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Orally Tolerized T Cells Are Only Able to Enter B Cell Follicles Following Challenge with Antigen in Adjuvant, but They Remain Unable to Provide B Cell Help[1]

Karen M. Smith, Fiona McAskill, and Paul Garside[2]

Although it is well documented that feeding Ag can tolerize or prime systemic humoral and cell-mediated immune responses, the mechanisms involved remain unclear. Elucidation of these mechanisms remains, in part, complicated by the inability to assess responses by individual lymphocyte populations. In the past, in vivo studies have examined T cell responses at the gross level by examining their ability to support B cell Ab production. However, as the fed Ag has the capacity to affect B cells directly, analyzing the functional capacity of a single Ag-specific T cell population in vivo has been difficult. Using a double-adoptive transfer system, we have primed or tolerized T cells, independently of B cells with a high dose of fed Ag, and examined the ability of these primed or tolerized T cells to support B cell clonal expansion in response to a conjugated Ag in vivo. We have been able to show that primed T cells support B cell clonal expansion and Ab production whereas tolerized T cells do not. Thus, we have provided direct evidence that tolerized T cells are functionally unable to help B cells in vivo. Furthermore, we have shown that this inability of tolerized T cells to support fulminant B cell responses is not a result of defective clonal expansion or follicular migration, since following challenge tolerized T cells are similar to primed T cells in both of these functions. *The Journal of Immunology,* 2002, 168: 4318–4325.**

A lthough it has been documented that feeding Ag can tolerize or prime systemic humoral (1–10) and cell-mediated (9–16) immune responses, the mechanisms involved remain unclear. Furthermore, the efficacy of these approaches remains controversial with some studies suggesting Ab responses can be tolerized by feeding (17–19), whereas others propose priming (6, 20) or no effect at all (17, 21). Investigation of the effects of orally delivered Ag on systemic B cell responses and elucidation of the mechanisms underlying these effects remain, in part, complicated by the inability to assess responses by Ag-specific lymphocyte populations after feeding and have usually relied on indirect measurements by delayed-type hypersensitivity or Ab production in vivo or proliferation or cytokine production in vitro.

Recently, the adoptive transfer of TCR transgenic (tg)[3] lymphocytes has improved our ability to assess the effect Ag has on a single Ag-specific population of T cells in vivo (22). However, functional analysis of these T cells has usually required additional in vitro restimulation. Although these in vitro techniques have proved useful in the past, they remain unphysiological and recent studies have questioned their relevance (23). Furthermore, in vivo functional studies of Ag-specific populations are technically challenging and often rely upon indirect assays of T cell function. One method of assessing T cell functional capacity in vivo is to analyze Ag-specific Ab production to demonstrate the capacity of T cells to provide B cell help (24). However, the potential for fed Ag to directly inactivate B cell responses may confound the interpretation of the effect feeding has on an Ag-specific T cell's ability to provide cognate help. Using a double tg T and B cell adoptive transfer system, we have previously shown that expansion of Ag-specific B cells is dependent on cognate interactions with Ag-specific T cells (24, 25). In this study, we have modified this system to assess the ability of orally primed or tolerized T cells to provide B cell help. DO11.10 OVA-specific T cells were transferred into naive recipients which were subsequently primed by feeding a high dose of OVA in the presence of the mucosal adjuvant cholera toxin (CT), or tolerized by feeding a high dose of OVA alone. Subsequently, the animals received MD4 hen egg lysozyme (HEL)-specific B cells before being challenged in the periphery with conjugated OVA-HEL in adjuvant. Thus, we have an in vivo system to assess the functional ability of primed or tolerized T cells to provide cognate help for B cell responses. Furthermore, because B cells were not present at the time of primary Ag exposure and because they are specific for a different Ag (HEL vs OVA) the priming or tolerizing treatments should have no direct affect on the tg B cells. Using this approach, we have been able to show that, in contrast to orally primed T cells, orally tolerized T cells display an initial inability to enter B cell follicles and support primary Ab responses. Following challenge in the periphery, primed and tolerized T cells are both able to enter B cell follicles but tolerized T cells remain unable to provide adequate B cell help. However, this failure of B cell help is not a result of reduced T cell clonal expansion or migration.

Materials and Methods

Animals

BALB/c (H-2$^{d/d}$, IgM^a) and C57BL/6 (H-2$^{b/b}$, IgM$^{b/b}$) mice were purchased from Harlan-Olac (Bicester, U.K). Congenic IgH^b BALB/c (H-2$^{d/d}$, IgM^b) and (C57BL/6 × IgH^b BALB/c)F$_1$ (H-2$^{b/d}$, IgM^b) recipient mice

were bred in the Central Research Facility of the University of Glasgow. Mice homozygous for the chicken OVA (cOVA) peptide$_{323–339}$/I-A^d-specific DO11.10 TCR transgenes on the BALB/c background (26) were crossed to C57BL/6 mice to produce animals on an F$_1$ background, which were used as TCR tg T cell donors. Similarly, mice heterozygous for the anti-HEL IgM^a and IgD^a transgenes on the C57BL/6 background (MD4) (27) were crossed to congenic IgH^b BALB/c to produce animals on an F$_1$ background. Offspring were screened for the expression of the MD4 transgenes by flow cytometry and tgs were used as BcR tg B cell donors. Six-week-old male (C57BL/6 × IgH^b BALB/c) F$_1$ mice were used as recipients. All animals were specified pathogen free and were maintained under standard animal house conditions in accordance with Home Office regulations.

