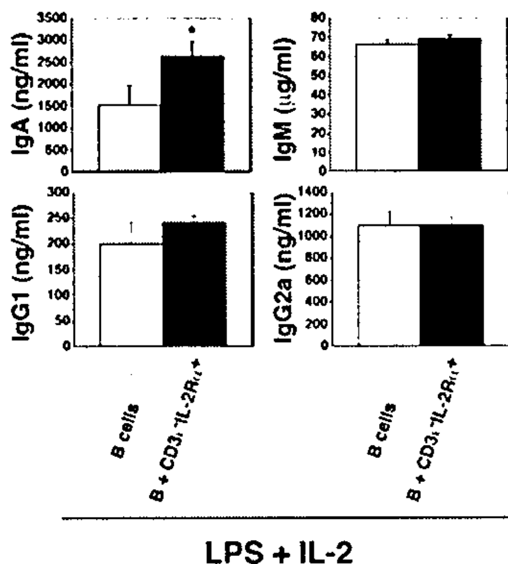

Fig. 8. CD3ε⁻IL-2Rα⁺ cells enhance IgA production by PP B cells. B220⁺ B cells were prepared from PP cells by MACS and cultured in a 96-well plate (2×10^5 cells/well) with (filled bar) or without (open bar) PP c-kit⁻CD3ε⁻IL-2Rα⁺ cells (2×10^4 cells/well) purified by FACS sorting in the presence of LPS (20 μg/ml) plus IL-2 (250 U/ml). Culture supernatants were collected at 7 days and analyzed for secreted Ab by ELISA. The results are expressed as the mean of triplicate cultures ± SD. *, $p < 0.05$ when compared to cultures without PP c-kit⁻CD3ε⁻IL-2Rα⁺ cells. Similar results were obtained from two independent experiments.

ophils [23], and epithelial cells [25]. Characterization of CD3ε⁻IL-2Rα⁺ cells shows that these cells do not belong to the T lineage because they lack CD3ε, TCRβ and TCRγδ. The lack of expression of CD23, c-kit, DX5 and NK1.1 suggests that CD3ε⁻IL-2Rα⁺ cells are not mast cells, NK cells or NKT cells. Moreover, immunohistochemical analysis reveals that CD3ε⁻IL-2Rα⁺ cells show a lymphoid morphology, therefore eliminating the possibility that they are eosinophils or epithelial cells with the potential to produce cytokines. Thus CD3ε⁻IL-2Rα⁺ cells do not belong to a previously characterized population with an ability to produce IL-5.

Yoshida et al. [26] reported that embryonic intestinal CD3⁻IL-7Rα⁺ cells, which they call PP inducers, play an essential role in PP organogenesis and that administration of anti-IL-7Rα mAb to mice during gestation disrupted PP development in the progeny. CD3⁻IL-7Rα⁺ cells are subdivided into CD4⁺ or CD4⁻ populations, and further study showed that CD4⁺CD3⁻IL-7Rα⁺ cells were able to activate VCAM-1⁺ICAM-1⁺ mesenchymal cells through LTβR to express adhesion molecules and chemokines [27]. A more recent report by Finke et al. [28] revealed that CXCL13 signaling via its receptor CXC chemokine receptor 5 (CXCR5) activated α4β1 expression on fetal CD4⁺CD3⁻ cells and induce PP development. CD4⁺CD3⁻ cells are also considered critical for secondary lymphoid tissue development including LN [29, 30] and nasopharyngeal-associated lymphoid tissue [31]. CD4⁺CD3⁻ cells are thought to be derived from fetal liver [32, 33] and migrate to developing LN [34] or intestine [33]. The migrated CD4⁺CD3⁻ cells can give rise to APC, NK cells and follicular cells but not T or B cells [33, 35]. More recently, Kim et al. [36] described a CD4⁺CD3⁻ population in adult mice which is able to support the survival of CD4 T cells. Although these populations are phenotypically similar to the CD4⁻CD3ε⁻IL-2Rα⁺ cells described here, it differs from our CD4⁻CD3ε⁻IL-2Rα⁺ cells since CD4 is expressed on these cells. On the other hand, the function of CD4⁻CD3⁻IL-7Rα⁺ cells is still unclear, though Yoshida et al. [33] suggested that these cells may have the potential to give rise to NK cells. It is possible that CD4⁻CD3⁻IL-7Rα⁺ cells in the embryonic intestine and CD4⁻CD3ε⁻IL-2Rα⁺ cells described here may represent related populations, although they do have some differences such as expression of IL-2Rα and c-kit. They may be distinct subsets, or alternatively they may be derived from a common precursor that colonizes the PP and matures to lose c-kit expression and gain IL-2Rα expression and the ability to produce cytokines in adult. Clearly, more investigations are needed to determine whether or not they are related populations.

Although at present the function of our CD4⁻CD3ε⁻IL-2Rα⁺ cells *in vivo* is unclear, our *in vitro* data (Fig. 8) suggests that these cells enhance IgA secretion by B cells. IL-5 induces maturation and differentiation of sIgA⁺ B cells into IgA-producing plasma cells, therefore it may be possible that CD3ε⁻IL-2Rα⁺ cells secrete IL-5 in response to IL-2 and help B cells to produce IgA. We also observed that CD3ε⁻IL-2Rα⁺ cells prepared from mesenteric LN secrete IL-5 in response to IL-2 (data not shown). Since PP and mesenteric LN have been considered to be inductive sites for initiation of secretory IgA immune responses in the gastrointestinal tract, CD3ε⁻IL-2Rα⁺ cells may help IgA induction at these sites. B220⁻CD3ε⁻IL-2Rα⁺ cells also exist in the gut lamina propria, known for the effector site of IgA and IgM Ab, by FACS analysis (data not shown). B1 cells, which express IL-5Rα spontaneously on their surface and respond to IL-5 to mature or differentiate into Ab-secreting plasma cells, exist in these sites so it is possible that CD3ε⁻IL-2Rα⁺ cells help in the Ab production by lamina propria B1 cells. We are now currently studying whether B220⁻CD3ε⁻IL-2Rα⁺ cells of the lamina propria could have a potential to produce IL-5 and might affect IgA or IgM production by the lamina propria B1 cells.

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We observed IL-5 production by CD3ε⁻IL-2Rα⁺ cells in response to IL-2 (Fig. 3C and 6C). IL-2 is a principal cytokine secreted by naive CD4⁺ T cells, so it could be considered that CD4⁻CD3ε⁻IL-2Rα⁺ cells rapidly produce IL-5 in response to IL-2, which may be produced by naive CD4⁺ T cells at the beginning of immune responses. The fact that we could not detect IL-5 production by CD3ε⁻IL-2Rα⁺ cells stimulated with anti-CD3 mAb, whereas IL-5 production by PP cells was significantly reduced by a depletion of CD3ε⁻IL-2Rα⁺ cells when stimulated with anti-CD3 mAb, are compatible with this hypothesis (Fig. 7A and data not shown).

In summary we have described a unique subset of lymphoid cells in PP from adult mice. The population is characterized by a high potential to produce IL-5, by the expression of IL-2Rα and IL-7Rα, and by an absence of CD3ε, CD4 and c-kit expression. Our data also indicate that CD4⁻CD3ε⁻IL-2Rα⁺ cells are not T cells, NK cells, NKT cells, mast cells or eosinophils, all of which are capable of producing IL-5. We observed that CD4⁻CD3ε⁻IL-2Rα⁺ cells produced IL-5 in response to IL-2, and that elimination of these cells from PP resulted in a marked reduction of IL-5 production *in vitro*. Furthermore, CD4⁻CD3ε⁻IL-2Rα⁺ cells increased IgA secretion by B cells. Our hypothesis is that CD4⁻CD3ε⁻IL-2Rα⁺ cells produce IL-5 in response to IL-2 produced by naive CD4⁺ T cells and help IgA secretion by B cells in gut-associated lymphoreticular tissue.

4 Materials and methods

4.1 Mice

Female BALB/c and C57BL/6N mice (6–8 weeks old) were purchased from CLEA Japan (Tokyo, Japan).

4.2 Ab

The following Ab were used for staining or stimulating cells. Anti-IL-7Rα Ab (A7R34) was a kind gift from Dr. Ishikawa (Keio University School of Medicine). Anti-CD11c Ab (N418), anti-CD62L Ab (MEL-14) and anti-CD16/32 Ab (2.4G2) were purified from hybridoma culture supernatant in our laboratory. Anti-IL-2Rα (7D4), anti-CD3ε (145-2C11), anti-CD4 (H129.120), anti-CD28 (37.51), anti-Thy1.2 (30-H12), anti-B220 (RA3–6B2), anti-c-kit (2B8), anti-NK1.1 (PK136), anti-CD23 (B3B4), anti-TCR-αβ (H57-597), anti-CD8α (53–6.7), anti-B7-1 (16-10A11), anti-B7-2 (GL1), anti-CD40 (3/23), anti-CD44 (IM7), anti-LFA-1 (2D7), anti-Fas (Jo2), anti-β7 (M293) and anti-Syndecan-1 (281-2) were purchased from BD PharMingen (San Diego, CA). Anti-Mac-1 (M1/70; Caltag, San Francisco, CA), anti-DX5 (DX5 [24]; eBioscience, San Diego, CA), anti-TCR-γδ (GL3; Cedarlane, Ontario), anti-sIgD (11-26; Southern Biotechnology Associates, Birmingham, AL), anti-sIgM (1B4B1; eBioscience) and anti-α4 (R1-2; Cedarlane) were also purchased. Upon flow cytometric analysis, we checked that these Ab were able to detect surface antigens expressed on positive control cell populations. Surface phenotype of CD3ε⁻IL-2Rα⁺ cells was analyzed using a FACSort with CellQuest software (BD Biosciences, Mountain View, CA). Before staining, Fcγ R was blocked using 2.4G2 (anti-CD16/CD32).

4.3 Cell preparation

Both PP and SPL were digested with collagenase Type I (Sigma, St. Louis, MO) and single-cell suspension was prepared. CD4⁺ T cells, B220⁺ B cells and Thy1.2⁻ cells were isolated using magnetic beads (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's protocol. CD3ε⁻IL-2Rα⁺ cells were prepared from the Thy1.2⁻B220⁻ fraction of PP and SPL in parallel as follows. Thy1.2⁻B220⁻ cells were isolated by MACS and were incubated with mAb. After washing, stained cells were analyzed using a FACSort or CD3ε⁻IL-2Rα⁺ cells were purified using a FACS Vantage (BD Biosciences). Further, CD4⁺CD3ε⁻IL-2Rα⁺ cells, CD4⁻CD3ε⁻IL-2Rα⁺ cells, c-kit⁺CD3ε⁻IL-2Rα⁺ cells or c-kit⁻CD3ε⁻IL-2Rα⁺ cells were sorted using a FACS Vantage. The purity of sorted cells was routinely > 98%.

