell division and cytokine production by IELs, LPLs, and PBMCs. LG has characteristics of a mitogen: the precursor frequencies are similar and the peak response occurs early, on day 3. It is unlikely to be a superantigen since its effects are independent of MHC class II. Rather, LPL proliferation to LG was reduced by the CTLA4/Fc chimera and by MAbs against CD80 and CD86, antigens found on APCs, as well as MAbs against CD2 and CD58. LG may stimulate the CD28⁺ LPLs through their CD2 pathway, known to be a potent stimulus for secretion of IL-2 and IFN- γ (20–22). While the proliferative responses of LPLs to LG or PHA were of similar magnitude to those of PBMCs, limiting dilution analysis showed a lower frequency of proliferating CD4⁺T cells in the lamina

RESPONSE OF LPLs TO LG

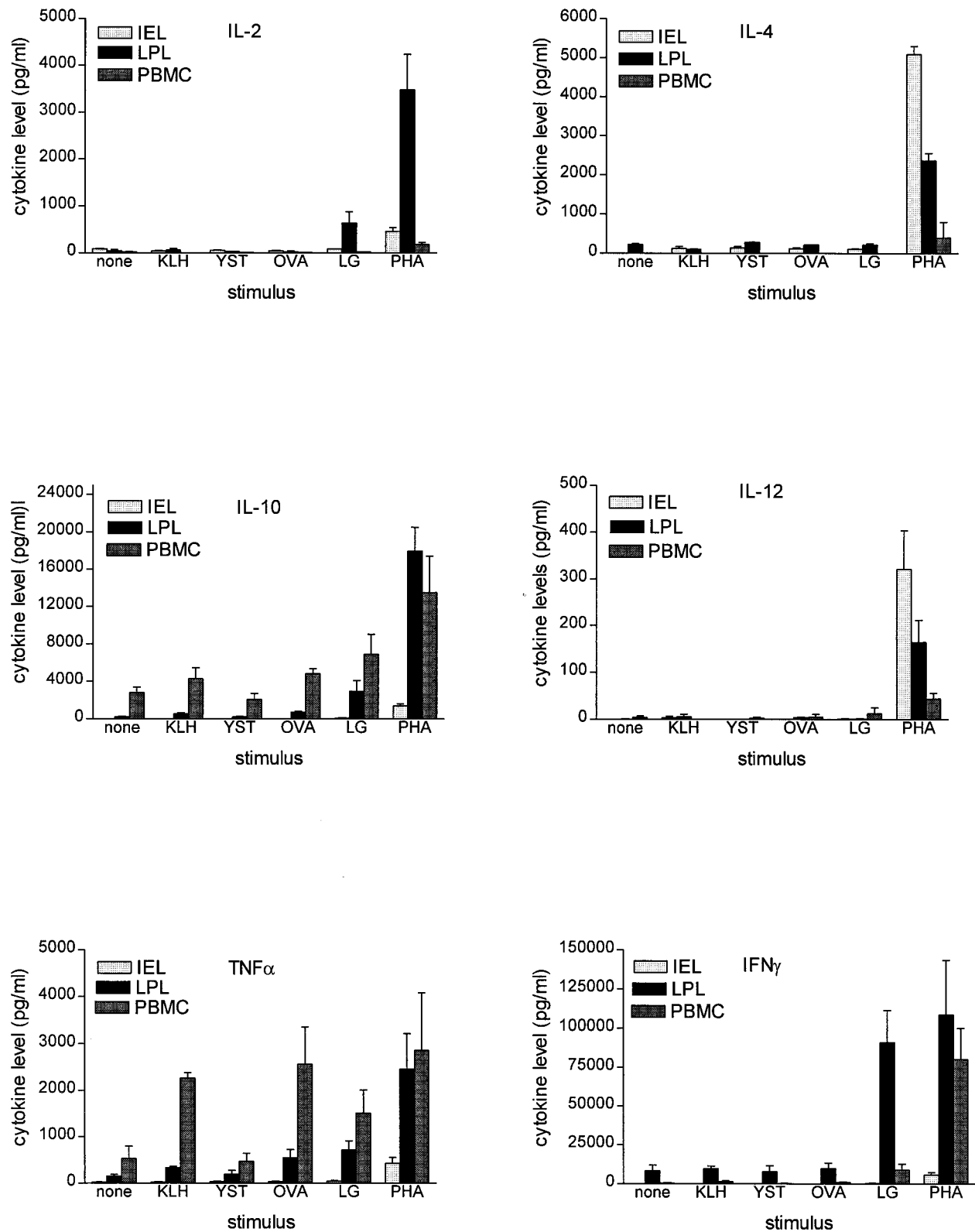


Fig 4. Cytokine levels produced by each lymphocyte type with each stimulus. Culture supernates were collected after three days (for IFN- γ) or one day (for the remaining cytokines) (each representing the day of maximal production). Cytokine levels were measured by ELISA.