Preparation of cell suspensions for adoptive transfer

Peripheral lymph nodes (PLN: axillary, inguinal, and cervical), mesenteric lymph nodes (MLN), and spleens from DO11.10 F$_1$ and MD4 F$_1$ mice were pooled and forced through Nitex mesh (Cadisch Precision Meshes, London, U.K.) using a syringe plunger. Suspensions were washed in RPMI 1640 (Life Technologies, Paisley, U.K.). The percentage of CD4$^+$KJ1.26$^+$ T cells and IgM^a+B220$^+$ B cells were determined by flow cytometric analysis as described below. TCR tg T cells (1–6 × 10^6) were injected i.v. into age- and sex- matched IgH^b (BALB/c × C57BL/6)F$_1$ recipients as described previously (25). Ten days later, 1–6 × 10^6 IgM^a+B220$^+$ B cells were similarly transferred.

Ag Administration

cOVA (fraction V) and CT were obtained from Sigma-Aldrich and HEL from Biozyme (Gwent, U.K.). Following adoptive transfer, recipient mice were exposed to Ag by feeding PBS, 100 mg OVA, 100 mg OVA with 20 μg CT (OVA/CT; Sigma-Aldrich, Poole, U.K.) or by s.c. injection with 100 μg OVA in 100 μl saline/50% CFA (OVA/CFA; Sigma-Aldrich). cOVA-HEL was prepared by coupling HEL to cOVA using glutaraldehyde as described previously (24). Twenty-four hours after transfer of tg B cells, secondary immunizations were performed by injecting 130 μg OVA-HEL in 100 μl saline/50% IFA (Sigma-Aldrich) s.c.

Flow cytometry

PLN were harvested between days 1 and 10 after Ag challenge. Cell suspensions were prepared as described above. Aliquots of cells were incubated with FcR blocking buffer (anti-CD16/32 hybridoma supernatant, 10% mouse serum (Diagnostic Scotland, Edinburgh, U.K.) and 0.1% sodium azide (Sigma-Aldrich) for 10 min at 4°C to prevent binding of Ab to cells via Fc regions.

For two-color analysis of CD4$^+$ tg T cells, cells were incubated with PE-conjugated anti-CD4 (BD PharMingen, Oxford, U.K.) and biotinylated clonotypic anti-TCR Ab, KJ1.26 (28), for 40 min at 4°C. Cells were washed in FACS buffer (PBS, 2% FCS, and 0.05% sodium azide) and then incubated with FITC-conjugated streptavidin (BD PharMingen) for 40 min at 4°C. tg B cells were detected with PE-labeled anti-B220 (BD PharMingen) in combination with biotinylated anti-IgM^a (BD PharMingen) followed by FITC-labeled streptavidin. Following a wash in FACS buffer, cells were resuspended in FACS flow (BD PharMingen) for analysis with a FACScan and CellQuest software (BD PharMingen). Two-color analysis was performed on 20,000 events.

ELISAs

To detect anti-HEL Abs in serum, Immulon 2 plates (Costar; Corning Glass, Corning, NY) were coated with HEL and then blocked with PBS-10% FCS for 1 h at 37°C. Serum samples were added for 3 h at 37°C before incubation with biotinylated anti-IgM^a (BD PharMingen) for 1 h at 37°C. Plates were then incubated with extravidin-peroxidase (1/1000; Sigma-Aldrich) for 1 h at 37°C. TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to detect enzymatic activity as described previously (25). Anti-OVA serum Abs were detected similarly but plates were coated with OVA and Abs were detected with biotinylated anti-IgG1 or biotinylated anti-IgG2a (both BD PharMingen).

Immunohistochemistry

PLN and MLN were frozen in liquid nitrogen in OCT embedding medium (Miles Diagnostic Division, Elkart, IN) and stored at −70°C. Six- to 10-μm sections were cut then stored at −20°C. For staining, sections were incubated in acetone for 10 min, air dried, and rehydrated with PBS before incubation in 0.1% azide/3% H$_2$O$_2$ for 45 min. Avidin solution (Vector Laboratories, Burlingame, CA) was added for 15 min and then biotin so-

lution (Vector Laboratories) was added and finally Fc block for 30 min. Sections were washed in PBS after each treatment.

Single staining for the DO11.10 TCR

To detect tg T cells, sections were stained with 1/1600 KJ1.26 in TNB (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.5% blocking reagent (NEN Life Science, Boston, MA)) for 30 min, before being washed twice in TNT (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20). Subsequently, sections were incubated with streptavidin-HRP (1/100 in TNB block; NEN Life Science) for 30 min before washing as before. Biotinyl-tyramide (1/50 in TNB; NEN Life Science) was then added for 10 min, followed by two washes in TNT. Streptavidin-HRP was added again for 30 min before washing twice in TNT. Enzymatic activity was detected with 3,3′-diaminobenzidine substrate (Vector Laboratories) before washing in H$_2$O, followed by incubation with diaminobenzidine-enhancing solution (Vector Laboratories) for ~10 s and a wash in H$_2$O. Harris hematoxylin (Vector Laboratories) was used to counterstain before rinsing in H$_2$O and dipping in acid alcohol, tap water, bicarbonate, then tap water. Sections were subsequently exposed to 70% ethanol, 95% ethanol twice, then 100% ethanol for dehydration before clearing in Histoclear (BS & S, Edinburgh, U.K.) and immediate mounting in Histomount (BS & S).