To deplete IL-5-producing CD3ε⁻IL-2Rα⁺ cells, we incubated PP cells with FITC-conjugated anti-B220, PE-conjugated anti-CD3ε and biotinylated anti-IL-2Rα Ab, followed by streptavidin-conjugated red 670 (Life Technologies, Gaithersburg, MD). B220⁻CD3ε⁻IL-2Rα⁺ cells were then depleted from PP cells using a FACS Vantage.

In some experiments, we used dispase (grade II, Boehringer Mannheim, Mannheim, Germany) for collagenase to prepare PP cells and splenocytes, or PP cells were prepared by crushing PP mechanically (by non-enzymatic technique).

4.4 Cell culture

Cells were cultured at various concentrations as indicated in the figure legends in RPMI 1640 containing 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 0.03% L-glutamine and 0.2% $NaHCO_3$ for 48 h or 7 days. For cell stimulation, 50 U/ml or 250 U/ml human rIL-2 (Takeda, Tokyo, Japan), 50 ng/ml PMA (Wako, Osaka, Japan) plus 250 ng/ml calcium ionophore A23187 (Wako), 1 μg/ml anti-CD3ε Ab or 20 μg/ml LPS (Sigma) were added. To stimulate CD4⁺ T cells plate-bound anti-CD3ε Ab (3 μg/ml) and anti-CD28 Ab (5 μg/ml) were used. The culture supernatants were collected and kept frozen until use.

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4.5 ELISA

Cytokine levels and Ab titers in culture supernatants were determined by ELISA as described [37].

4.6 Cytostaining

Purified PP CD3ε⁻IL-2Rα⁺ cells were stimulated with PMA and ionomycin for 24 h and Golgi Stop™ (BD PharMingen) was added in the final 6 h. The cells were collected and fixed with 4% paraformaldehyde/PBS for 15 min. After washing, the cells were permeabilized in PBS/0.5% BSA/0.5% saponin/0.2% azide. The cells were then incubated with PE-conjugated anti-IL-5 Ab (TRFK5, BD PharMingen) or PE-conjugated isotype-matched IgG (BD PharMingen). After washing carefully, stained cells were analyzed using a FACSort.

4.7 Immunohistochemistry

Freshly purified c-kit⁻CD3ε⁻IL-2Rα⁺ cells were centrifuged at 800 rpm for 5 min onto glass slides and air-dried. The cells were then stained with Diff-Quik (International Reagents, Kobe, Japan).

4.8 Statistics

Data were expressed as the mean ± SD and compared by the Student's *t*-test.

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Candida albicans and *Saccharomyces cerevisiae* induce interleukin-8 production from intestinal epithelial-like Caco-2 cells in the presence of butyric acid

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Abstract

Intestinal epithelial cells (IEC) are important in initiation and regulation of immune responses against numerous foreign substances including food, microorganisms and their metabolites in the intestine. Since the responses of IEC against yeasts have not yet been well understood, we investigated the effects of *Candida albicans*, *Saccharomyces cerevisiae*, and their cell wall components on interleukin-8 (IL-8) secretion by the IEC-like Caco-2 cells. Live cells of both yeast species stimulated Caco-2 cells to produce IL-8 only in the presence of butyric acid, which is a metabolite produced by intestinal bacteria. *S. cerevisiae* zymosan and glucan also enhanced IL-8 secretion. Treatment of Caco-2 cells with butyric acid increased the expression of mRNAs coding for Toll-like receptor 1 (TLR1), TLR6 and dectin-1, which recognize zymosan. *C. albicans* induced more IL-8 secretion and also decreased transepithelial electrical resistance more rapidly than *S. cerevisiae*. These results suggest that both yeasts in the intestine stimulate the host's mucosal immune systems by interacting with IEC.
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Keywords: *Candida albicans*; *Saccharomyces cerevisiae*; Caco-2 cells; Interleukin-8; Butyric acid

1. Introduction

Intestinal epithelial cells (IEC), which line the inner surface of the intestinal lumen, function as the physical barrier of this organ. These cells are exposed to many foreign substances, including food, microorganisms and their metabolites. IEC are also important in the initiation and regulation of mucosal immune responses to foreign substances [1].

Many kinds of microorganisms, including yeasts, reside in the human intestine. In healthy humans, *Candida albicans* is a commensal yeast, but in immunosuppressed patients it becomes an opportunistic pathogen that causes mucosal candidiasis [2]. This species of yeast is also thought to participate in atopic dermatitis [3] and asthma [4]. In contrast, *Saccharomyces cerevisiae* is not a commensal yeast, although it may be transiently present in the intestine following oral ingestion. *S. cerevisiae* is recognized as nonpathogenic and is utilized for food production and as a probiotic [5,6]. Live *S. cerevisiae* cells have been found in the intestinal tract of gnotobiotic mice and human feces after oral ingestion [7]. By being present in the human intestine, *C. albicans* and *S. cerevisiae* may react with IEC and stimulate the mucosal immune system.

It has been reported that the production of cytokines, such as interleukin-1β (IL-1β), IL-6, IL-8, IL-15, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α), by IEC and IEC-like cell lines increases in response to both pathogenic [8–10] and

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nonpathogenic [11–13] bacteria. While recent studies have shown that *C. albicans* enhances the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) by oral and vaginal epithelial cells [14,15], the response of IEC to yeasts is not known.

These responses are believed to be induced by the binding of foreign substances to receptors on IEC, among which are the Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs). To date, 10 of these receptors, termed TLR1 to TLR10, have been identified, and seven of them have been shown to recognize specific PAMPs. Of these, TLR2, in cooperation with TLR1 or TLR6, has been found to recognize zymosan on yeast cells and peptidoglycan on Gram-positive bacteria [16,17]. Four other receptors, TLR3, TLR4, TLR5 and TLR9 have been shown to recognize double-stranded RNA, lipopolysaccharide on Gram-negative bacteria, bacterial flagellin, and unmethylated CpG DNA, respectively [18]. An IEC-like cell line, Caco-2 is known to express mRNAs and/or proteins of TLR1, TLR2, TLR4, TLR5 and TLR6 [19–21]. In addition to TLRs, other pattern recognition receptors (PRRs) are shown to participate in responses to yeast components. Dectin-1, which binds to β-glucan contained in zymosan, mediates cytokine production by macrophage in response to zymosan [22–24].

Butyric acid is produced during carbohydrate fermentation by intestinal bacteria such as *Clostridium* spp., *Eubacterium* spp. and *Butyrivibrio* spp. [25–27]. Butyric acid has been reported to inhibit the proliferation and induce differentiation and apoptosis in the IEC-like cell lines, HT29 and Caco-2 [28,29]; hence, its presence in the intestine may have profound health consequences for the host. Because IEC are exposed to butyric acid, the synergistic action of yeasts with this metabolite may have some effects on the immune responses of the intestinal lumen. We therefore investigated whether *C. albicans*, *S. cerevisiae*, or their cell wall components induces IL-8 production by Caco-2 cells in the presence or absence of butyric acid. We also examined whether butyric acid and yeast cells alter the expression of mRNAs coding for TLRs and dectin-1.

2. Materials and methods

2.1. Microorganisms

Candida albicans JCM 1542^T and *S. cerevisiae* JCM 7255^T, purchased from the Japan Collection of Microorganisms (JCM; Wako, Japan), were grown in YM broth (Difco Laboratories, Detroit, MI) at 30 °C for 3 days. Cell suspensions of each yeast were aliquoted, washed three times with phosphate-buffered saline (PBS) by centrifugation at $1000 \times g$ at 4 °C for 10 min, resuspended in the same buffer, and kept at 4 °C for no more than 3 h prior to use. The number of viable cells in each suspension was estimated by the pour-plate count method using Potato dextrose agar (Eiken Chemical, Tokyo, Japan).

Killed organisms were prepared by heating cell suspensions in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) for 30 min at 65 °C. To confirm that all treated cells were killed, a 100 μl aliquot containing 1×10^8–3×10^8 colony-forming units (CFU) ml^{-1} prior to treatment was added to a Potato dextrose agar plate and incubated at 30 °C for 2 days.

2.2. Culture of Caco-2 cells

Caco-2 (RCB0988) cells [30,31] were purchased from the Riken Cell Bank (Tsukuba, Japan) and cultured in DMEM, supplemented with 1 mM L-glutamine (Wako Pure Chemical Industries, Osaka, Japan), 10 mM HEPES (Dojindo Molecular Technologies, Kumamoto, Japan) and 15% heat inactivated fetal bovine serum (FBS; PAA Laboratories, Linz, Austria), in 6-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) at 37 °C in an atmosphere of 5% CO_2. For cytokine assays and transepithelial electrical resistance (TER) measurements, 1.5×10^5 cells of passages 20–35 in 0.5 ml of medium were seeded onto a 12-mm Millicell-HA filter (Millipore, Bedford, MA). Each filter was placed into a well of a 24-well culture plate (Becton–Dickinson Labware) containing 0.5 ml medium, and the filters were cultured for 6–7 days in the same medium, changing the medium every day. For mRNA assays, 6.0×10^5 cells in 2.5 ml of medium were seeded onto a 45-mm filter. Each filter was placed in a well of a 6-well culture plate (Becton–Dickinson) containing 2.5 ml medium. The cells were incubated for 4 days in media containing various concentrations of butyric acid (0–20 mM) (Wako Pure Chemical Industries), with adjustments of the media to pH 7.4 with sodium bicarbonate. The media were refreshed every day. Caco-2 cells were subsequently co-cultured with various concentrations of *S. cerevisiae* or *C. albicans* cells or with various concentrations of the *S. cerevisiae* cell wall components, zymosan, glucan or mannan (all from Sigma, St. Louis, MO) in 0.5 ml (12-mm filter) or 2.5 ml (45-mm filter) medium, with or without butyric acid, which was added to the apical side of the filter. An identical volume of fresh medium without yeast cells or cell wall components was added to the basolateral side of the filter. Yeast cells and cell wall components were left in each well throughout the 3, 7, or 24 h co-culture period.

2.3. *Measurement of secreted IL-8 and TER*

For cytokine assays, basolateral supernatants were collected after co-culture for 7 or 24 h, centrifuged for 5 min at 1000*g*, divided into aliquots, and stored below −20 °C until assayed. Secreted IL-8 was determined using a human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA). TER of Caco-2 cell monolayers was assayed using a Millipore electrical cell resistance meter and probe (Millipore).

