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Enteric Infection Acts as an Adjuvant for the Response to a Model Food Antigen

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Oral administration of soluble protein Ags typically induces Ag-specific systemic nonresponsiveness. However, we have found that feeding a model food protein, OVA, to helminth-infected mice primes for a systemic OVA-specific Th2 response. In this report we show that, in addition to creating a Th2-priming cytokine environment, helminth infection up-regulates costimulatory molecule expression on mucosal, but not peripheral, APCs. To examine the consequences of mucosal infection for the T cell response to orally administered Ag, we adoptively transferred transgenic, OVA-specific, T cells into normal mice. We found that helminth infection enhances the expansion and survival of transgenic T cells induced by Ag feeding. Transfer of 5,6-carboxyfluorescein diacetate succinimidyl ester-labeled donor cells showed that T cell proliferation in response to Ag feeding takes place primarily in the mesenteric lymph nodes. Upon subsequent peripheral exposure to Ag in adjuvant, the proliferative capacity of the transferred transgenic T cells was reduced in noninfected mice that had been fed OVA. Helminth infection abrogated this reduction in proliferative capacity. Our data suggests that enteric infection can act as an adjuvant for the response to dietary Ags and has implications for allergic responses to food and the efficacy of oral vaccination. The Journal of Immunology, 2000, 165: 6174–6182.

A number of recent studies have shown that oral or i.v. administration of soluble Ags results in transient T cell activation and proliferation, followed by functional inactivation or cell death. The sustained T cell proliferation and subsequent development of immunological memory that characterizes the generation of an adaptive immune response requires the potentiation of the T cell recognition signal, induced by a variety of inflammatory stimuli. Experimentally, immune responses toward otherwise nonimmunogenic Ags are achieved through the coadministration of adjuvants, such as CFA (a mixture of killed mycobacteria in oil), which have been thought to mimic the inflammation induced by microbial Ags or products. Indeed, recent reports have directly demonstrated that other heat-killed bacteria (Listeria monocytogenes, Ref. 1) or microbial products, such as bacterial LPS (2), pertussis toxin (3), cholera toxin (CT) (4, 5), and bacterial DNA/synthetic oligonucleotides containing CpG motifs (6), can act as adjuvants to alter the cytokine profile and/or enhance the magnitude of the response to coadministered soluble Ags. The effects of adjuvants on the immune response are 3-fold: they influence Ag presentation, clonal expansion, and T cell differentiation (reviewed in Ref. 7). We have previously found that oral administration of a soluble model food Ag, OVA, at the peak of the mucosal immune response to an enteric infection with the helminth parasite Heligmosomoides polygyrus, elicits a Th2-biased immune response to this normally tolerogenic form of Ag (8). Although the polarized, parasite-induced, Th2 cytokine response provides the priming microenvironment for T cell differentiation, other stimuli are needed to tilt the response toward immunity rather than tolerance. To explore whether helminth infection can act as an adjuvant for the response to oral Ag, we examined the ability of H. polygyrus infection to alter Ag presentation and enhance the clonal expansion of Ag-specific T cells. To our knowledge, our study is the first to demonstrate an adjuvant effect, directly ex vivo, in the context of an on-going natural infection.

Materials and Methods

Mice and parasitic infection

BALB/cByJ mice (8–10 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained in a specific viral pathogen-free facility at Massachusetts General Hospital. To examine the influence of parasitic infection on the expression of the costimulatory molecules B7.1 and B7.2, groups of mice were infected with 200 third-stage larvae as previously described (8) and sacrificed at 8 days postinfection (p.i.). To follow the response of transgenic, OVA-specific T cells in vivo, BALB/c mice were adoptively transferred with cells from age- and sex-matched homozygous DO.11.10 mice on a BALB/c background as described below. The adoptive transfer recipients were either not infected or were orally inoculated with 200 third-stage larvae 2 days after transfer. Groups of mice were then fed and/or immunized with OVA using four different experimental protocols as indicated in Fig. 2. Where indicated, 25 mg of OVA (fraction VII; Sigma, St. Louis, MO) or PBS was administered intragastrically using a ball-tipped feeding needle. In some experiments, mice were immunized at day 8 p.i. by the injection of 100 μg of OVA in CFA or IFA in the hind footpads.