TABLE 1. ANTIBODY INHIBITION OF CYTOKINE PRODUCTION BY LACTOGLOBULIN-STIMULATED LPLS*

Cell type	Cytokine assayed	Antibody specificity (% inhibition)		
		IL-12	CD80/CD86	CD40/CD40L
LPL	IFN- γ	75 $\pm$ 7a	69 $\pm$ 10a	60 $\pm$ 10a
LPL	IL-2	74 $\pm$ 23	46 $\pm$ 19	6 $\pm$ 20
LPL	TNF- α	6 $\pm$ 7	39 $\pm$ 6	-9 $\pm$ 17
PBMC	IFN- γ	46 $\pm$ 10	96 $\pm$ 7b	30 $\pm$ 10

*LPLs or PBMCs (2×10^6) were cultured for three days with LG (100 μ g/ml) and blocking MAbs as indicated. The amounts of cytokines produced by each cell type in culture supernates were measured by ELISA ($N = 4$). Significant inhibition is indicated: a, $P < 0.05$; and b, $P < 0.01$.

propria, probably since fewer LPLs than PB T cells express CD28 (23).

When exposed to LG, LPLs produced large amounts of IFN- γ , moderate amounts of TNF- α , and little IL-2, IL-4, IL-10, or IL-12. Quantitating cytokines in the culture medium is affected by their utilization, so the true amount of cytokine produced may be greater, especially with IL-2. However, it is a good estimate of the amount available to neighboring cells. The pattern of cytokine synthesis may represent the normal response to the bit of LG that routinely enters the lamina propria; the LPLs are probably already sensitized to this antigen. Other studies of intestinal immunological tolerance also showed a predominance of IFN- γ , rather than IL-2, being produced in response to food antigens soon after immunization, while later measurements showed reduced production of IFN- γ (24–26). IFN- γ has many proinflammatory effects on the immune system, such as its increase

in expression of surface markers on APCs. While IFN- γ normally increases IL-12 levels that aid in development of Th-1 effector function, critical for reversal of mucosal tolerance (27), here IL-12 was low, presumably preserving tolerance.

LPL synthesis of IFN- γ depended upon IL-12, CD40-CD40L, and CD80/86-CD28, similar to that reported in other systems (28). This outpouring of IFN- γ by LPLs may be a normal response to any stimulus, maintaining the normal lymphocytic infiltrate in the mucosa. When a segment of bowel is excluded from the flow of food, as in the afferent loop of Billroth II operations, the mucosal infiltrate is reduced and the villi become shortened. These changes are reversed by the reintroduction of food antigens. On the other hand, when the inflammation is intense, as in active inflammatory bowel disease, a standard treatment is elimination of food. Food clearly has an active role in mucosal health, perhaps by promoting IFN- γ production when food antigens enter the lamina propria.

Hypersensitivity responses to food antigens may be due to those containing mitogens or highly stimulatory substances, such as LG. Other mitogens in food include peanuts, (*Arachis hypogaea*) soybeans, (*Glycine max*) and wheat germ (*Triticum vulgaris*). Children, for example, may have immature epithelium, permitting entry of food stuffs, such as milk products, into the lamina propria. LPLs may proliferate to LG, producing IFN- γ which increases permeability of the epithelial lining, permitting other antigens to enter the mucosa. Patients with milk allergy may carry more than the normal number of LG memory cells in the lamina propria, so reintroduction of small amounts of the antigen would trigger large responses.

After the limited IFN- γ synthesis, LPLs subsequently developed suppressor activity with LG, as previously shown with stimulation of the CD2 pathway (24). In fact, the effect of LG may be mediated

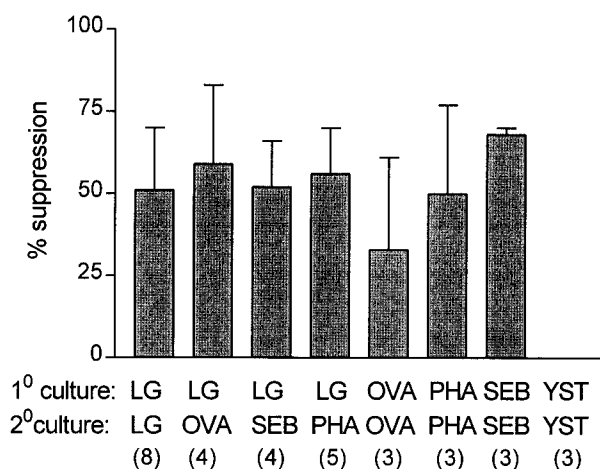


Fig 5. Development of suppression with food antigens. LPLs, cultured with each stimulus for three days, were irradiated and added to naïve LPLs in secondary cultures with various stimuli. The percent suppression was calculated. The number of experiments performed is in parentheses.

through CD2. This suppression is of the bystander type since LG-stimulated LPLs inhibited activation of naive autologous LPLs in response to various stimuli. This suppression is not found with PBMCs, whose response to foreign antigens should be stimulatory.

The IFN- γ synthesis and suppressor activity by LPLs in this study are antigen nonspecific, and so are not examples of oral tolerance in the classical sense. These activities, however, are probably occurring continuously in the upper small bowel with exposure to food.

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