2.4. *Assay of IL-8, TLR and dectin-1 mRNAs*

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) from each Caco-2 cell culture before (0 h) or after co-culture with yeasts for 3 or 7 h. An aliquot of each RNA was treated with amplification grade DNase I (Invitrogen), and cDNA was synthesized using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) and oligo $d(T)_{12-18}$ primers (Amersham Bioscience, NJ). The LightCycler real-time PCR system (Roche, Mannheim, Germany) was utilized for PCR amplification of the cDNAs, using a QuantiTect SYBR Green PCR Kit (Qiagen) and oligonucleotide primer pairs specific for IL-8, TLR1, TLR2, TLR6, dectin-1 and GAPDH mRNA (Nihon Gene Research Laboratories, Sendai, Japan). The primer sequences, PCR conditions and expected amplicon size are shown in Table 1. The relative concentration of each message in the cell cultures was calculated using a standard curve for each, generated from serially diluted known standards, and the level of each transcript was normalized to that of GAPDH in the same sample. The specificity of each PCR primer pair was confirmed by observing a single peak by melting curve analysis with the LightCycler system (Roche) and by a single band of expected size by electrophoresis in 2% agarose gels (Agarose L 03, Takara Bio, Otsu, Japan), using a DNA ladder of 100–1500 bp (Takara Bio) as the molecular weight standards.

2.5. *Statistical analysis*

Statistical analysis was performed using SPSS software (Statistical Package for Social Sciences, Chicago, IL). When group variances were not significantly different, the statistical significance of differences between means was calculated using the two-sided Dunnett method for multiple comparisons. When the group variances were unequal, the statistical significance of differences between means was calculated using the Dunnett T3 method. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Candida albicans *enhances IL-8 secretion from Caco-2 cells only in the presence of butyric acid*

Caco-2 cells were co-cultured with or without *C. albicans* (3.2×10^5 CFU ml^{-1}) for 24 h in the presence or absence of butyric acid to determine whether this species of yeast alters IL-8 secretion. Butyric acid increased dose-dependently IL-8 secretion, which was further enhanced when *C. albicans* was included in the culture medium (Fig. 1). Optimum IL-8 secretion, in the presence or absence of *C. albicans*, was observed in media containing 10 mM butyric acid (Fig. 1).

3.2. Saccharomyces cerevisiae *also enhances IL-8 production by Caco-2 cells in the presence of butyric acid*

We subsequently compared the ability of *C. albicans* and *S. cerevisiae* to induce IL-8 secretion from Caco-2 cells. Caco-2 cells were co-cultured with 1.6×10^7–1.6×10^3 CFU ml^{-1} *C. albicans* or 3.8×10^7–3.8×10^3 CFU ml^{-1} *S. cerevisiae*, with or without butyric acid. *S. cerevisiae* as well as *C. albicans* had no significant effect on IL-8 secretion from Caco-2 cells cultured in the

Table 1
Primer sequences and amplification conditions

Target gene	Primer sequence (5′ – 3′)		Annealing temperature (°C)	Extension time (s)	Amplicon size (bp)
GAPDH	Sense	tgaacgggaagctcactgg	63	30	307
	Antisense	tccaccaccctgttgctgta			
IL-8	Sense	tggctctcttggcagccttc	63	30	238
	Antisense	tgcacccagttttccttggg			
TLR1	Sense	ccaaggaaaagagcaaacgtg	60	15	130
	Antisense	gcagcaatatcaacaggaggaa			
TLR2	Sense	tgcggaagataatgaacacc	60	15	140
	Antisense	gatcccaactagacaaagactg			
TLR6	Sense	aagcaaacgtgggctctt	56	20	156
	Antisense	cgactgtactattcaccatcatcc			
Dectin-1	Sense	tcaatgtaagaggaagggtg	52	15	127
	Antisense	gccaagctctctaaacattt			

The amplification protocol consisted of an initial heat activation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at the temperature shown for 20 s, and extension at 72 °C for the time shown.

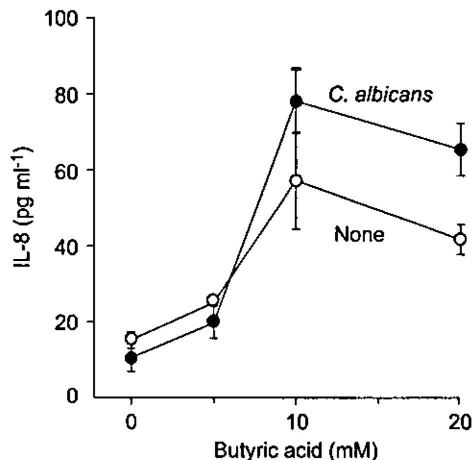

Fig. 1. IL-8 secretion from Caco-2 cells in response to intact *C. albicans* in the presence of various concentrations of butyric acid. Caco-2 cells were seeded at 1.5×10^5 cells well^{-1} on a 12-mm Millicell-HA filter and pre-cultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 0–20 mM butyric acid. The cells were cultured for 24 h in the presence or absence of 3.2×10^5 CFU ml^{-1} *C. albicans* in 0.5 ml of fresh, identical medium, which was added to the apical side of the filter. IL-8 secreted into the basolateral supernatants (0.5 ml) was measured by ELISA. Data are presented as means ± SD of triplicate cultures. Data shown are representative of at least three independent experiments.

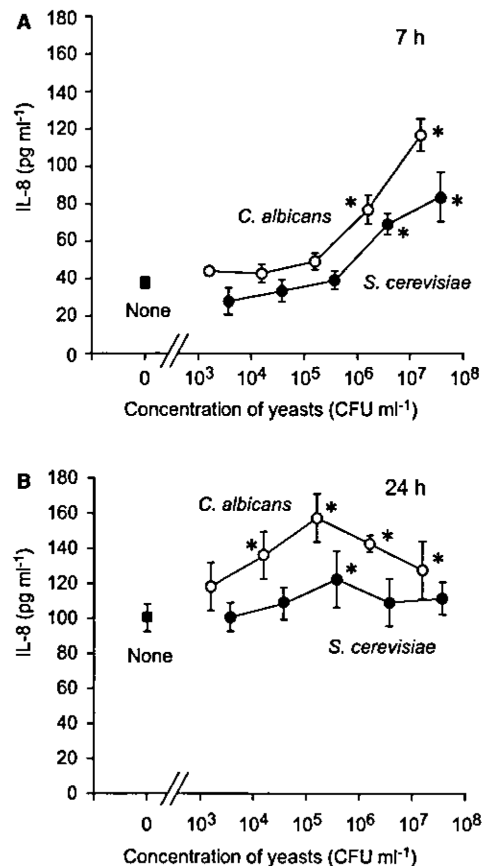

Fig. 2. IL-8 secretion from Caco-2 cells in response to intact *C. albicans* and *S. cerevisiae* in the presence of butyric acid. Caco-2 cells were seeded at 1.5×10^5 cells well^{-1} on a 12-mm Millicell-HA filter and pre-cultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 10 mM butyric acid. The cells were cultured for 7 (A) or 24 h (B) in the presence or absence of *C. albicans* (1.6×10^7–1.6×10^3 CFU ml^{-1}) or *S. cerevisiae* (3.8×10^7–3.8×10^3 CFU ml^{-1}) in 0.5 ml of fresh, identical medium, which was added to the apical side of filter. IL-8 secreted into the basolateral supernatants (0.5 ml) was assayed by ELISA. Data are presented as means ± SD of five cultures. Data shown are representative of at least three independent experiments. $^*P < 0.05$ compared with untreated cells.

medium without butyric acid after co-culture for 7 or 24 h (data not shown). In the presence of 10 mM butyric acid, however, *C. albicans* concentrations of at least 1.6×10^6 CFU ml^{-1}, and *S. cerevisiae* concentrations of at least 3.8×10^6 CFU ml^{-1} induced dose-dependent secretion of IL-8 after 7 h (Fig. 2A). In contrast, after 24 h, maximal IL-8 secretion was seen at 1.6×10^5 CFU ml^{-1} *C. albicans* and at 3.8×10^5 CFU ml^{-1} *S. cerevisiae* (Fig. 2B). Heat-killed cells of both species had no effect on IL-8 secretion (data not shown).

To determine whether yeast-induced enhancement of IL-8 secretion is accompanied by increased levels of IL-8-specific mRNA, we assayed IL-8 message in these cultures by quantitative real-time PCR. Although Caco-2 cells pre-cultured with butyric acid for 4 days in the absence of yeast (0 h) showed enhanced levels of IL-8 mRNA, the addition of *C. albicans* (3.8×10^7 CFU ml^{-1}) or *S. cerevisiae* (1.4×10^7 CFU ml^{-1}) for 3 or 7 h further increased the IL-8 mRNA production (Table 2).

3.3. *TER of Caco-2 cell monolayers is decreased by* C. albicans *more than by* S. cerevisiae

We also compared the effects of *C. albicans* and *S. cerevisiae* on TER of Caco-2 cell monolayers cultured in the medium containing 10 mM butyric acid. After 7 h in culture, higher concentrations of *C. albicans* ($\geqslant 1.6\times10^4$ CFU ml^{-1}), but not *S. cerevisiae*, significantly decreased TER (Fig. 3A). After co-culturing for 24 h, *S. cerevisiae* had some effect, but the effect of *C. albicans* was still significantly greater (Fig. 3B).

3.4. Saccharomyces cerevisiae *cell wall components also enhance IL-8 secretion*

We assayed the effect of the *S. cerevisiae* cell wall components, zymosan, glucan and mannan (0, 10, 100 and 1000 μg ml^{-1}), to induce IL-8 secretion by Caco-2 cells in the presence of 10 mM butyric acid. We found that zymosan and glucan each had a significant, dose-dependent effect on IL-8 secretion after co-culture for 7 and 24 h. In contrast, mannan did not enhance IL-8

Table 2
Effect of butyric acid and intact yeast cells on expression of IL-8 mRNA in Caco-2 cells

Butyric acid	Microorganism (CFU ml^{-1})		IL-8		
			0 h	3 h	7 h
0 mM	None		1.0	1.0	1.5
0 mM	*C. albicans*	(6.0 × 10^6)	–	1.2	1.2
0 mM	*S. cerevisiae*	(2.2 × 10^6)	–	0.6	1.6
10 mM	None		37	42	33
10 mM	*C. albicans*	(6.0 × 10^6)	–	221	663
10 mM	*S. cerevisiae*	(2.2 × 10^6)	–	130	435

Caco-2 cells, seeded at 6 × 10^5 cells well^{-1} on a 45-mm Millicell-HA filter, were pre-cultured for 4 days in medium with or without 10 mM butyric acid and cultured for 0, 3, or 7 h in the presence or absence of *C. albicans* or *S. cerevisiae* in 2.5 ml of identical medium, which was added to the apical side of the filters. Data are shown as expression of IL-8 mRNA relative to that in Caco-2 cells solely pre-cultured for 4 days in medium without butyric acid. The level of each transcript was normalized to that of GAPDH in the same sample. The results shown are representative of four independent experiments.