Double staining tg T cells and B cell areas or tg B cells and T cell areas

To detect tg T or B cells, sections were stained with KJ1.26 (1/400) or bio-IgM^a, respectively, for 30 min before being washed in PBS for 15 min. Sections were then incubated with avidin-biotin complex-labeled alkaline phosphatase (Vector Laboratories) for 30 min before being washed as before. Sections were next incubated with 5-bromo-4-chloro-3-indolyl phosphatase/nitroblue tetrazolium substrate (Vector Laboratories) for 45 min and then washed. To detect T or B cell areas (paracortex vs follicle), sections were next incubated with biotinylated anti-Thy1.2 or anti-CD45R/B220 (1/500; BD PharMingen), respectively, for 30 min before washing. Avidin-biotin complex-labeled peroxidase (Vector Laboratories) was then added for 30 min before washing. Sections were finally incubated in 3,3′-diaminobenzidine substrate for 10 min before a final wash, dehydration in 95% ethanol followed by 100% ethanol and clearing in Histoclear (BS & S) before mounting in Histomount (BS & S).

Statistics

Results are expressed as mean ± SEM or mean + range. To test significance, Student's unpaired t test was performed; a p value of ≤0.05 was regarded as significant.

Results

Serum Ab production in response to feeding tolerogenic or immunogenic forms of OVA

To confirm the ability of orally delivered Ag to prime or tolerize systemic Ab responses, animals were fed OVA/CT or OVA and serum was collected and analyzed for OVA-specific Abs by ELISA. Serum was assessed from adoptively transferred mice that had been fed OVA or OVA/CT or immunized with OVA/CFA s.c. 19 days previously. Mice fed OVA alone did not produce any detectable OVA-specific IgG1 (Fig. 1A) or IgG2a (Fig. 1B). In contrast, those fed OVA/CT or immunized with OVA/CFA produced anti-OVA Abs of both the IgG1 and IgG2a subclasses (Fig. 1). OVA/CFA-immunized animals produced higher levels of both isotypes than the OVA/CT-fed animals. No anti-OVA Abs of either subclass were observed in unimmunized controls (data not shown).

Orally administered Ag can enhance or suppress systemic responses to subsequent challenge

To further confirm that feeding OVA vs OVA/CT resulted in tolerance or priming, respectively, the response to antigenic challenge was assessed by serum Ab production. Adoptively transferred mice were fed OVA or OVA/CT, or immunized s.c. with OVA/CFA. These animals were challenged 12 days later with OVA/IFA and serum was collected 7 and 14 days after challenge. As CT is reported to preferentially induce Th2 responses (29),
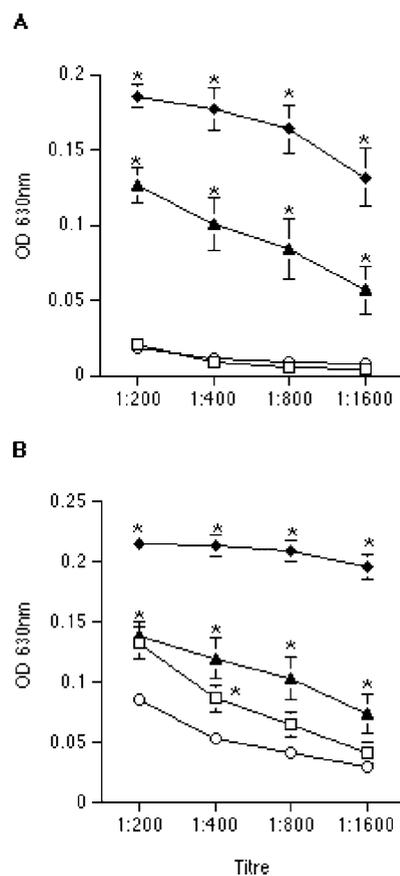
A

B
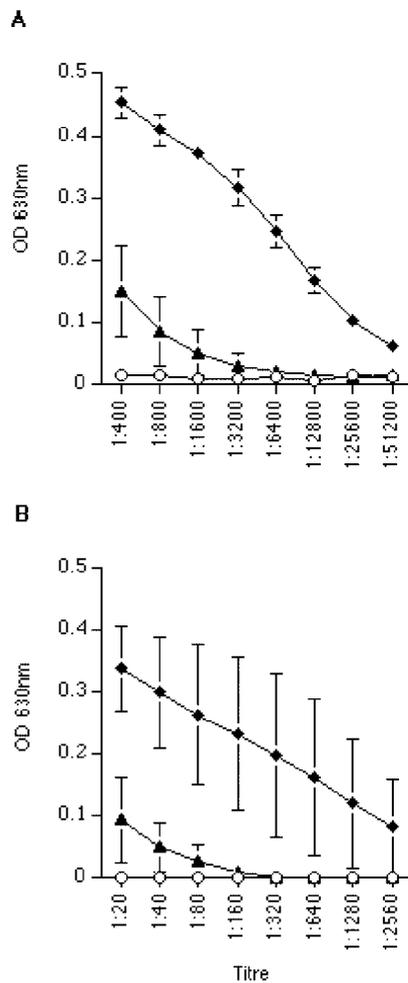
FIGURE 1. Ag-specific Ab responses after a single feed of OVA. $KJ1.26^+CD4^+$ T cells ($2.0–3.0 \times 10^6$) were transferred into naive recipients 1 day before feeding with 100 mg OVA (○) or 100 mg OVA plus 20 μg CT (OVA/CT, ▲) or s.c. injection with 100 μg OVA/CFA (◆). Nineteen days later, serum was collected and analyzed for OVA-specific IgG1 (*A*) and IgG2a (*B*) by ELISA. No OVA-specific IgG1 or IgG2a was detected in PBS-fed animals. Results represent two animals per group and are presented as the mean OD plus range. Similar results were obtained in one further experiment.