Examination of costimulatory molecule expression by two-color flow cytometric analysis

The expression of the costimulatory molecules B7.1 and B7.2 was analyzed on APC populations isolated from both noninfected mice and from mice at 8 days p.i. Spleen, mesenteric lymph nodes (MLN), and Peyer’s patches (PP) were pooled from 5 to 15 mice/group and pressed through nylon mesh to prepare single-cell suspensions. To enrich for macrophages,
the cell suspensions were incubated in complete DMEM (Life Technologies, Grand Island, NY) containing 10% FCS (HyClone Laboratories, Logan UT), 10 mM HEPES, 2 mM L-glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml, 50 mM 2-ME, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate for 1 h at 37°C, and the plastic adherent cells were removed for analysis. To enrich for dendritic cells, each of the tissues was digested with collagenase (200 U/ml; Worthington Biochemical, Lakewood, NJ). The low-density cell population was obtained by centrifugation in an OptiPrep gradient (Life Technologies). In each of the tissue preparations, the various APC populations were identified with FITC-labeled Abs (purchased from PharMingen, San Diego, CA) to B cells (CD45R/B220, clone RA3-6B2), macrophages (CD11b, Mac-1, or clone M1/70), and dendritic cells (CD11c, HL3) or with a mixture of FITC-labeled rat and hamster Ig isotype controls. B7.1 and B7.2 were identified with PE-labeled anti-CD80 (16-10A1) or CD86 (GL1, R1) for Ab staining, the clonotypic transgenic TCR (identified with PE-labeled KJ1-26 (Caltag, San Francisco, CA)). The cell counts were then adjusted so that each transfer recipient received the same number of CD4<sup>+</sup> transgenic donor cells (which varied from 2 to 5 × 10<sup>6</sup>/mouse, depending on the experiment). In some experiments the cell suspensions were incubated in complete DMEM with or without OVA (10 µg/ml). The cultures were pulsed at 72 h with 1 µCi/well of [3H]Thy (DuPont NEN, Boston, MA) and harvested 16 h later. [3H]Thymidine incorporation was determined by liquid scintillation counting (LS 1801; Beckman Coulter, Fullerton, CA). Additional PLN cells from each mouse were cultured in 24-well plates (4 × 10<sup>5</sup> cells/ml) in complete DMEM, with or without 100 µg/ml of OVA. Cytokine secretion into the supernatants 72 h after the initiation of the culture was determined by ELISA, as previously described (8, 14). Immuno II plates (Dynatech Laboratories, Chantilly, VA) were coated with capture Abs (BVD4-1D11 for IL-4 and R4-6A2 for IFN-γ) overnight at 4°C, followed by blocking in PBS with 3% FCS at 37°C for 1 h. After washing with PBS with 0.05% Tween, the culture supernatants, or recombinant murine IL-4 or IFN-γ (Genzyme, Cambridge, MA), were incubated, in triplicate, overnight at 4°C. The plates were then washed and incubated with biotinylated secondary Abs (BVD6-24G2 for IL-4 and XMG1.2 for IFN-γ), followed by peroxidase-conjugated strepavidin (Zymed, San Francisco, CA) and developed with O-phenylenediamine (Zymed). The reaction was stopped with 2 N H<sub>2</sub>SO<sub>4</sub>, and the plates were read at 490 nm using an Exmap Microplate reader (Molecular Devices, Sunnyvale, CA). The concentrations of cytokine in each sample were calculated from standard curves using SOFTmax PRO software (Molecular Devices).

Statistical analyses were performed using a two-tailed Student’s t test. A value of p < 0.05 was considered significant.

Results

APCs in mucosal, but not peripheral, lymphoid tissues up-regulate expression of the committed molecules B7.1 and B7.2 at day 8 p.i. with H. polygyrus

At least two signals are required to induce T cell activation and proliferation: 1) recognition of peptide/MHC complexes by the Ag-specific TCR, and 2) the signal delivered by costimulatory molecules on APCs. Among the most potent costimulatory molecules are two members of the B7 family, B7.1 and B7.2. Gause and colleagues have shown that the Th2 response to H. polygyrus infection, like that to other inflammatory stimuli, is B7 dependent and can be blocked by treating mice with Abs to B7.1 and B7.2 (15). To examine the levels of costimulatory molecules present on potential APC populations at day 8 p.i., the point at which we administer oral Ag, we looked at the expression of B7.1 and B7.2 on B cells (Fig. 1A), dendritic cells (Fig. 1B), and macrophages (Fig. 1C) in the PP, MLN, and spleen of both infected and non-infected mice. LPS blasts served as a positive control for B7 staining. Fig. 1A shows that B7.2 is markedly up-regulated on B cells, particularly in the MLN, at day 8 p.i., with little up-regulation in the PP and little or none in the spleen. B7.1 expression is not greatly up-regulated at any site. As we have previously noted (8), the proportion of B cells in the MLN of helmhnt-infected mice is also dramatically altered. By day 8 p.i., the enlarged MLN has shifted from an organ containing 70% T cells and 30% B cells (in noninfected mice) to one that contains 70% B cells and 30% T cells and is the primary site for the induction of the polyclonal parasite-induced IgG1 and IgE response. Fig. 1B shows that both B7.1 and B7.2 expression is up-regulated on dendritic cells in the MLN and PP of infected mice, but not in the spleen. The proportion of macrophages in the PP is increased, but B7 expression is not altered (Fig. 1C). By contrast, B7.2 (but not B7.1) expression is up-regulated on macrophages in the MLN of infected mice, and both B7.1 and B7.2 are down-regulated in the spleen. Together these results suggest that H. polygyrus infection dramatically up-regulates costimulatory molecule expression for each of the professional APC populations examined. This effect is most prominent in the MLN, detectable in the PP, and absent in peripheral lymphoid tissues such as the spleen (Fig. 1) and peripheral LN (data not shown).