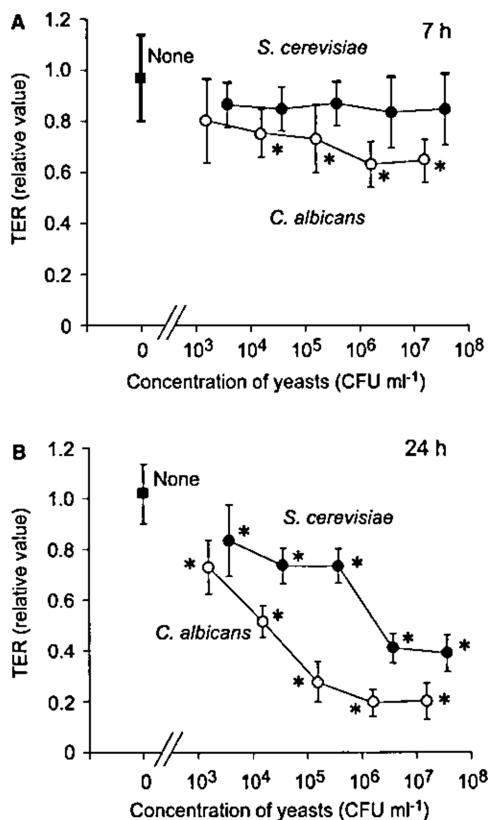

Fig. 3. Effects of intact yeasts on transepithelial electrical resistance (TER) of Caco-2 monolayers. Caco-2 cells were seeded at 1.5 × 10^5 cells well^{-1} on a 12-mm Millicell-HA filter and pre-cultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 10 mM butyric acid. The cells were cultured for 7 (A) or 24 h (B) in the presence or absence of *C. albicans* (1.6 × 10^7–1.6 × 10^3 CFU ml^{-1}) or *S. cerevisiae* (3.8 × 10^7–3.8 × 10^3 CFU ml^{-1}) in 0.5 ml of fresh, identical medium, which was added to the apical side of the filter. The ratios of TER before and after culture with yeast are shown. Data are presented as means ± SD of five cultures. Data shown are representative of at least three independent experiments. $^*P < 0.05$ compared with untreated cells.

secretion (Fig. 4). When these cells were pre- and co-cultured in medium in the absence of butyric acid, however, 7 or 24 h culture with zymosan, glucan or mannan had no effect on IL-8 secretion (data not shown).

3.5. Butyric acid enhances TLR1, TLR6 and dectin-1 mRNAs

Since cytokine response to zymosan has been shown to be mediated by TLR2 with either TLR1 or TLR6 [16,17] and dectin-1 [22–24], we assayed the effects of butyric acid and yeast cells on the levels of TLR and dectin-1 mRNAs. In the absence of butyric acid and in the absence of yeast stimulation (0 h), Caco-2 cells expressed TLR1, TLR2, TLR6 and dectin-1 mRNAs as shown by RT-PCR (Fig. 5), and quantitative RT-PCR using the LightCycler system showed that the expression of each message was not affected by stimulation with yeast cells for 7 h (Table 3). In contrast, culture of Caco-2 cells for 4 days in medium containing 10 mM butyric acid without stimulation with yeasts (0 and 7 h) increased expression of TLR1, TLR6 and dectin-1 mRNA, but had no effect on TLR2 message (Table 3). These results shown in Table 3 suggest that, in the presence of 10 mM butyric acid, stimulation of Caco-2 cells with yeast cells decreased TLR1 and TLR6 mRNAs, but increased dectin-1 mRNA. Data from repeated experiments, however, showed that, when stimulated with yeasts, the relative amounts of TLRs and dectin-1 mRNA increased or decreased less than threefold compared with expression in unstimulated Caco-2 cells (data not shown).

4. Discussion

Our finding, that both *C. albicans* and *S. cerevisiae* enhance the production of IL-8 by IEC-like Caco-2 cells in the presence of butyric acid suggests that some yeast

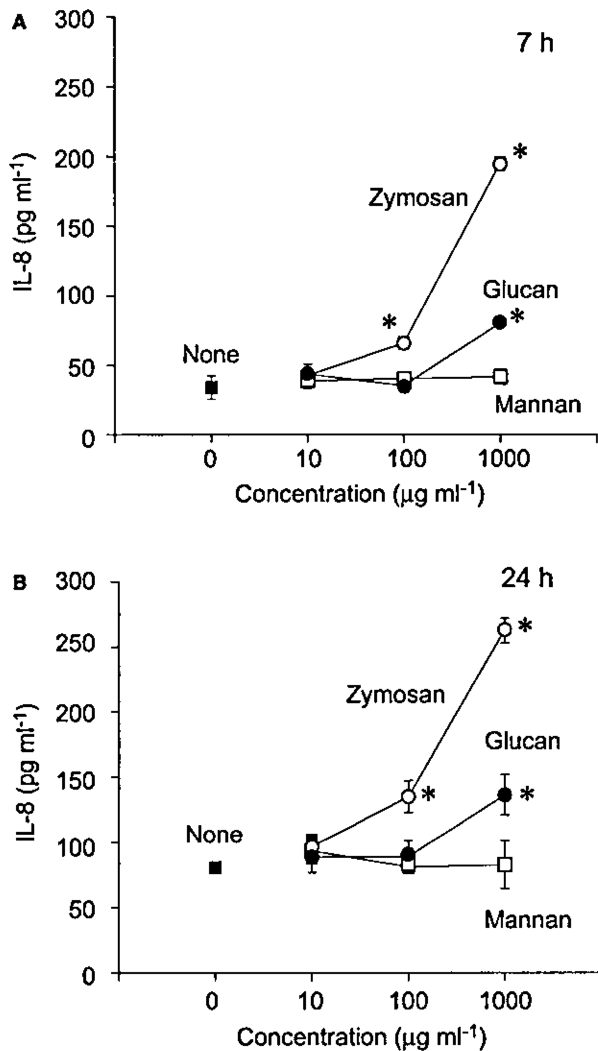

Fig. 4. IL-8 secretion from Caco-2 cells in response to *S. cerevisiae* cell wall components in the presence of butyric acid. Caco-2 cells were seeded at 1.5×10^5 cells well^{-1} on a 12-mm Millicell-HA filter and pre-cultured for 4 days in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, also containing 10 mM butyric acid. The cells were cultured for 7 (A) or 24 h (B) in the presence or absence of various concentrations of zymosan, glucan or mannan, in 0.5 ml identical medium added to the apical side of filter. IL-8 secreted into the basolateral supernatants (0.5 ml) was assayed by ELISA. Data are presented as means ± SD of triplicate cultures. Data shown are representative of at least three independent experiments. $^*P < 0.05$ compared with untreated cells.

cells stimulate the intestinal immune system by inducing cytokine secretion from IEC. The observation that *S. cerevisiae* cells enhanced IL-8 production, albeit to a lesser extent than *C. albicans*, suggests that both opportunistic and nonpathogenic yeasts in the intestine stimulate the immune system via cytokine secretion from IEC. Under normal conditions, the weak stimulation of the host's mucosal immune system by nonpathogenic microorganisms may have favorable effects

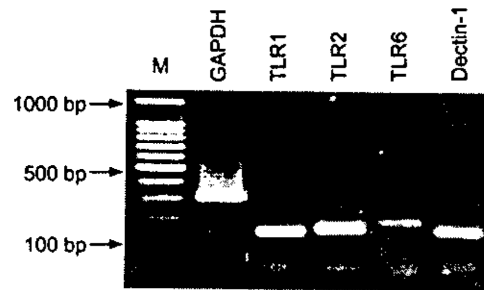

Fig. 5. RT-PCR assay of TLR1, TLR2, TLR6, and dectin-1 mRNAs in Caco-2 cells cultured in DMEM, supplemented with 1 mM L-glutamine, 10 mM HEPES and 15% heat inactivated fetal bovine serum, in the absence of butyric acid and in the absence of yeast stimulation (0 h). Lane M, 100 bp DNA ladder. Expression of mRNA for GAPDH, a housekeeping gene, was also detected as a positive control.

on the development and maintenance of this immune system.

Candida albicans is known to produce phospholipomannan, mannoprotein, proteinases, and high-affinity iron permease, all of which are considered virulent factors [32–34]. In addition, *C. albicans* has been shown to form germ tubes in response to environmental factors, such as temperature, pH, hemin, or some serum components [35–37]. These substances produced by *C. albicans* and/or these morphological changes are thought to be involved in yeast adhesion to host cells, cytokine production, and/or yeast pathogenicity. In this study, we confirmed that *C. albicans* germinated during the 7 and 24 h co-culture period (data not shown). The substances expressed by, or the morphological changes induced in, *S. cerevisiae* may be different from those in *C. albicans*, which may account for the different effects of these yeasts on cytokine secretion and TER in Caco-2 cells. The more rapid decrease in TER induced by *C. albicans* compared with *S. cerevisiae* suggests that the former induces a greater degree of damage on Caco-2 cells.

We found that the yeast cell wall component, zymosan, enhanced IL-8 secretion from Caco-2 cells. The zymosan used in our study was supposed to expose glucan to the outer side, according to the product information [38,39]. Zymosan has been reported to be recognized by TLR2, together with either TLR1 or TLR6 [16,17]. We also found that glucan enhanced IL-8 secretion. Since the inflammatory response of macrophages to β-glucan has been reported to be inhibited in a dominant-negative mutant of MyD88, a mediator of signaling through the Toll-like receptor/interleukin-1 receptor-like (TIR) domain, glucan can be considered to induce cytokine responses through TLRs [40]. However, β-glucan is also known to bind to receptors other than TLRs, including dectin-1, complement receptor 3 and scavenger receptor [23,24,41,42]. Thus, the glucan in zymosan may have been recognized by TLRs and other receptors, such as dectin-1, leading to enhanced IL-8 secretion from Caco-2 cells.