A

B

FIGURE 2. A single feed of Ag can enhance or suppress systemic responses to subsequent challenge. $KJ1.26^+CD4^+$ T cells ($2.0–3.0 \times 10^6$) were transferred into naive recipients 1 day before feeding with PBS (□), 100 mg OVA (○), or 100 mg OVA plus 20 μg CT (OVA/CT, ▲) or injection with 100 μg OVA/CFA (◆). Twelve days later, animals were injected s.c. with 100 μg OVA/IFA. Seven (*A*) or 14 (*B*) days later, serum was collected and analyzed for anti-OVA IgG1 by ELISA. Results represent four to five animals per group and are presented as the mean OD ± SEM. Similar results were obtained in one further experiment (∗, $p \leq 0.05$).

serum from these animals was analyzed for OVA-specific IgG1 to assess the humoral immune response to challenge. Seven days after challenge the OVA/CT- and OVA/CFA-primed groups produced significantly more OVA-specific IgG1 compared with the OVA-fed (tolerized) and PBS-fed (primary response) groups (Fig. 2*A*). Fourteen days after challenge the primed responses remained significantly higher than those of the tolerized group. At this time point, the primary T cell response had begun to support Ab production with PBS-fed animals displaying OVA-specific IgG1 levels similar to the OVA/CT group. The amount of Ab produced by the OVA-fed group had also increased slightly but still remained significantly less than that of the PBS-fed primary immune response group at the higher titers analyzed (Fig. 2*B*).

Ag-specific $KJ1.26^+CD4^+$ T cell responses following challenge: clonal expansion

Having confirmed our recent studies that our feeding regimens induced priming or tolerance in adoptively transferred mice (30), we initially examined the effects of oral priming and tolerance on Ag-specific T cells by assessing their clonal expansion. The re-

sponse of previously primed or tolerized T cells to challenge was analyzed by flow cytometry. Adoptively transferred mice were primed or tolerized as described above. Nine days later, all animals received tg B cells before being challenged with cOVA-HEL/IFA s.c. Control groups were injected with PBS, as opposed to OVA/IFA, and results from these groups at each time point were averaged and are represented as day 0. The primary T cell response (represented by the PBS-fed group) peaked on day 3 with a 7-fold increase in tg T cell numbers and declined thereafter (Fig. 3), as described previously (24, 25). All other groups, which had previously been exposed to Ag before challenge, failed to achieve the same level of T cell clonal expansion as naive cells at any time point examined. Following challenge the OVA-tolerized and OVA/CT-primed tg T cells displayed similar levels of clonal expansion 3 days after challenge. However, the OVA/CT-primed T cell clonal expansion continued to rise and peaked on day 5, whereas the OVA response declined after day 3 (Fig. 3). Animals initially primed with OVA/CFA showed a peak clonal expansion 3 days after challenge, which remained at a similar level until day 5 (Fig. 3). There were more tg T cells present after challenge in OVA/CFA-primed animals than in those primed with OVA/CT or tolerized with OVA alone (Fig. 3). However, the starting frequency of tg T cells in OVA/CFA-primed animals was higher than
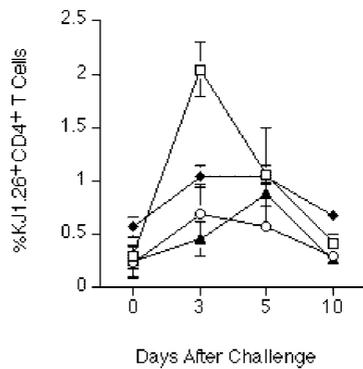
FIGURE 3. Ag-specific T cell proliferation following challenge. KJ1.26^{+}CD4^{+} T cells ($2.0–3.0 \times 10^6$) were transferred into naive recipients 1 day before feeding with PBS (□), 100 mg OVA (○), or 100 mg OVA plus 20 μg CT (OVA/CT, ▲) or s.c. injection with 100 μg OVA/CFA (◆). Nine days later, all animals received HEL-specific B cells i.v. A further 24 h later, animals were challenged with OVA-HEL/IFA s.c. Three, 5, and 10 days after challenge, animals were sacrificed and the PLN were analyzed for the presence of OVA-specific tg T cells by FACS. A control group received a s.c. injection of PBS; results from these animals were averaged and represented as day 0. Results represent two animals per group and are presented as the mean plus range. Similar results were obtained in two additional experiments.

in other experimental groups. Thus, following challenge, all groups that had previously been exposed to OVA exhibited only a 2- to 3-fold clonal expansion relative to their respective starting levels. Since TCR tg T cells previously exposed to Ag in immunogenic or tolerogenic forms do not clonally expand as well in response to OVA-HEL/IFA as the tg T cells in PBS-fed animals, these results imply that T cells in Ag-experienced animals do not have the same capacity to expand in response to challenge as naive cells, as has previously been suggested (31–34), and that an apparent defect in clonal expansion is not indicative of tolerance.