Therefore, H. polygyrus infection fulfills one of the criteria expected of an adjuvant by inducing the expression of costimulatory molecules on APCs.
T cells in these mice is specific for the chicken OVA peptide 323–339 in the context of I-A<sup>κ</sup> (9) and is detectable with the clonotypic anti-TCR Ab KJ1-26 (19). This model has been used extensively to track the response to tolerogenic and immunogenic forms of OVA in vivo (2, 7, 10, 16–18, 20–25). Four different types of adoptive transfer experiments were performed, as indicated in the experimental protocols outlined in Fig. 2.

In the first set of experiments, BALB/c mice were adoptively transferred with transgenic, OVA-specific T cells (Fig. 2, protocol A). Two days after transfer, one group of mice was infected with <i>H. polygyrus</i>. At 8 days p.i., groups of both infected and noninfected mice were immunized in the footpads with OVA in CFA. We used two-color flow cytometric analysis to look at the percentage of clonotype-positive cells in the PLN (Fig. 3). Very few, if any, clonotype (KJ1-26)-positive cells are detected in the PLN of normal BALB/c mice. After transfer, but without Ag, we can reproducibly detect a small (0.4%) population of KJ1-26<sup>+</sup> cells. If we immunize with OVA/CFA in the footpad and take the draining PLN 3 days later we see a 10-fold expansion in the OVA-specific KJ1-26<sup>+</sup> T cells. This clonal expansion is further enhanced (about 2-fold) in the presence of <i>H. polygyrus</i> infection. Therefore, enteric helminth infection potentiates the response to footpad immunization with OVA in CFA.

To examine whether <i>H. polygyrus</i> infection can act as a mucosal adjuvant without administration of exogenous adjuvants like CFA, we examined the Ag-specific expansion of transgenic T cells by calculating the absolute number of CD4<sup>+</sup> KJ1-26<sup>+</sup> cells in the MLN (Fig. 4A) and PLN (Fig. 4C) at 0, 2, 3, and 7 days after OVA feeding according to the protocol in Fig. 2B. Fig. 4A shows that intragastric administration of OVA to adoptively transferred BALB/c mice induces the Ag-specific expansion of transgenic, OVA-specific T cells in the MLN with a peak at day 3, followed by a decline. However, in infected mice, the absolute number of CD4<sup>+</sup> KJ1-26<sup>+</sup> cells increases in the MLN of both OVA- and PBS-fed mice. The proportion of CD4<sup>+</sup> KJ1-26<sup>+</sup> cells in the infected MLN is actually lower than that in noninfected mice (Fig. 4B), but the dramatic parasite-induced enlargement of the MLN results in greater absolute numbers of clonotype-positive cells in this tissue. By day 7, the numbers of CD4<sup>+</sup> KJ1-26<sup>+</sup> cells have declined in the MLN of normal mice but remain elevated in infected mice. There is a statistically significant difference in the numbers of transgenic T cells that have survived in the MLN of infected and noninfected mice 7 days after OVA feeding (p = 0.03). No expansion of transgenic T cells is observed in the PLN after oral administration of Ag, and both the numbers (Fig. 4C) and percentage (Fig. 4D) of CD4<sup>+</sup> KJ1-26<sup>+</sup> cells in the PLN decrease slightly over 7 days.