Table 3
Effect of butyric acid and intact yeast cells on TLR and dectin-1 mRNA levels in Caco-2 cells

Butyric acid	Microorganism ($CFU\ ml^{-1}$)		Relative mRNA expression index							
			TLR1		TLR2		TLR6		Dectin-1	
			0 h	7 h	0 h	7 h	0 h	7 h	0 h	7 h
0 mM	None		1.0	1.2	1.0	1.1	1.0	2.4	1.0	0.2
0 mM	*C. albicans*	(2.4×10^6)	–	1.8	–	1.8	–	2.0	–	2.1
0 mM	*S. cerevisiae*	(2.4×10^6)	–	1.4	–	0.8	–	2.8	–	0.4
10 mM	None		24	38	0.4	0.6	49	66	7.3	4.7
10 mM	*C. albicans*	(2.4×10^6)	–	14	–	0.3	–	22	–	15
10 mM	*S. cerevisiae*	(2.4×10^6)	–	26	–	0.7	–	32	–	11

Caco-2 cells, seeded at 6×10^5 cells $well^{-1}$ on a 45-mm Millicell-HA filter, were pre-cultured for 4 days in medium with or without 10 mM butyric acid cultured for 0 or 7 h in the presence or absence of *C. albicans* or *S. cerevisiae* in 2.5 ml of identical medium, which was added to the apical side of the filters. Data are shown as expression of each specific mRNA relative to that in Caco-2 cells pre-cultured for 4 days in medium without butyric acid. The level of each transcript was normalized to that of GAPDH in the same sample. The experiments were repeated at least three times.

On the other hand, intact yeast cells contain glucan in the inner layer of cell walls. We found that intact cells enhanced IL-8 secretion. Since some TLRs are expressed on vesicle membranes as well as on the cell surface [43,44] and recruited to phagosomes [16,17], intact yeast cells may have been taken up by Caco-2 cells and degraded, thus exposing their inner cell wall components, including glucan, stimulating TLR signaling inside Caco-2 cells. It is also possible that components other than glucan on the surface of yeast cells may induce various responses of mammalian cells. *C. albicans* phospholipomannan has been reported to be an important TLR2 ligand [45], and *C. albicans*- and *S. cerevisiae*-derived mannan have been shown to activate TLR4 and CD14 on human monocytes [46]. Furthermore, other components of yeast cells may bind to other PRRs, eliciting immune responses such as cytokine production. Further work is needed to determine the mechanism of yeast recognition by mammalian cells and the ensuing induction of cytokine responses.

We found that butyric acid increased the expression of TLR1, TLR6 and dectin-1 mRNAs in Caco-2 cells (Table 3). Thus, the increased expression of these molecules may be responsible for the enhanced response of Caco-2 cells to the yeast cells caused by butyric acid. Otherwise, butyric acid may affect intracellular signaling pathways downstream of some PRRs, leading to the enhancement of cytokine production. In the presence of butyric acid, stimulation of Caco-2 cells with *C. albicans* or *S. cerevisiae* either increased or decreased the expression of TLR1, TLR6 and dectin-1 mRNAs within a three-fold range, compared with expression in Caco-2 cells not stimulated with yeasts. Since the expression of TLR mRNA is known to be regulated by stimulation with the lipopolysaccharide, lipoarabinomannan and certain cytokines [47–49], it is possible that yeast stimulation directly altered mRNA expression levels. Otherwise, these alterations in mRNA expression in the presence of yeasts found in our experiment may be caused by slight differences in the characteristics of the Caco-2 cells used in each experiment, and/or analytical errors in the real-time RT-PCR method. Further experiments are required to determine the effects of yeast cells on the PAMP receptor expression.

The concentration of butyrate in the human large intestine has been estimated at 5–24 mM, depending on daily food intake, and higher than its concentration in the small intestine [50,51]. In Caco-2 cells, butyrate has been reported to affect the cell cycle, alkaline phosphatase activity, cell migration, cytokine secretion, and TER, depending on the extent of differentiation [28,52–56]. There have been conflicting reports on the effect of butyrate on IL-8 production by Caco-2 cells, with some groups reporting enhancement [53,54] and others reporting inhibition [55,56]. We confirmed that pre-culture of Caco-2 cells with 10 mM butyric acid for 4 days but not for 1 day enhanced the subsequent IL-8 secretion from Caco-2 cells stimulated with yeast cells in the presence of 10 mM butyric acid. It seems that the effects of butyric acid on cytokine secretion from IEC vary depending on the concentration of butyric acid, incubation time, and extent of IEC differentiation. Further analyses are necessary to determine the effects of butyric acid on Caco-2 cells.

Live yeast cells induced IL-8 secretion, but heat-killed cells of both species had no effect. This confirms previous findings that live *C. albicans* cells, but not heat-killed cells, induce IL-1α, IL-1β, IL-8, TNF and GM-CSF production by oral epithelial cells [14,15]. The inability of heat-killed yeasts to induce cytokine production may be due to the modification and/or destruction of the substance(s) responsible for cytokine induction, or by the absence of metabolite(s) secreted from live cells that participate in cytokine production. The induction of IL-8 by zymosan and glucan, which are extracted under harsher conditions than those we used to kill yeasts, suggests that our heat treatment may have destroyed labile substance(s) on the surface of these cells, without exposing any internal active substance(s) that retain the ability to induce cytokine production.

IL-8 is a chemokine, or chemoattractant cytokine, which has been shown to recruit neutrophils and trigger the firm adhesion of monocytes to vascular endothelial cells [57,58]. Although IL-8 secretion is often detected in proinflammatory responses, the ability of yeasts to enhance IL-8 secretion from IEC does not necessarily mean that yeasts in the intestine are promoters of inflammation. Triggering of the immune system by nonpathogenic microorganisms in the absence of inflammation may serve to enhance host defenses against occasional pathogenic microorganisms. For example, it has been reported that probiotic *Streptococcus thermophilus* and nonpathogenic *E. coli* induce IL-8 production from IEC line HT29/19A [13]. In the presence of underlying leucocytes, nonpathogenic *Lactobacillus sakei* has been shown to induce IL-8 mRNA expression by Caco-2 cells [11]. In addition, a commensal oral bacterium, *Fusobacterium nucleatum*, was found to induce IL-8 production, independent of inflammation, from gingival epithelial cells [59]. Thus, our finding, that *S. cerevisiae* induce IL-8 secretion weakly, does not suggest that this yeast provoke inflammation; rather, the microorganism may serve to prime the intestinal immune system against pathogenic organisms.

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Vaccination with an immunodominant peptide of bovine type II collagen induces an anti-TCR response, and modulates the onset and severity of collagen-induced arthritis

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Abstract

T cell responses directed toward TCR-derived peptides have been shown to be an important regulatory mechanism of protection against autoimmunity. Here, we show that a naturally induced TCR-directed immune response can delay the onset of collagen-induced arthritis (CIA), an animal model of autoimmune rheumatoid arthritis in humans. DBA/1 mice were pretreated with an immunodominant peptide, p245–270, from bovine type II collagen (bCII) and were subsequently immunized with whole bCII for the induction of arthritis. The results showed that preactivation of p245–270-reactive cells delayed the onset and reduced the severity of CIA, compared with animals in the control group. Interestingly, the serum antibody response to bCII and the bCII-specific cytokine were not affected under these conditions. This result indicates that the observed protection was neither directly due to a lower antibody response nor due to the immune deviation of the anti-bCII T cell response. Furthermore, immunization with p245–270, but not bCII, induced a strong response to the B5 peptide, an immunodominant region of the TCR $V_{\beta}8.2$ (amino acids 76–101) that binds very strongly to I-A^q. These data suggest that at a critical phase in the loss of self-tolerance, an effective anti-TCR response, induced naturally, can regulate the pathogenic autoimmune response and thus may provide protection against autoimmunity.

Introduction

Collagen-induced arthritis (CIA) is a disease model of human autoimmune rheumatoid arthritis and can be induced by injection of susceptible animals with heterologous type II collagen (CII) in adjuvant. Both MHC and non-MHC genes are involved in the susceptibility to CIA, and H-2^q and H-2^r mice are the most susceptible haplotypes (1). As in most autoimmune disease models, autoreactive T cells are essential in CIA. CII-reactive $CD4^+$ T cells, derived from DBA/1 mice, were shown to develop attenuated CIA by passive transfer (2,3). Also, by immunohistological techniques, $CD4^+$ and IL-2 receptor-expressing T lymphocytes were regularly detected in the affected joints (4). Antibody to collagen molecules can also transfer attenuated disease (5), indicating that a humoral response to CII is also involved in this disease. Early studies of Osman *et al.* showed that CII-reactive T cell hybridomas in DBA/1LacJ mice preferentially use the TCR $V_{\beta}8.2$ gene

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segment (6). Thus, anti-$V_\beta 8.2$ antibody treatment also resulted in a significant reduction in the incidence of arthritis in DBA/1LacJ mice.

Self-reactive T cells bearing the $V_\beta 8.2$ gene segment are often utilized in experimental autoimmune disease models, such as experimental autoimmune encephalomyelitis (EAE) (7) or diabetes in non-obese diabetic (NOD) mice (8). In earlier studies it was shown that natural remission of EAE was accompanied by an immune response against the TCR peptide from the $V_\beta 8.2$ chain (9). Furthermore, we investigated the T cell proliferative response against various TCR $V_\beta 8.2$-derived peptides and found that the immune response against the B5 peptide corresponding to residues 76–101 of the TCR $V_\beta 8.2$ chain was spontaneously induced in EAE-susceptible mice (10). We found that $CD4^+$ regulatory T cells that recognize B5 and $CD8^+$ regulatory T cells that were reactive to a different $V_\beta 8.2$ determinant from the CDR1/2 region (corresponding to 41–50) controlled the disease. Furthermore, we found that injection of B5 peptide has a suppressive effect against CIA (11) as well as EAE. In this study we show that CIA was significantly delayed and inhibited by the injection of p245-270, an immunodominant determinant of bovine CII (bCII) in DBA/1 mice, through activation of the B5-specific regulatory T cells. Notably, the B5 peptide binds to the $I\text{-}A^q$ molecule with a high binding affinity. These data suggest that during an autoimmune response to self-CII, feedback TCR-peptide-reactive regulatory T cell responses are also induced which are involved in the maintenance of peripheral self-tolerance.

Methods

Mice

Female DBA/1LacJ and DBA/1JNCrj (referred to as DBA/1J) mice (6–8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and Charles River Japan (Yokohama, Japan) respectively.