Ag-specific B cell responses following challenge: clonal expansion

As primed or tolerized T cells displayed similar clonal expansion in response to challenge, we next directly examined their capacity to provide help for humoral immune responses. Previously, naive tg T cells (PBS-fed group) helped B cells with the same kinetics as previously described (24, 25), with the B cell response first apparent at 3 days but peaking 5 days after challenge (Fig. 4) and declining thereafter. Primed animals (exposed to OVA/CFA or OVA/CT) also exhibited B cell clonal expansion following challenge (Fig. 4). tg T cells previously primed by OVA/CFA injection were able to help B cells clonally expand more quickly than T cells undergoing a primary immune response, with B cell proliferation peaking 3 days after challenge. However, the OVA/CT-primed group helped B cell clonal expansion with similar kinetics as T cells undergoing a primary immune response (Fig. 4), with the B cell response peaking 5 days after challenge and declining thereafter. Tolerized tg T cells appeared to support some clonal expansion of B cells on day 3. However, the initial B cell clonal expansion supported by tolerized T cells was impaired and at subsequent time points tolerized T cells do not help B cells to expand. Therefore, in sharp contrast to previously primed T cells or T cells undergoing a primary immune response, tolerized T cells display a marked defect in this ability.
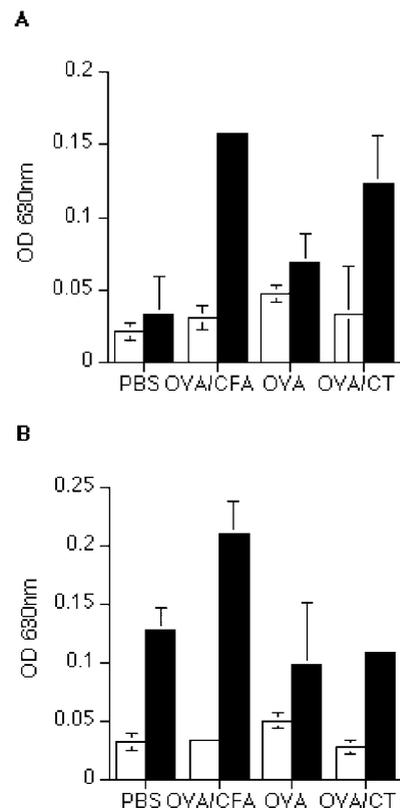
Ab production

The functional ability of primed and tolerized T cells was also assessed by comparing whether the two types of T cells could
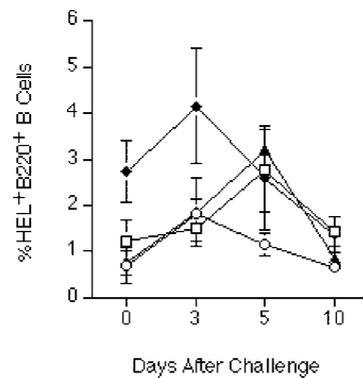
FIGURE 4. Ag-specific B cell expansion following challenge. The PLN from mice described in Fig. 3 legend were analyzed for the presence of HEL-specific B cells by FACS. A control group received a s.c. injection of PBS; results from these animals are averaged and represented as day 0. Results represent two animals per group and are presented as the mean plus range. Similar results were obtained in two additional experiments.

support Ab production by tg B cells. T cells undergoing a primary immune response took 5 days to help B cells to produce maximal anti-HEL IgM[a] titers (Fig. 5*B*) as previously described (24, 25). However, tg T cells previously primed with OVA/CFA or OVA/CT were able to support maximal Ab production by tg B cells 3 days after challenge (Fig. 5*A*). By day 5 the OVA/CT primed Ab responses had greatly diminished, whereas the OVA/

FIGURE 5. Support of HEL-specific IgM[a] production by primed and tolerized tg T cells. Serum was collected from mice described in Fig. 3 legend 3 days (*A*) and 5 days (*B*) after challenge with PBS (□) or OVA-HEL/IFA (■) s.c. Results represent two animals per group and are represented by the mean plus range. Similar results were obtained in two additional experiments.

CFA-primed responses remained high. In contrast to all other experimental groups, tolerized T cells were unable to support Ab production by tg B cells at any time point examined.

HEL-specific Tg B cell localization following challenge

To confirm the flow cytometric analysis (Fig. 4) which indicated that tolerized T cells were defective in providing support for B cell clonal expansion in response to challenge, IgM^{a+} tg B cells were visualized directly by immunohistochemistry. Before challenge, all groups (data not shown) exhibited tg B cell accumulation similar to that of OVA-fed animals (Fig. 6A). However, following challenge, tg B cell accumulation appeared to increase only in the PBS, OVA/CFA, and OVA/CT groups (Fig. 6, *B*, *C*, and *E*). In confirmation of FACS data (Fig. 4), there was little accumulation of tg B cells in the OVA-fed group in response to challenge (Fig. 6D).