Adaptively transferred transgenic T cells in the mesenteric (but not the peripheral) LN of both normal and <i>H. polygyrus</i>-infected mice proliferate in response to OVA feeding

To examine whether mucosal or peripheral CD4<sup>+</sup> KJ1-26<sup>+</sup> cells proliferate in response to OVA feeding, transgenic donor cells were labeled with the vital dye CFSE before transfer according to the protocol in Fig. 2C. CFSE segregates equally into daughter cells upon cell division, allowing proliferation to be measured as a reduction in CFSE fluorescence intensity using flow cytometric analysis. This powerful technique provides a proliferative history of subpopulations of cells in response to in vivo Ag challenge, without manipulation in vitro. In the representative histograms in Figs. 5 and 6 proliferating cells are indicated by the peaks of reduced CFSE fluorescence intensity to the left of the dotted lines. Fig. 5 shows the proliferative response of gated CD4<sup>+</sup> KJ1-26<sup>+</sup> cells to a peptide-containing OVA fragment, obtained by homogenizing mice 5 days after OVA feeding (20 mg/mouse) and by staining with a mAb to the chicken OVA peptide 323–339.

**FIGURE 1.** APC in mucosal, but not peripheral, lymphoid tissues up-regulate expression of the costimulatory molecules B7.1 and B7.2 at day 8 p.i. with <i>H. polygyrus</i>. B cells, dendritic cells, and macrophages were isolated from the PP, MLN, and spleen of infected and noninfected mice and stained with FITC-labeled Abs to B220 (B cells), CD11c (dendritic cells), and Mac-1 (macrophages) and with PE-labeled Abs to B7.1 and B7.2. The results shown are a single histogram overlay of two-color flow cytometric analysis of gated B220<sup>+</sup> (A), CD11c<sup>+</sup> (B), and Mac-1<sup>+</sup> (C) cells. Heavy solid lines indicate cells from infected mice, and noninfected mice are indicated by dotted lines and shaded histograms. B7 staining of 48-h B220<sup>+</sup> LPS blasts is included as a positive control.

Enteric helminth infection acts as an adjuvant for the expansion of OVA-specific TCR-transgenic T cells

It is difficult, in general, to follow Ag-specific responses induced in vivo without in vitro restimulation, in part because the numbers of cells stimulated in response to a nominal Ag like OVA are too small to follow in vivo in normal mice. To examine the consequences of mucosal infection for the T cell response to orally administered Ag, we tracked the response of OVA-specific lymphocytes directly isolated ex vivo, using the adoptive transfer system originally described by Kearney et al. (16). In this model, transgenic T cells are transferred into normal BALB/c mice in numbers large enough to track in vivo with an anti-TCR-specific Ab, but small enough to simulate the normal physiological response to Ag (17, 18). The transgenic TCR expressed on 70–80% of the CD4<sup>+</sup> T cells in these mice is specific for the chicken OVA peptide 323–339 in the context of I-A<sup>κ</sup> (9) and is detectable with the clonotypic anti-TCR Ab KJ1-26 (19). This model has been used extensively to track the response to tolerogenic and immunogenic forms of OVA in vivo (2, 7, 10, 16–18, 20–25). Four different types of adoptive transfer experiments were performed, as indicated in the experimental protocols outlined in Fig. 2.
cells in the MLN and peripheral LN of infected and noninfected mice 3 days after intragastric administration of OVA or PBS (i.e., at the peak of clonal expansion; see Fig. 4). OVA feeding induces the proliferation of CD4^{+} KJ1-26^{+} cells in the MLN (Fig. 5A) but not peripheral LN (Fig. 5C). In Fig. 5B, the data obtained from the CFSE fluorescence histograms of CD4^{+} KJ1-26^{+} cells from the MLN of individual mice in each of the four groups is averaged and plotted as the mean percentage of undivided cells and the mean percentage of cells with greater than four divisions (±SEM). Fig. 5B shows that, in the absence of infection, most (82 ± 1.4%) of the CD4^{+} KJ1-26^{+} cells in PBS-fed mice do not divide, while only 25 ± 3.5% of the transgenic T cells are undivided in OVA-fed mice (p = 0.0001). A total of 41 ± 1.4% of the cells in the MLN of OVA-fed mice have undergone greater than four divisions, compared with only 7 ± 0.9% in PBS-fed mice (p < 0.0001). A total of 62 ± 8% of the CD4^{+} KJ1-26^{+} cells from the MLN of helminth-infected, PBS-fed mice did not divide, whereas only 32 ± 1.1% had not divided after OVA feeding (p = 0.021). The mean percentage of CD4^{+} KJ1-26^{+} cells in the MLN of OVA-fed, infected mice that have undergone greater than four divisions (33 ± 0.6) resembles that seen in OVA-fed, noninfected mice but is not significantly different from that seen in PBS-fed, infected

**FIGURE 2.** Four different experimental protocols for examining the response of adoptively transferred OVA-specific TCR transgenic T cells to orally administered Ag in mice with and without helminth infection.