Protein and peptide

bCII used for experiments with DBA/1LacJ mice was purchased from the Institute Jacques (Paris, France). bCII used for experiments with DBA/1J was purified by the salting-out technique after the digestion of bovine joint cartilage with pepsin. Its purity was confirmed by SDS-PAGE analysis. B1 (amino acid 1–30 with an additional C-terminal leucine) and B5 (amino acids 76–101) peptides from mouse TCR $V_\beta 8.2$, chain, hen egg lysozyme (HEL) 74–90 and β-Lg119–133 were synthesized by Dr S. Horvath at the California Institute of Technology (Pasadena, CA) and purified by reversed-phase HPLC, as described earlier (12). Peptides of residues 245–270 (p245-270) and 316–333 (p316-333) from bCII were synthesized with a 430A machine (Applied Biosystems, Foster City, CA), and purified with reversed-phase HPLC.

The sequences of peptides used in this study were: p245-270 (bCII), ATGPLGPKGQTGEPGIAGFKGEQGPK; p316-333 (bCII), GFBGADGIAGPKGPBGER; B1 (TCR$V_\beta 8.2$ 1–30L), EAAVTQSPRNKVAVTGGKVTLSCNQTNNHNL; B5 (TCR$V_\beta 8.2$ 76–101), LILELATPSQTSVYFCASGDAGGGYE; HEL74-90, NLCNIPCSALLSSDITA; HEL93-113, NCAKKIVSDGNGMNAWVAWRN; β-Lg119-133, CQCLVRTPEVDDEAL.

CIA induction

Ten DBA/1J mice in each group were injected intradermally at the base of the tail with 100 µl of an emulsion containing (i) bCII p245-270 (15 µg/mouse) plus complete Freund's adjuvant (CFA; Difco, Detroit, MI), (ii) bCII p316-333 (10 µg/mouse) plus CFA, (iii) PBS/CFA or (iv) nothing, and 14 days later they were further injected with bCII (100 µg/mouse) plus incomplete Freund's adjuvant (IFA; Difco). The peptides and bCII were dissolved in PBS and 0.06% acetic acid respectively, and emulsified with the same volume of adjuvant. The clinical severity of the arthritis was assessed according to an arthritis scale for each limb, which was subjectively graded on a scale of 0–3: 0 = absence of arthritis, 1 = one finger swelling or mild swelling, 2 = two fingers swelling or swelling of tarsus and ankle, and 3 = hard swelling or bony deformity. A sum of the scale for four paws of a single mouse was calculated and a total of this sum in a group of mice was obtained. The arthritic index was defined by dividing this total by the number of mice in the group. Blood was taken every 10 days during the observation period to measure the antibody in the serum. Ten mice in each group were injected with bCII p245-270/CFA, bCII p316-333/CFA, PBS/CFA or nothing and then all the mice were s.c. immunized with bCII for the induction of the disease. We determined the severity of the arthritis by calculating the arthritic index for these four groups of mice (Fig. 1). We also compared the incidence of arthritis by calculating the percentages of arthritic mice and arthritic legs in each group. A mouse was regarded as arthritic when an individual had swelling in at least one leg. The percentage of arthritic mice was obtained by dividing the number of arthritic mice by the total number of mice in each group. The percentage of arthritic legs was obtained by dividing the number of arthritic legs by the number of all mice for each group; arthritic legs were those having swelling or deformity.

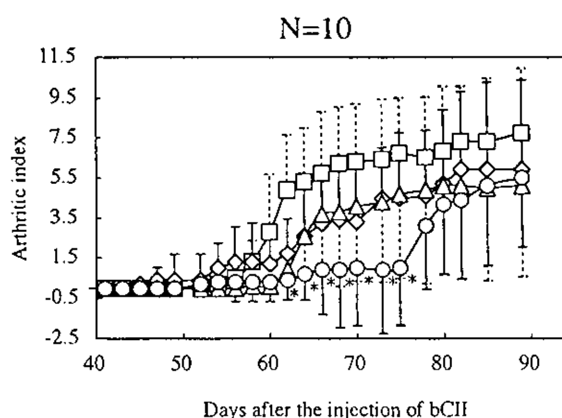

Fig. 1. The arthritic index of CIA after preimmunization with bCII-derived peptide. Groups of DBA/1J mice (10 in each) were preimmunized with p245-270 (circles) or p316-333 (inverted triangles) or PBS (diamonds) or were not preimmunized (squares) on day 0. CIA was induced on day 14 by injecting mice with bCII plus IFA, as described in the Methods. The data were analyzed by the Mann–Whitney *U*-test. *Significant difference at $P < 0.05$.

ELISA for detecting anti-bCII antibody in the serum

The titer of anti-bCII antibody in serum from mice treated for the CIA induction previously described was determined by ELISA. bCII was dissolved in PBS at a concentration of 100 μg/ml by heating; it was incubated in the wells of Maxisorp plates (Nunc, Roskilde, Denmark) overnight at 4°C. The solutions were removed and the wells were washed with PBS-Tween (0.05% Tween 20 in PBS). The serum samples were diluted with PBS-Tween and incubated in the wells for 2 h at room temperature. Bound antibody to the solid phase was detected by two sequential sets of incubation with alkaline phosphatase (ALP)-labeled anti-mouse subclass antibody for 2 h and its substrate of *p*-nitrophenylphosphate disodium salt for ~30 min after washing the wells with PBS-Tween. The conjugates used here were ALP-labeled anti-mouse IgG1, IgG2a and IgG2b (all from Zymed, South San Francisco, CA). Color development was measured at 405 nm.

Cell preparation and culture for proliferation and cytokine release assay

DBA/1J mice were s.c. injected with bCII p245–270 plus CFA H37Ra, PBS plus CFA or nothing. Seven days following the preinjection, the mice were immunized with 100 μg of bCII plus IFA in the base of the tail or treated with nothing. After 10 days, lymphocytes were removed from popliteal and inguinal lymph nodes and cultured at 5×10^5 in each well of a 96-well plate with 200 μl of RPMI 1640 containing 10% FBS with or without B5 (amino acids 76–101, $TCRV_{\beta}8.2$) peptide or p245–270. IFN-γ in the supernatant was determined by sandwich ELISA using R4-6A2 (PharMingen, San Diego, CA) as the first antibody and XMG1.2 (PharMingen) as the second antibody. In other experiments, DBA/1LacJ mice were s.c. vaccinated with p245–270 (10 μg/mouse) plus CFA or PBS plus CFA as controls. Lymphocytes were taken and cultured in the protein-free medium X-vivo 20 (Biowhittaker, Walkersville, MD) with either B5 or B1 (amino acids 1–30, 31L, $V_{\beta}8.2$), or without any antigenic peptide. Three days later, 1 μCi [^{3}H]thymidine was added to the culture and incubated overnight. The incorporation of thymidine into cultured cells was measured by liquid scintillation.

Competitive-binding assay of B5 (amino acids 76–101, $TCRV_{\beta}8.2$) to I-A^q molecules

I-A^q molecules were purified from spleens of untreated DBA/1J mice as previously described (13). In brief, splenocytes from DBA/1J mice were solubilized and I-A^q molecules were purified with affinity column using anti-I-A^q antibody M5/114.15.2. Binding assay was performed as previously reported (14). The purified I-A^q molecule (14 nM), 2 μM or absence of biotinylated β-Lg119–133 with various concentrations of B1, B5, β-Lg119–133 or without competitor peptide were preincubated in 50 mM citrate/phosphate buffer, pH 5.0 containing 0.2% NP-40 and 2 mM EDTA at 37°C for 48 h. Meanwhile, Nunc Maxisorp plates were coated with anti-rat Ig at room temperature for 2 h and with M5/114.15.2 for another 2 h. Non-specific binding was blocked at 4°C for overnight with 50 mM Tris-HCl, pH 7.5 containing 0.3% BSA and 0.1% Tween 20. The incubated mixtures were transferred to the plate and incubated for 1.5 h at room temperature. Biotinylated peptide bound to the I-A^q molecule was detected with AP-streptavidin (Zymed) by development with its substrate, *p*-nitrophenylphosphate disodium. Color development was measured at 405 nm.

Results

Suppression of CIA by preimmunization with bCII p245–270

CIA can be induced in H-2^q DBA/1 mice by injection with bCII plus adjuvant. In our case, a single injection of 6- to 8-week-old female DBA/1J with bCII plus IFA could efficiently induce CIA with almost 100% incidence. Previous reports (15,16) and our results (data not shown) had indicated that p245–270 contained a dominant T cell determinant, judged from the significant proliferative response to p245–270 in lymph node cells from mice immunized with bCII. DBA/1J mice were preimmunized with p245–270 from bCII plus CFA in order to activate T cells specific for the immunodominant determinant in this region. We studied the effect of this preactivation of the dominant T cell population on the incidence of CIA. The data in Fig. 1 indicate that p245–270 preimmunization delayed the onset of arthritis and reduced the severity of the disease, compared with the other three groups of mice. The percentages of arthritic legs and arthritic mice for the four groups showed a similar pattern to that of the arthritic index. We also tested another schedule for studying the effect of p245–270 preimmunization. Before injection with bCII, the mice were treated twice with p245–270 or other peptides. Also, in this experimental set-up (data not shown), the onset of CIA was much delayed and the severity was reduced compared with mice in the other three groups. When we preinjected mice with p245–270, 35 days before injection with bCII (data not shown), the onset of CIA was also suppressed. However, the efficiency of the delay was less than with other protocols having shorter intervals between the p245–270 and bCII injections.

Suppression of CIA is independent of alterations in the antibody response or immune deviation of the anti-bCII response

We next examined the antibody response to bCII in the serum of individual mice from the four groups. The antibody titer versus the days after the first treatment of mice was plotted. After 21 days from the first treatment, the IgG1 and IgG2a titers to bCII increased. Although the onset and the severity of CIA were altered by bCII p245–270 preimmunization, the IgG1 antibody response to bCII was similar to that of the other three groups of mice (Fig. 2A). The response of IgG2a possessing complement-binding activity has been proposed to be more relevant to CIA than the IgG1 response (17). However, the mice preinjected with p245–270 showed an IgG2a titer specific for bCII that was similar to the other groups (Fig. 2B). These observations indicate that the suppression of CIA by p245–270 preimmunization was independent of the antibody responses to bCII.