Localization of tg T cells

Because primed or tolerized tg T cells were capable of achieving similar levels of clonal expansion in response to challenge but displayed marked differences in their ability to support B cell responses, we examined whether these cells traffic to different locations during primary and secondary immune responses. Inguinal lymph nodes were taken from the mice described above and examined for the presence of tg T cells. In animals fed PBS (data not shown) or OVA (Fig. 7), tg T cells were primarily localized within the paracortical areas of PLN (Fig. 7A) or MLN (Fig. 7B) before challenge with few, if any, cells apparent in B cell follicles. However, accumulation of tg T cells was apparent in the paracortex of OVA-fed animals. In OVA/CT-fed (Fig. 7A, B) and OVA/CFA
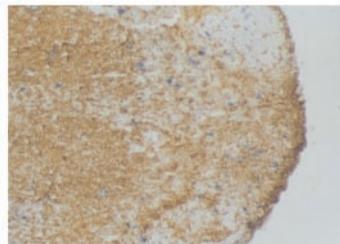
(data not shown)-primed animals, the situation observed was different. After both of these treatments, tg T cells accumulated in the paracortex and were visible in B cell follicles in the PLN (Fig. 7A) and MLN (Fig. 7B) during the primary response. Following challenge in the periphery, tg T cells from OVA/CFA-primed, OVA/CT-primed, and OVA-tolerized mice expanded in paracortical regions (Fig. 8), confirming FACS data. Interestingly, both primed and tolerized tg T cells were visible within B cell follicles after challenge (Fig. 8, *B–D*). Therefore, during the primary response T cells primed with Ag plus adjuvant are able to migrate to B cell follicles, and this property is not lost during the secondary response. However, tolerized T cells are unable to enter into B cell follicles during their primary exposure to Ag but are able to migrate to these locations following subsequent challenge.
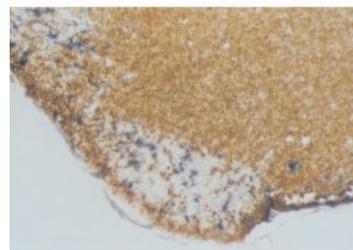
Discussion

In this report, we have demonstrated directly in vivo that in contrast to primed T cells, tg T cells tolerized by feeding a high dose of Ag are incapable of providing cognate help to support B cell clonal expansion and Ab production. Furthermore, we have also shown that this defect is not a result of a reduced ability to clonally expand or migrate as though orally tolerized T cells display a decrease in follicular migration following the primary exposure to fed Ag they are comparable to primed T cells in both clonal expansion and follicular migration upon challenge.

The mechanisms of oral tolerance of T and B cells are unknown. However, it is widely believed that the B cell unresponsiveness induced occurs because of a lack of T cell help and that the B cell remains potentially active. In support of this hypothesis, only T

A) FED OVA D5

FIGURE 6. Localization of HEL-specific tg B cells. PLN were harvested from mice described in Fig. 3 legend at 5 days after challenge with PBS (*A*) or OVA-HEL/IFA s.c. (*B–E*). tg B cells are stained blue and T cell areas are stained brown. Similar results were obtained in two additional experiments.
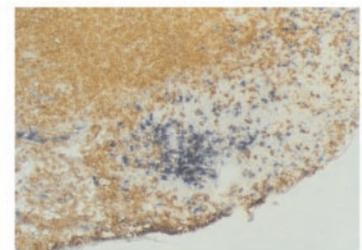
B) FED PBS/ OVA – HEL + IFA s.c.

C) OVA-CFA s.c./OVA-HEL + IFA s.c.

D) FED OVA/OVA-HEL + IFA s.c.

E) FED OVA + CT/OVA-HEL + IFA s.c.

A) PLN

FED OVA D3

FED OVA/CT D3

B) MLN

FED OVA D3

FED OVA/CT D3

FED OVA/CT D5
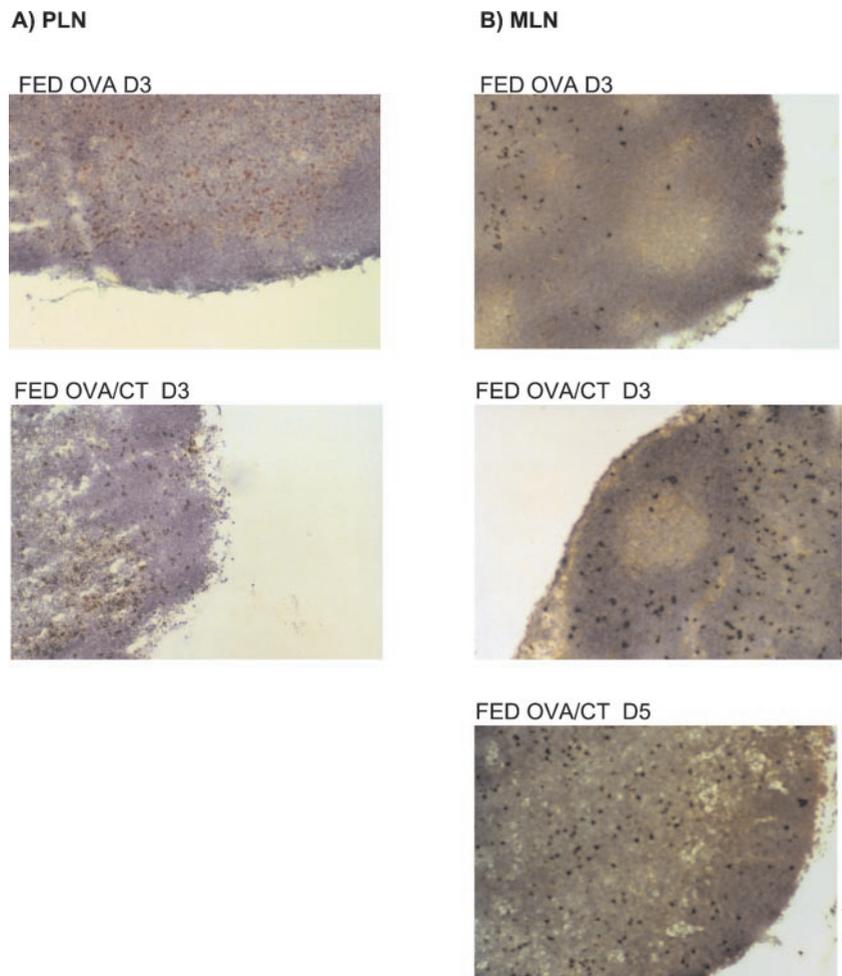
FIGURE 7. Localization of primed or tolerized OVA-specific tg T cells. PLN (*A*) or MLN (*B*) were harvested from mice described in Fig. 1 legend 3–5 days after Ag exposure. tg T cells are stained brown and Harris hematoxylin was used as a counterstain. Similar results were obtained in two additional experiments.