**FIGURE 3.** Enteric helminth infection enhances the expansion of OVA-specific T cells induced by immunization with OVA in CFA. OVA-specific, TCR-transgenic T cells from DO.11.10 mice were adoptively transferred into normal BALB/c mice (as indicated in the experimental protocol outlined in Fig. 2B). Two days later, some of the transfer recipients were infected with H. polygyrus. At day 8 p.i., groups of both infected and noninfected mice were immunized in the footpads with OVA in CFA. PLN were pooled from two mice in each treatment group 3 days after immunization and stained with a CyChrome-labeled Ab to CD4 and a PE-labeled Ab to the clonotypic TCR expressed by the transgenic DO.11.10 T cells (KJ1-26). The small boxes within the upper right quadrant of each panel identify KJ1-26^{+} T cells. The percentage of total cells in the PLN that are KJ1-26^{+} is indicated in bold type.

**FIGURE 4.** Enteric helminth infection acts as an adjuvant for the expansion of OVA-specific TCR-transgenic T cells. BALB/c mice were adoptively transferred with 5 × 10^6 CD4^{+} KJ1-26^{+} T cells from DO.11.10 OVA TCR-transgenic mice. Two days later the transfer recipients were infected with H. polygyrus. Groups of infected and noninfected mice were fed either PBS or 25 mg of OVA at day 8 p.i. and were sacrificed before feeding and on days 2, 3, and 7 after feeding. The percentages of CD4^{+} KJ1-26^{+} cells in the MLN (B) and PLN (D) were determined by two-color flow cytometric analysis and were multiplied by the cell counts to obtain the absolute number of CD4^{+} KJ1-26^{+} cells in the MLN (A) and PLN (C). The data presented are the mean of three individual mice in each treatment group ± SEM. Open symbols represent noninfected mice, filled symbols are infected mice: □ and ■, OVA-fed; ○ and ●, PBS-fed.
mice (22 ± 9). The polyclonal immune activation induced by helminth infection is apparently inducing some nonspecific activation and proliferation in the CD4+ KJ1-26+ cells in the PBS-fed mice, which varied from mouse to mouse (note the larger error bars for PBS-fed, infected mice in Fig. 5B). This Ag-nonspecific proliferation of the transgenic T cells is consistent with the expansion of CD4+ KJ1-26+ cells in the MLN of both PBS- and OVA-fed mice noted in Fig. 4. By contrast, no proliferative response to OVA feeding was observed in the peripheral LN of either infected or noninfected mice (Fig. 5C).

The proliferative capacity of transgenic CD4+ KJ1-26+ T cells responding to challenge with OVA in IFA is reduced by OVA feeding in normal, but not helminth-infected, mice

Our earlier work had shown that in vitro restimulation of cells from the draining PLN of helminth-infected mice immunized in the footpads with OVA in CFA resulted in a Th2-biased proliferative and cytokine response to OVA instead of the systemic nonresponsiveness normally induced by orally administered Ags (Ref. 8). Yet our experiments to this point indicate that the initial peripheral and mucosal response to orally administered OVA is the same in both infected and noninfected mice. Therefore, we next examined the response of helminth-infected mice given oral Ag to immunization in the footpad with OVA plus IFA. We chose s.c. immunization with Ag in IFA because previous reports had shown that this protocol elicits a T cell response in the draining LN while minimizing the inflammation and Th1 bias that would be induced by Ag in CFA (16, 23). BALB/c mice were adoptively transferred with CFSE-labeled transgenic donor cells as in Fig. 5. However, in this set of experiments, 5 days after oral administration of OVA or PBS the mice were immunized in the footpads with OVA in IFA and sacrificed 3 or 10 days later (according to the protocol outlined in Fig. 2D). As in the experiments shown in Fig. 5, the orally administered soluble OVA induced the proliferation of CD4+ KJ1-26+ cells in the MLN of both noninfected and infected mice. Footpad immunization drains predominantly to the PLN and does not induce a response in the MLN (data not shown). A much more pronounced proliferative response is induced in the PLN by immunization with OVA in adjuvant (Fig. 6). By 3 days after immunization, most of the CD4+ KJ1-26+ cells have undergone some division. However, the histogram overlays shown in Fig. 6 demonstrate that CD4+ KJ1-26+ cells from the PLN of OVA-fed noninfected mice (shaded) proliferate less well than those from OVA-fed infected mice (heavy lines, no shading) or PBS-fed mice with or without infection. About 60% of the CD4+ KJ1-26+ cells from OVA-fed noninfected mice underwent greater than four cell divisions in B. The data shown represent the mean ± SEM for three mice in each group.