Furthermore, we measured the amount of IFN-γ secreted by T cells specific for p245–270 (Fig. 3). Since the balance of the cytokines produced by T_h1/T_h2 subsets of the T_h cells plays an important role in the development of CIA (18), we considered that the regulation caused by preimmunization with p245–270

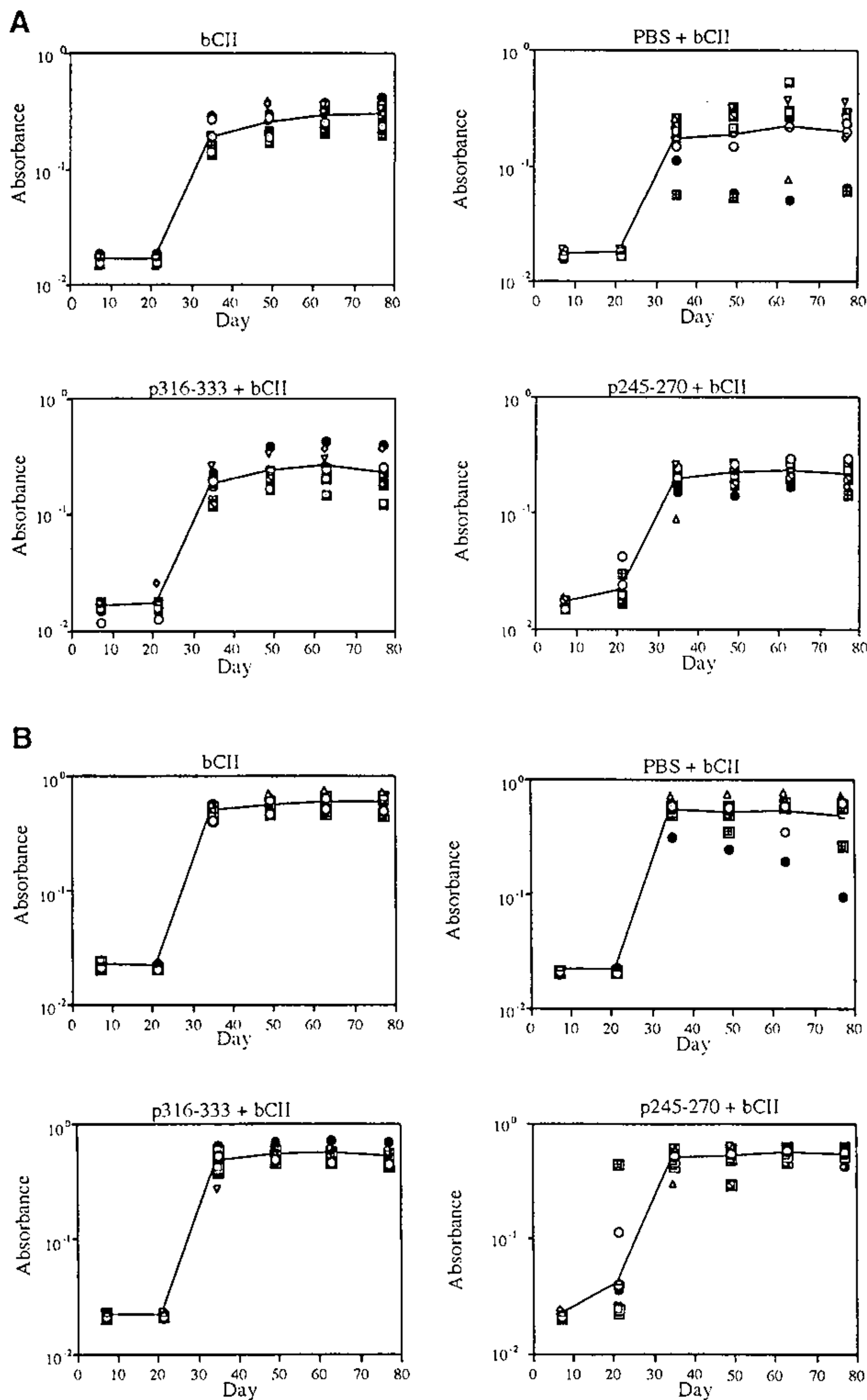

Fig. 2. Antibody responses against bCII. Anti-bCII IgG1 (A) and IgG2a (B) antibody amounts in the serum of individual mice were measured. Mice were the same as those shown in Fig. 1. Each symbol indicates absorbance for each individual mouse by ELISA and the average values for each day are linked with a straight line. Each panel indicates the following group: 'bCII' = mice immunized with bCII plus IFA without any preimmunization; 'PBS + bCII' = mice preimmunized with PBS plus CFA on day 0 and immunized with bCII on day 21; 'p316-333 + bCII' = mice preimmunized with p316-333 on day 0 and immunized with bCII on day 21; 'p245-270 + bCII' = mice preimmunized with p245-270 on day 0 and immunized with bCII on day 21.

might have been due to T_h1/T_h2 deviation. However, the IFN-γ response against p245-270 was clearly shown in mice preimmunized with p245-270 before the immunization with bCII in a dose-dependent manner. The IFN-γ response against

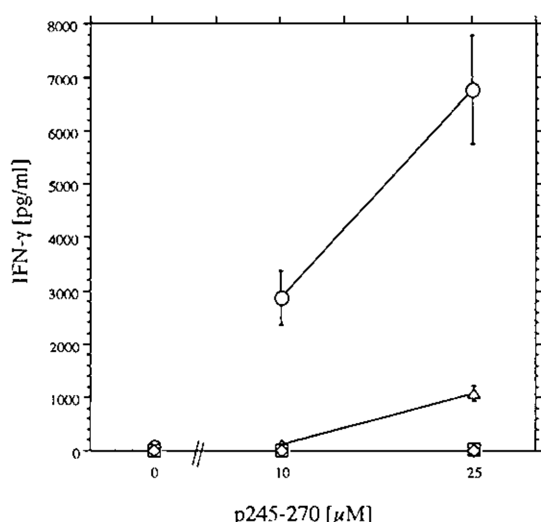

Fig. 3. The IFN-γ production of lymphocytes specific for p245–270. The triangles indicate the response of DBA/1J mice immunized with p245–270 on day 0 without further treatment; circles, preimmunized with p245–270 on day 0 and immunized with bCII on day 7; squares, immunized with bCII without preimmunization; diamonds, preimmunized with PBS on day 0 and immunized with bCII on day 7. Lymphocytes were taken on day 17 and cultured with different concentrations of p245–270 or without antigen.

p245–270 could not be detected in mice immunized with bCII without preimmunization with p245–270. A slight response was shown in mice immunized with p245–270 without further immunization. We also tested IL-2 and IL-4 included in the same supernatant with a bioassay. No significant difference between the two groups was apparent (data not shown). These results implied that regulation of CIA by preimmunization with p245–270 was not due to T_h1/T_h2 deviation.

We then considered the possibility that preimmunization with p245–270 regulated CIA via the activation of a natural immune regulatory system (11). We have shown earlier that a TCR-specific immune regulatory response inhibited CIA. We set out to examine whether immunization of DBA/1J mice with p245–270 could activate a TCR-specific response.

Immunization of DBA/1J mice with bCII p245–270 induces a T cell response against the dominant TCR peptide (B5: amino acids 76–101, $V_\beta8.2$)

Lymphocytes from individual DBA/1J mice immunized with p245–270 or control mice were examined for a TCR-specific proliferative response (Fig. 4A and B). Mice immunized with p245–270 showed proliferative responses to the B5 peptide, but not to the control peptide B1 (amino acids 1–30L, $V_\beta8.2$). We found similar data indicating a response to B5 in a repeated experiment using pooled lymphocytes from another set of three mice. Interestingly, lymphocytes from mice injected with PBS plus CFA also proliferated against B5, although the response was much weaker than those from mice immunized with p245–270. This result is consistent with the data in Fig. 1, which showed that PBS plus CFA or control peptide plus CFA delayed the onset of CIA. We also found that splenocytes from some, but not all, individuals showed T cell proliferative responses against B5 even without any treatment (unpublished data). These facts imply that the TCR-specific response represents a physiological immune regulatory system that is very easily triggered. Previous reports suggested that B5-specific CD4+ regulatory T cells are required to produce a T_h1 response for effective regulation and prevention of autoimmune disease (19). We measured the B5-specific IFN-γ response of lymphocytes from the mice that were treated with the same schedule as the CIA induction (Fig. 4C). The result indicated that immunization only once with p245–270 induced a significant IFN-γ response to B5 and that preimmunization with p245–270 before immunization with bCII induced the strong IFN-γ response to the B5 peptide, suggesting that immunization with p245–270 before the induction of CIA induced an effective regulatory response. On the other hand, no IFN-γ was detected from other groups, those preinjected with PBS or nothing, before the immunization with bCII.

B5 (amino acids 76–101, $V_\beta8.2$) peptide binds to MHC class II I-A^q molecules

Next, we investigated whether TCR peptide B5 is able to bind to I-A^q molecules by a competitive-binding assay using the biotinylated β-Lg119–133 peptide (Fig. 5). In this experiment, increasing amounts of B1 peptide ($V_\beta8.2$, amino acids 1–30L), B5 or non-labeled β-Lg119–133 peptide were added as competitors to the mixture of I-A^q and biotinylated β-Lg119–133. The result indicated that peptides of B5 and β-Lg119–133 could bind to I-A^q molecules, while B1 did not (the slight inhibition by B1 peptide is a background effect). It could be shown that B5 and β-Lg119–133 competed for binding to I-A^q, suggesting that this TCR peptide binds well to I-A^q molecules and, thereby, can be presented to appropriate CD4+ regulatory T cell populations, as has been shown in EAE (12).

Discussion

Regulation of immune responses using peptides derived from the determinant region of CII has been shown as an effective method to control CIA. Development of CIA likely requires responses of both T cells and antibodies. Although p245–270 contains the dominant determinant of CII in H-2^q mice (20), the peptide fragment that contains p245–270 cannot induce CIA (21) since it does not contain the appropriate B cell epitope of CII. However, i.v. or neonatal administration of peptide containing this determinant from chicken CII could inhibit CIA in DBA/1 mice (16). It has also been shown that oral administration of human CII or chicken CII determinant peptide inhibited CIA in DBA/1 mice (22). Previously we screened overlapping peptides derived from bCII by intranasal administration and found that the peptide corresponding to 253–272 of bCII could most effectively inhibit CIA (23). In these cases, the administration of peptides reduced either the T cell response or the antibody response against CII or the immunizing peptide due to tolerization of pathogenic lymphocytes.