cell-dependent Ags induce oral tolerance and after feeding hapten-protein conjugates the tolerance generated shows complete carrier specificity (1, 4, 7, 35). Furthermore, tolerance in vivo can be bypassed by stimulating OVA-reactive B cells with Ag plus LPS or an unrelated carrier (7, 36). In this study, we have provided direct in vivo evidence that orally tolerized T cells are incapable of providing cognate help for B cells, suggesting that the B cell unresponsiveness observed after feeding may indeed result from a lack of T cell help.

Since we have shown that our feeding regimens induce systemic priming and tolerance (30), we decided to examine whether primed and tolerized tg T cells were functionally different in their ability to support B cell responses. Initially, we assessed the clonal expansion of primed, tolerized, or naive tg T cells in response to challenge. T cells undergoing a primary immune response displayed a 7-fold clonal expansion, whereas tg T cells primed via the oral route with OVA/CT or systemically with OVA/CFA s.c. had expanded only 2- to 3-fold. Somewhat surprisingly, OVA-tolerized T cells also showed a 2-fold increase in tg cell numbers, similar to primed cells. These results are in accordance with previous studies of secondary responses of systemically primed or i.v. tolerized T cells. Merica et al. (32) showed that OVA/CFA-primed T cells clonally expand poorly upon re-exposure to Ag in adjuvant, while other workers demonstrated that i.v. tolerized T cells exhibited a previously activated phenotype and were capable of as much clonal expansion in response to challenge as T cells primed with Ag in adjuvant (33). It has long been hypothesized that T cells tolerized by feeding Ag do not elicit productive immune responses

because they proliferate poorly in response to subsequent antigenic challenge (6, 7, 37, 38). Importantly, we have shown directly that in vivo orally tolerized T cells are capable of as much clonal expansion as orally primed T cells. However, although the latter T cells also display reduced clonal expansion in comparison to naive T cells activated in a primary immune response they are able to support immune responses equally well. Thus, our data suggest that orally tolerized T cells are qualitatively distinct from primed cells but the critical difference is not in their ability to clonally expand in response to subsequent challenge.

Because orally primed and tolerized T cells displayed similar abilities to clonally expand in response to challenge, we next examined their functional capacities directly in vivo. Studies have demonstrated that although primed and tolerized T cells do not differ in their clonal expansion, the ability of the former to make effector cytokines is greatly increased in comparison to the latter (33). Since one of the functions of such T cell-derived cytokines is to support B cell responses (39), we examined this ability directly in vivo. We used an adoptive transfer system whereby the response of BcR tg B cells to a coupled Ag is exquisitely dependent upon TCR tg T cells (24, 25). However, we modified this system so that initial priming of tolerizing treatments had no effect upon the B cells because they were physically absent and do not recognize the uncoupled T cell epitope. T cells undergoing a primary response or T cells previously primed with OVA/CFA or OVA/CT were capable of supporting fulminant B cell clonal expansion and Ab production, whereas orally tolerized T cells were unable to support either of these functions adequately. The B cell response was also
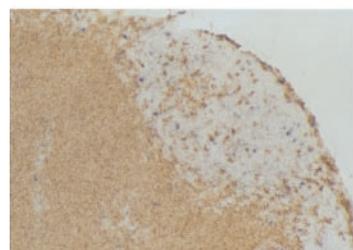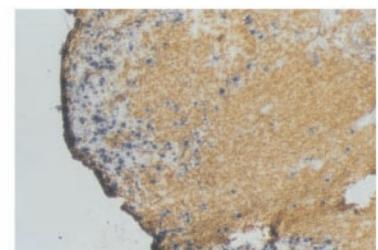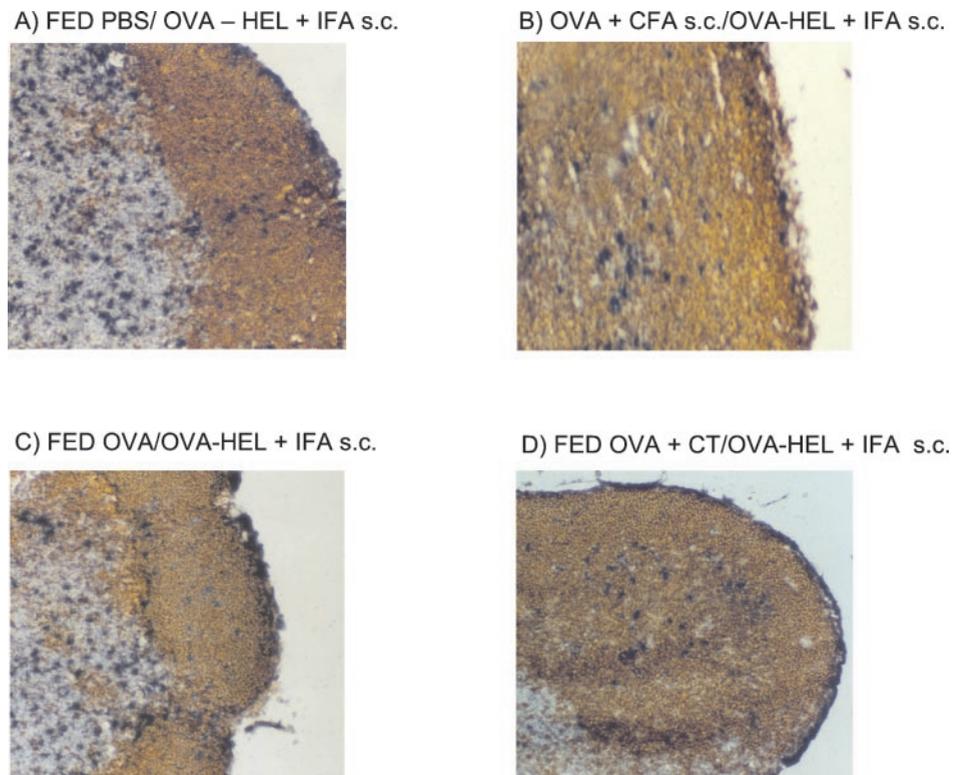
A) FED PBS/ OVA – HEL + IFA s.c.　　　　　　B) OVA + CFA s.c./OVA-HEL + IFA s.c.