FIGURE 5. Adoptively transferred transgenic T cells in the mesenteric (but not the peripheral) LN of both normal and H. polygyrus-infected mice proliferate in response to OVA feeding. Pooled cells from DO.11.10 donors were labeled with CFSE as described in Materials and Methods, and 5 × 10^6 CD4+ KJ1-26+ cells were transferred into each BALB/c recipient. Two days after transfer, one-half of the mice were infected with H. polygyrus. At day 8 p.i., groups of mice were fed PBS or 25 mg of OVA and were sacrificed 3 days later (i.e., 13 days after CFSE labeling and adoptive transfer). CFSE fluorescence was analyzed in CD4+ KJ1-26+ cells from three individual mice in each group. The representative histograms shown display the CFSE fluorescence of gated CD4+ KJ1-26+ cells from the MLN (A) and pooled peripheral (axillary, inguinal, and popliteal) LN (C) for one mouse from each of the treatment groups. Undivided cells are to the right of the dotted line, and the peaks to the left of the dotted line represent cell divisions. The CFSE proliferative response in the MLN is plotted as the percentage of undivided cells and the percentage of cells with greater than four divisions in B. The data shown represent the mean ± SEM for three mice in each group.
To exclude the possibility that helminth infection is simply altering the kinetics of the response to OVA in IFA, we examined the proliferation of CFSE-labeled CD4+ KJ1-26+ T cells at both early (3 days) and late (10 days) time points after footpad immunization. This data is shown in Table I, where the events under each peak in the histograms of CFSE fluorescence have been used to calculate the responder frequency (the proportion of cells that participate in clonal expansion) and the proliferative capacity (the number of daughter cells generated by each precursor T cell) of the CD4+ KJ1-26+ T cells (Refs. 13 and 12; see Materials and Methods). The mean frequency of CD4+ KJ1-26+ cells that responded by dividing in the PLN of PBS-fed, noninfected mice was 66% (at day 3) and 59% (day 10), similar to the responder frequency of 65% reported by Turka and colleagues (12, 13). The responder frequency was somewhat reduced in OVA-fed mice (between 45 and 57%) but did not significantly differ from PBS-fed controls in either infected or noninfected mice. Moreover, the mean proliferative capacity of CD4+ KJ1-26+ cells in the PLN of OVA-fed, noninfected mice was significantly reduced at both 3 and 10 days postimmunization when compared with PBS-fed controls. This reduction in proliferative capacity was abrogated in helminth-infected mice. To directly link the reduction in proliferative capacity measured directly ex vivo in CFSE-labeled cells to our previous observations on the influence of helminth infection on the response to oral Ag, we also restimulated cells from the draining PLN, at both time points postimmunization, with OVA in vitro. Both proliferative responsiveness (as assessed by incorporation of [3H]thymidine) and cytokine secretion into the culture supernatants (as measured by ELISA) were examined. At 3 days postimmunization, cells from the draining PLN proliferated poorly when restimulated with OVA in vitro (Fig. 7E) and secreted low levels of IFN-γ (Fig. 7A). Interestingly, however, although the IFN-γ response was undetectable, IL-4 was detectable only in the supernatant of PLN cells from helminth-infected, OVA-fed mice (Fig. 7C). By day 10 postimmunization PLN cells from PBS-fed mice proliferated and secreted IFN-γ in response to restimulation with OVA in vitro. In both infected and noninfected mice OVA feeding virtually abrogates the ability of these cells to secrete IFN-γ in response to OVA restimulation in vitro (Fig. 7B), in agreement with our previous report (8). T cells from OVA-fed, noninfected mice proliferate weakly in response to OVA in vitro, whereas the proliferative responsiveness of cells from the PLN of OVA-fed, helminth-infected mice was only partially reduced (Fig. 7F).

Taken together, our data suggest that, in helminth-infected mice, the proliferative capacity of the transgenic T cells is restored and, in this Th2-biased cytokine environment, OVA feeding primes for an OVA-specific Th2-type (IL-4) response (Fig. 7). As can be seen in Figs. 5 and 6, as well as in previous reports (12, 13), division of

### Table I. The proliferative capacity of transgenic CD4+ KJ1-26+ T cells responding to challenge with OVA in IFA is reduced by OVA feeding in normal, but not helminth-infected, mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Responder Frequencya</th>
<th>Proliferative Capacitya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 10</td>
</tr>
<tr>
<td>Noninfected OVA-fed</td>
<td>0.57 ± 0.05</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>Noninfected PBS-fed</td>
<td>0.64 ± 0.01</td>
<td>0.59 ± 0.03</td>
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<tr>
<td>Infected OVA-fed</td>
<td>0.56 ± 0.04</td>
<td>0.45 ± 0.06</td>
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<tr>
<td>Infected PBS-fed</td>
<td>0.59 ± 0.02</td>
<td>0.55 ± 0.02</td>
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* Responder frequency and proliferative capacity of CD4+ KJ1-26+ T cells were calculated using the events under each peak of fluorescence histograms of CFSE-labeled cells as described in Materials and Methods. The data shown are the mean ± SEM of three mice in each group.