Administration of peptide with adjuvant is a general protocol for induction of antigen-specific T cells. In this report we induced activation of bCII-reactive T cells by immunization of DBA/1 mice with peptide plus adjuvant (Fig. 3). Thus, regulation of CIA in our case was caused by the activation of

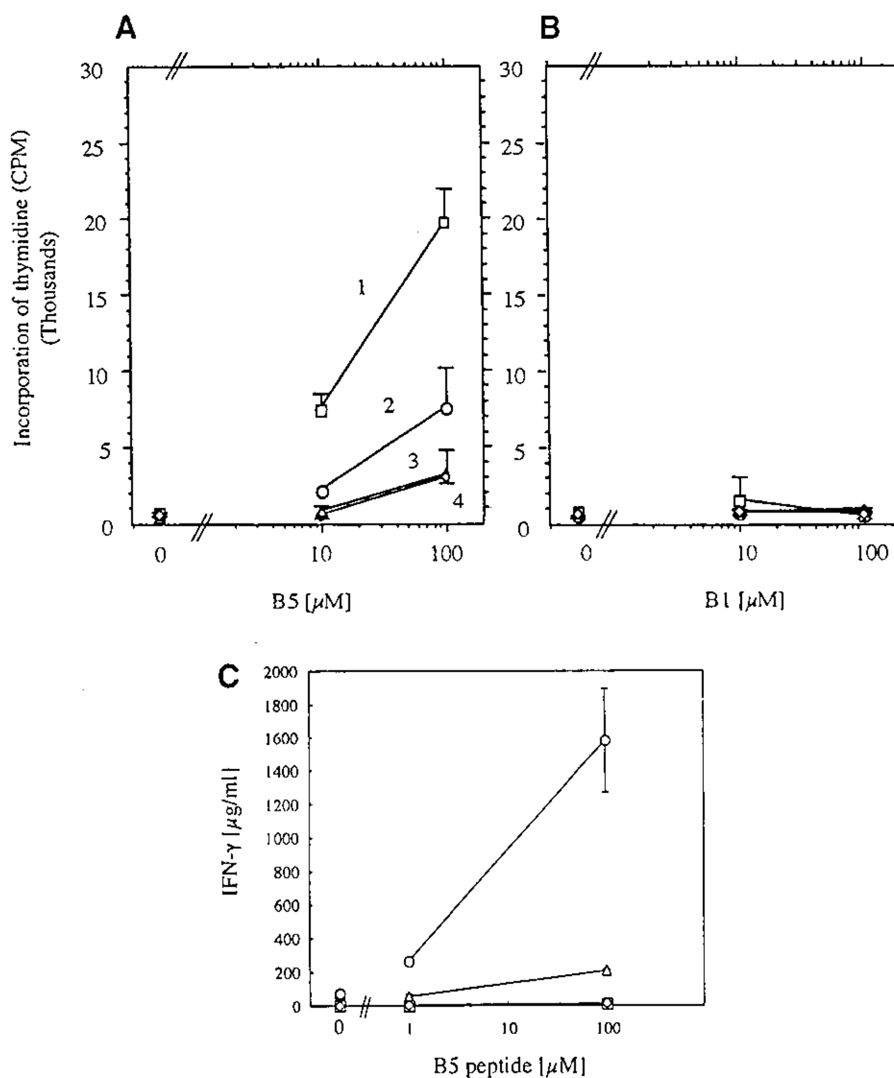

Fig. 4. B5-specific proliferative responses of lymphocytes. The assay was started 10 days after immunization of four individual mice with p245–270 (mouse 1: squares; mouse 2: circles) or PBS plus CFA (mouse 3: triangles; mouse 4: diamonds). The cells were incubated with various concentrations of B5 peptide $V_\beta8.2$ (amino acids 76–101, TCR $V_\beta8.2$) (A) or B1 (amino acids 1–30, L31, TCR $V_\beta8.2$) (B). B5-specific IFN-γ production of lymphocytes from DBA/1J mice immunized with p245–270 on day 0 without further treatment (triangles), preimmunized with p245–270 on day 0 and immunized with bCII on day 7 (circles), immunized with bCII without preimmunization (squares) or preimmunized with PBS on day 0 and immunized with bCII on day 7 (diamonds). Lymphocytes were taken on day 17 and cultured with graded concentrations of B5 peptide or without antigen (C).

regulatory T cells, but not by the induction of tolerance. Several reports showed that regulation of CIA due to tolerization accompanies reduction of the CII-specific IgG2a response (16,24). However, we have shown that bCII-specific IgG1 and IgG2a did not change by immunization with bCII p245–270 (Fig. 2), which also shows that our protocol induced the activation of T cells. $CD4^+$ T cells mainly differentiate into either T_h1- or T_h2-type effectors. T_h1-type cells produce IL-2, IFN-γ and tumor necrosis factor-β, and support cell-mediated immunity, whereas T_h2-type cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, and enhance humoral immune responses (25–27). The T_h1 response has been considered to play a major role in the pathogenesis of CIA (28). However, the role of IFN-γ may be different at different stages of the diseases, as the administration of IFN-γ can inhibit CIA (29). It is suggested from our data that regulation of CIA through activation of CII-reactive pathogenic T cells by immunization with the dominant CII peptide may not be due to the immune deviation of the anti-bCII response. Notably, the IFN-γ response raised by p245–270 was shown to be increased in the mice preimmunized with p245–270, and yet the disease was significantly delayed and inhibited.

Our data suggest that TCR peptide-reactive regulatory T cells that are naturally induced following expansion of bCII-reactive $V_\beta8.2^+$ cells are able to inhibit the disease. Osman *et al.* established T cell clones that were specific for p245–270

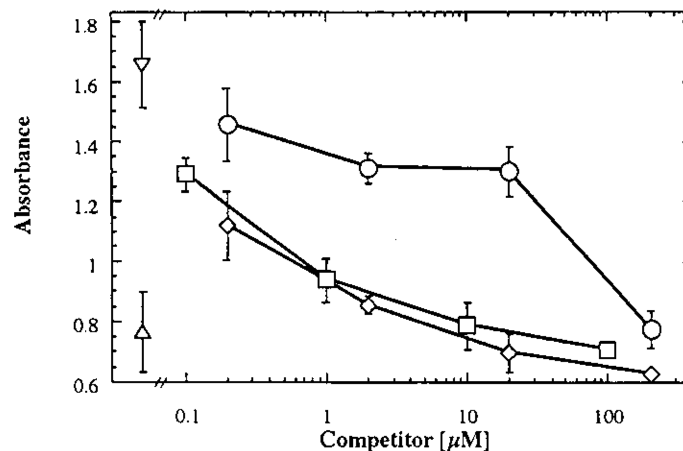

Fig. 5. Competitive-binding assay of I-A^q. The amount of a biotinylated peptide bound to I-A^q in the presence of B1 (circles), B5 (squares), β-Ig119–133 (diamonds), without biotinylated peptide (triangle) or competitor (inverted triangle).

from DBA/1 mice for the characterization of the pathogenic TCR repertoire and found that the V_β8.2 gene segment was preferentially utilized (58.3%) (6). It has also been demonstrated that the TCR V_β8.2 gene segment is preferentially utilized in the EAE of B10.PL mice (H-2^u) (7), in the uveoretinitis of B10.A (H-2^k) mice (30) as well as the EAE of Lewis rats (31). Other studies also implicate the role of $V_\beta8.2^+$ T cells in the pathogenesis of NOD mice, a model for insulin-dependent juvenile diabetes (32). Both in EAE and CIA, it has been shown that administration of antibodies specific for V_β8.2 significantly protects animals from disease (7,33,34). Therefore, we have examined the possibility that CIA was inhibited by an immune regulatory mechanism involving TCR V_β8.2 determinants.

We have already shown that immunization of DBA/1LacJ mice with B5 peptide, corresponding to amino acids 76–101 of the TCRV_β8.2 framework region, results in significant protection of the animals from CIA (11). Response to the B5 peptide was found to be naturally induced during the course of EAE in B10.PL mice. Both $CD4^+$ and $CD8^+$ regulatory T cells were necessary when remission of EAE was to occur. Furthermore, $CD4^+$ regulatory T cells were shown to be specific for the B5 peptide, whereas $CD8^+$ regulatory T cells were specific for a distinct peptide from the CDR1/2 region of the V_β8.2 chain. EAE was inhibited by priming a T_h1-type response against B5, whereas EAE was enhanced by T_h2-type priming (19). We have hypothesized that both $CD4^+$ and $CD8^+$ regulatory T cells recognize each of the TCR-derived peptides that were processed in professional antigen-presenting cells (APC) through the turnover of effector T cells. IFN-γ secreted by $CD4^+$ regulatory T cells was considered to be necessary for the recruitment/activation of $CD8^+$ regulatory T cells and the consequent inhibition of effector T cells. $CD8^+$ T cell activation probably occurs through a CD40 signal that enhances antigen processing and up-regulates co-stimulatory molecules on APC.

Our results show that immunization of DBA/1J mice with p245–270 induces an anti-B5 response accompanied by IFN-γ secretion. As shown earlier (11), the immunization of DBA/1J mice with B5 down-regulated the immune response against bCII and p245–270. We have also established B5-specific T cell lines from DBA/1 LacJ. Their responses to B5 were inhibited by anti-CD4 or anti-MHC class II antibodies (Koh, unpublished data). We propose that the expansion of $V_\beta8.2^+$ effectors and their turnover *in vivo* lead to processing and presentation of a B5-like peptide by APC. It is evident from our data that B5 is likely to be presented by APC, because it displays a strong binding affinity for the I-A^q molecule (Fig. 5). The expansion of effector T cells bearing V_β8.2 was not strong enough to induce effective regulation through bCII immunization; however, it becomes strong when the dominant determinant is used as the immunogen.

Jolly *et al.* reported that the expression of TCR V_β8.2 from the unrearranged gene in a murine lymphoid precursor cell line is higher than that of other V_β members of the repertoire at an early phase of T cell differentiation (35). It may be possible that V_β8.2 plays an unknown role in the body and the anti-B5 response may be a general regulatory system for the control of the immune response.

In EAE, a B5-specific T cell response was induced during the natural remission of the disease. We induced such a response through the activation of $V_\beta8.2^+$ T cells specific for the dominant determinant region of the antigen, bCII. Induction of the regulation by activating the dominant determinant-specific response has not been reported by others. Considering both of these cases with EAE and CIA, we can propose that the TCR peptide B5-specific T cell represents an important focus of the immune regulation system. Thus, B5-specific responses could be frequently induced by immune responses towards self-antigen, especially when the V_β8.2 T cells comprise a significant portion of the response.

The inflammation of limbs in mice suffering from CIA usually settles gradually, although they cannot be completely cured due to irreversible bony deformities. However, a reduction in the inflammation is the important aspect for therapy of rheumatoid arthritis. It is possible that eventual reduction in the inflammation is caused by a naturally induced anti-TCR response following immunization with bCII. Immunization with bCII may only have induced a weaker anti-TCR response