C) FED OVA/OVA-HEL + IFA s.c.　　　　　　D) FED OVA + CT/OVA-HEL + IFA s.c.

FIGURE 8. Localization of primed or tolerized OVA-specific tg T cells following challenge. PLN were harvested from mice described in Fig. 3 legend 3–5 days after challenge with OVA-HEL/IFA s.c. tg T cells are stained blue and B cell areas are stained brown. Similar results were obtained from two additional experiments.

directly visualized in vivo. Again, it was noted that tg B cell numbers increase in PLN after challenge of both naive and primed groups. However, very little increase in tg B cells in tolerized groups following challenge was observed.

It has long been hypothesized that after tolerance induction the B cell unresponsiveness observed is due to functional defects in the capacity of tolerized T cells to provide B cell help (40). However, this assumption has been difficult to prove. Using several assays based upon the double adoptive transfer system described above, we have been able to follow cognate interactions among naive, primed, and tolerized T cells and fully competent B cells directly in vivo. These studies have shown that previously naive and primed T cells are capable of providing help for B cell clonal expansion and Ab production but tolerized T cells are unable to provide such help. These results support the hypothesis that the B cell unresponsiveness observed after feeding results from a defect in tolerized tg T cells to provide cognate help for B cells. However, whether fed Ag also directly inactivates B cells will require further study. This may be an important consideration if oral tolerance is to be used therapeutically to treat B cell-mediated autoimmune diseases such as myasthenia gravis.

An alternative explanation for the defective capacity of orally tolerized T cells to provide B cell help may lie in their relative ability to migrate to B cell follicles. Previous studies have shown that during primary immune responses T cells proliferate in T cell areas then move toward B cell follicles to help B cells (24, 31, 41, 42). T and B cells specific for the same Ag then meet at the border between the T and B cell areas (24, 43, 44). Ag-specific T cells subsequently provide cognate help for B cells which clonally expand and produce Ab (24). Interestingly, studies of i.v. administration of Ag have indicated that TCR tg T cells tolerized in this way are unable to undergo follicular migration (31). However, in contrast to the findings reported here, the previous studies did not examine follicular migration of tolerized T cells upon challenge. Following oral or systemic priming, we observed significant num-

bers of TCR tg T cells in follicular regions of local and PLN but such localization was not apparent following the induction of oral tolerance, probably explaining the lack of a primary Ab response in this group. However, following systemic challenge with Ag in adjuvant, T cells were apparent in B cell follicles of all experimental groups. These data not only suggest that tolerized T cells display an initial defect in migration which is overcome by subsequent challenge but also that the effects of orally administered Ag are apparent systemically in both situations.

The migration of T cells is controlled by chemokines. Naive T cells express the chemokine receptor CCR7, the ligands of which, secondary lymphoid tissue chemokine and EBI1 ligand chemokine, are produced by stromal cells in the T cell area (45). The CXCR5 chemokine receptor is expressed by Ag-specific T cells after exposure to Ag plus adjuvant but not Ag alone (46). The ligand for CXCR5, B lymphocyte chemoattractant, is produced by follicular stromal cells (45) and may be responsible for recruiting Ag-stimulated T cells to B cell areas. Interestingly, signals through CD28 and OX40 are required for CXCR5 induction on T cells and subsequent migration to follicles. Furthermore, the ligands for CD28 and OX40 (B7 and OX40 ligand) are induced on dendritic cells by inflammation (47). These findings may explain why tolerized T cells do not migrate into B cell follicles during primary immune responses (as no inflammation is present), whereas following challenge, tolerized T cells acquire the ability to enter B cell follicles, as a consequence of acquiring CXCR5 expression after interaction with adjuvant activated dendritic cells. However, despite being able to migrate into B cell follicles, tolerized T cells remain unable to support fulminant B cell clonal expansion and Ab production. The defect in tolerized T cells remains unclear but may lie in their inability to produce effector lymphokines. Indeed, we have previously shown that the production of Th2 cytokines proposed to be important in B cell help is dramatically reduced in orally tolerized animals (48), and we are now investigating this at the single cell level.

Our studies have begun to directly dissect the defects which may underlie the inability of orally tolerized T cells to provide B cell help in vivo. We have found that orally tolerized T cells display a reduced capacity to provide B cell help and to migrate to B cell follicles following initial exposure to Ag. However, although following challenge in the periphery tolerized T cells remain unable to support B cell responses, this is not a result of defective T cell clonal expansion or follicular migration, which are similar to primed cells.

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