* p < 0.05 compared to PBS-fed control group.
ELISA. The data is expressed as mean pg/ml of IFN- gamma supernatants 72 h after the initiation of the culture was determined by ELISA. The response to restimulation with OVA in vitro was determined by [3H]thymidine incorporation (E and F) and is expressed as the mean cpm (±SEM) for three mice in each group. *, p < 0.05 by Student’s t test.

FIGURE 7. OVA feeding induces systemic nonresponsiveness for a Th1-type (IFN-gamma) response by CD4+ KJ1-26+ T cells responding to in vitro challenge in both infected and noninfected mice. In helminth-infected mice, OVA feeding primes for an OVA-specific Th2-type (IL-4) response. PLN cells from each mouse were collected at both 3 (A, C, and E) and 10 (B, D, and F) days after immunization and cultured with or without OVA (100 μg/ml). IFN-gamma (A and B) and IL-4 (C and D) secretion into the culture supernatants 72 h after the initiation of the culture was determined by ELISA. The data is expressed as mean pg/ml of IFN-gamma or IL-4 produced per 10⁶ input CD4+ KJ1-26+ cells, ± SEM. The Ag-specific proliferative response to restimulation with OVA in vitro was determined by [3H]thymidine incorporation (E and F) and is expressed as the mean cpm (±SEM) for three mice in each group. *, p < 0.05 by Student’s t test.

clonotype-positive cells in this model is asynchronous; some cells have divided as many as eight times while others divide only two or three times, or not at all. The death and elimination of cells that have undergone multiple rounds of division, combined with the reduction in proliferative capacity induced by the tolerizing stimulus, may result in a cumulative loss of functional responsiveness that is compounded by each subsequent challenge (see Discussion). By contrast, an immunizing stimulus primes for the clonal expansion of Ag-specific cells that are further expanded with each challenge such that the loss of cells to clonal deletion is compensated for by cell division and the generation of more daughter cells.

Discussion

Unlike other murine models of helminth infection (e.g., Trichinella spiralis, Nippostrongylus brasiliensis, or Trichurus muris) most strains of mice become chronically infected with H. polygyrus, making it a particularly relevant model for the chronic helminth infections that afflict humans and domestic animals. The data presented in this report indicate that this strictly enteric infection upregulates costimulatory molecule expression on all subpopulations of professional APCs in the mucosal lymphoid tissue (particularly the MLN), with little up-regulation in the periphery. Tracking OVA-specific T cells in an adoptive transfer model showed that H. polygyrus infection enhances the clonal expansion of T cells in the draining PLN induced by immunization with OVA in CFA. In the absence of exogenous adjuvant, intragastric administration of OVA induces the expansion of OVA-specific transgenic T cells in the MLN, followed by a decline, in noninfected, adoptively transferred mice, as reported previously (26). Transgenic T cells also expand in the MLN of infected mice, and their survival is significantly enhanced over that seen in noninfected mice 1 wk after feeding. By labeling the transgenic donor cells with the vital dye CFSE, we showed that intragastric administration of OVA induces the proliferation of cells in the MLN, but not in the peripheral LN, of noninfected mice, in agreement with a previous report (10). Although we had previously shown that administration of soluble OVA to helminth-infected mice induces a Th2-biased response to OVA, instead of the peripheral nonresponsiveness that is normally induced (8), the data described herein shows that the initial cellular response to OVA feeding is similar in infected and noninfected mice. However, when OVA-fed noninfected mice, adoptively transferred with OVA-specific transgenic T cells, were immunized with OVA in IFA in the footpads, clonotype-positive cells from the draining PLN exhibited a reduced proliferative capacity to this primary Ag challenge. By contrast, the proliferative capacity of transgenic T cells from the PLN of OVA-fed helminth-infected mice was similar to that seen in PBS-fed infected and noninfected controls. Taken together, therefore, our results suggest that helminth infection acts as an adjuvant for the response to orally administered soluble OVA by enhancing the survival of transgenic T cells (Fig. 4) and up-regulating costimulatory signals (Fig. 1), which restore the capacity of KJ1-26+ T cells to proliferate in response to Ag challenge to that seen in PBS-fed mice (Fig. 6). In the presence of the parasite-induced, Th2-biased cytokine response, these adjuvant effects result in a Th2-biased immune response to OVA upon restimulation in vitro (Ref. 8 and Fig. 7).

Our results also have implications for the role of the gut-associated lymphoid tissue in the induction of oral tolerance, which remains controversial. Recent reports demonstrating peripheral T cell activation in response to oral Ag have reinforced the view that nonresponsiveness is due primarily to Ag uptake into the bloodstream and the direct induction of anergy in peripheral sites (27). However, subsequent work has demonstrated that this peripheral up-regulation of phenotypic markers of activation (e.g., CD69) is not accompanied by proliferation or clonal expansion, which occurs primarily in the MLN (10, 26). These observations have been confirmed, and extended, in this report. Moreover, because we have no indication of a proliferative response of cells in the peripheral LN to orally administered OVA (Fig. 5C), we suggest that at least some of the reduction in the proliferative capacity of KJ1-26+ cells in the peripheral LN of noninfected, OVA-fed mice represents the migration of cells from the MLN into the peripheral LN in response to footpad immunization with OVA in IFA. Other work has shown that T cell anergy is likely to be the consequence of the preferential binding of low levels of constitutively expressed B7 to its higher-affinity, inhibitory T cell ligand CTLA-4 (23). A recent report demonstrating that engagement of CTLA-4 induces TGF-beta secretion by CD4+ T cells provides a link between the induction of functional nonresponsiveness at low levels of costimulation and the generation of regulatory cells secreting TGF-beta.
which have been implicated in oral tolerance (28). TGF-β-secret ing cells have been thought to require the unique cytokine micro- environment of the gut-associated lymphoid tissue for their growth and differentiation (29). It is tempting to speculate that the reduced proliferative capacity of KJ1-26+ cells in the peripheral LN of OVA-fed mice reflects the migration of cells from the MLN, which have already transiently divided, but have received an inhibitory signal via CTLA-4. These functionally nonresponsive, gut-derived cells may further dampen the responsiveness of cells in the peripheral LN to subsequent challenge with Ag via their secretion of regulatory cytokines like TGF-β, resulting ultimately in systemic nonresponsiveness. This hypothesis would also be consistent with a recent report indicating that, in the periphery, CD4 T cell tolerance induction in vivo is not due simply to an insufficient proliferative response to initial TCR engagement (30).

Subcutaneous administration of LPS with soluble OVA, using the same adoptive transfer model, also elicits the clonal expansion of Ag-specific T cells (2). LPS stimulates macrophages to secrete the proinflammatory cytokines TNF-α, IL-1, and IL-6, and coadministration of soluble Ag with TNF-α and IL-1 mimics the T cell clonal expansion and follicular migration seen with OVA itself (2, 32). Cholera toxin (CT) is among the most potent of mucosal adjuvants (4, 5). Recent work has shown that immunity to orally administered soluble Ag plus CT can be enhanced by treatment with Flt3L, a dendritic cell growth factor (31). The potentiation of CT’s adjuvanticity resulted from the combined effects of DC expansion and CT’s ability to induce the up-regulation of B7 expression (and DC maturation) through the induction of proinflammatory cytokines, particularly IL-1 and IL-6 (31). Indeed, administration of Ag (OVA) plus IL-1 alone (without CT) was sufficient to induce a productive immune response to a normally tolerogenic form of Ag (31). The ability of IL-1 to act as an adjuvant to abrogate both peripheral and mucosal tolerance to soluble Ag has been documented in other reports as well (33). Helminth infection also induces the secretion of proinflammatory cytokines, at least one of which, TNF-α, has recently been shown to be essential in regulating the Th2 response involved in host protection against helminth infection (34). A recent report has also linked the polyclonal activation induced by helminth infection to the up-regulation of accessory cell production of IL-6. Helminth infection was shown to enhance the survival of activated T cells by increasing T cell proliferation and reducing activation-induced cell death (35).

It is increasingly clear that systemic and mucosal adjuvants are microbial products like CT (31, 33), bacterial CpG DNA (6), and LPS (2, 32), which elicit the secretion of proinflammatory cytokines by cells of the innate immune system. The resultant up-regulation of costimulatory molecule expression induces a response to a normally tolerogenic form of Ag (reviewed in Ref. 36). Therefore, the innate immune system can control the generation of the adaptive immune response through the up-regulation of B7 costimulatory molecule expression on APC. We report here the novel observation that an ongoing enteric infection appears to use a similar mechanism to act as an adjuvant for the response to an orally administered soluble Ag. Because parasitic infection is endemic in developing countries, our results also have important clinical implications for strategies for oral vaccination and the development of allergic responses to food Ags.

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References


