Expression of Intestine-Specific Antigen Reveals Novel Pathways of CD8 T Cell Tolerance Induction

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Summary

Reactivity to intestinal epithelium-specific antigen was studied by transgenic expression of cytosolic ovalbumin controlled by an enterocyte-specific promoter. Transferred OVA-specific CD8 cells (OT-I) preferentially expanded in mucosal lymphoid tissues and the epithelium but failed to cause tissue damage. In contrast, concomitant VSV-ova infection induced OT-I-mediated epithelial cell destruction that correlated with antigen density. OT-I cells retained in the epithelium exhibited high levels of lytic activity but were unable to produce cytokines. The mice were systemically tolerant to OVA since endogenous CD8 cells were nonresponsive to VSV-ova infection. Thus, intestinal antigen gained access to peripheral tissues via absorption from effete epithelial cells. This system demonstrated a requirement for inflammation to drive pathogenic autoreactivity against enterocytes and identified pathways of intestine-specific immunoregulation.

Introduction

Tolerance is an important requirement for the regulation of autoimmunity. Understanding tolerance induction, maintenance, and breakdown is of paramount importance, since absence or loss of tolerance is thought to result in organ damage leading to debilitating diseases such as diabetes, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease (IBD). Mechanisms are in place to delete or downregulate autoreactive T and B (Van Parijs and Abbas, 1998) lymphocytes, with negative selection in the thymus being the predominant process for eliminating self-reactive T cells (Blackman et al., 1990; Nossal, 1994). However, it is not clear how tolerance is maintained to developmentally regulated or tissue-specific proteins or what events control autoreactive T cells that have bypassed negative selection. Tolerance in these cases may be mediated by ignorance of antigen because such T cells may not migrate to the site of antigen expression (Van Parijs and Abbas, 1998) or may be due to cross-tolerance induced in lymph nodes draining the site of antigen expression (Kurts et al., 1997). These questions have not been addressed in the intestinal mucosa, which presents unique anatomical and physiological features that affect the interface of this organ with the immune system. The handling of large quantities of microbial and food antigens occurs at this surface and results in a symbiotic development of the mucosal immune system in which untoward responses to ingested antigens or to normal flora are prohibited. Although many activated lymphocytes reside in the intestinal mucosa (James et al., 1990; Huleatt and Lefrancçois, 1995; Lundqvist et al., 1996), tissue damage does not occur under normal circumstances. Thus, this organ provides a distinctive model for analysis of tolerance to a self-antigen in a dynamic system.

Generation and maintenance of tolerance to self-proteins found in the mucosa is crucial, since the gut serves the body in two distinctly important ways. First, nutrient and fluid absorption occurs at this site allowing for nourishment and homeostasis of all tissues. Second, the mucosa presents a barrier to normal and pathogenic microorganisms. This barrier function is manifested through physical means (e.g., mucus), innate immune mechanisms such as defensins, and adaptive antigenspecific immunity. A breach in any of these functions may lead to autoimmune-type reactions and subsequently IBD (Podolsky, 1991; Hermiston and Gordon, 1995; Mashimo et al., 1996). Recent analysis of animal models suggests that experimental IBD can occur as a result of dysregulation of the delicate balance between chronic activation of the mucosal immune system and control of autoreactivity (Strober and Ehrhardt, 1993). However, this is not the only potential mechanism underlying disease progression, as disruption of the integrity of barrier function at the level of the epithelial cell (Hermiston and Gordon, 1995) or nonspecific antimicrobial peptides (Mashimo et al., 1996) can result in IBD-like syndromes. Whether the tissue changes and destruction that ensue after the initiation of IBD is related to antigen-specific reactivity directed toward autoantigens is unknown. To begin to understand the regulation of tolerance to self in the intestinal mucosa, we have developed a transgenic mouse model in which expression of the nominal antigen ovalbumin (OVA) is intestine specific.

Results

Intestine-Specific Ovalbumin Transgene Expression

In order to test the immunological consequences of intestine-specific antigen expression, we developed two different lines of transgenic mice expressing a truncated, nonsecreted form of OVA (tOVA; Shastri and Gonzalez, 1993) under control of the intestinal fatty acid binding promoter (iFABP) (Figure 1A). This promoter directs protein expression to small intestinal mature enterocytes but not to crypt epithelial cells, and there is differential expression of the promoter along the length of the small intestine (Sweetser et al., 1988; Green et al., 1992). The expression pattern of mRNA encoding OVA was evaluated by mRNA dot blot analysis. OVA mRNA was detected only in the small intestine (Figure 1B) and was not detected in thymus, lung, kidney, liver, or brain (data not shown). mRNA quantitation revealed that line 232-4 expressed 10-fold higher levels of OVA mRNA than line 232-6, and this was confirmed by Northern blot analysis (data not shown). Highest mRNA levels
Tissue-Specific Activation of Adoptively Transferred Ova-Specific CD8 T Cells

In order to be able to track the immune response to OVA and determine the sites of immune reactivity to IEC-expressed OVA, we utilized a T cell adoptive transfer system (Pape et al., 1997; Kim et al., 1998). Small numbers ($5 \times 10^5$) of CD8$^+$ TCR transgenic T cells reactive with the OVA peptide SIINFEKL in the context of MHC class I H-2K$^b$ (OT-I cells) (Hogquist et al., 1994) were transferred to 232-4 and 232-6 mice. Four and five days later tissues were analyzed for the presence of donor cells. In normal, nontransgenic mice, OT-I cells comprised $\sim$0.1% of cells in spleen, mesenteric lymph node (MLN), peripheral lymph node (PLN), and Peyer’s patch (PP) but were not detectable in lamina propria (LP) or intraepithelial lymphocyte (IEL) compartment, in agreement with our previous results showing that OT-I cells traffic into the gut mucosa only after activation (Kim et al., 1997, 1998). By day 4 after transfer into 232-4 mice, a substantial expansion of OT-I cells had occurred in the PP and MLN, with on average 50% and 30% of CD8$^+$ cells, respectively, being donor (Figure 2). In LP and IEL, $\sim$15% of the CD8$^+$ subset were OT-I cells at day 4. In the nonmucosal lymph nodes and the spleen, $\sim$5% or less of CD8$^+$ cells were OT-I cells. By day 5 after transfer, large populations of OT-I cells ($\sim$50% of total CD8$^+$ cells) were present in the LP and the intestinal epithelium. OT-I cells in PP remained at high levels, while OT-I cells in MLN had decreased significantly at this time point, and there were small numbers of OT-I cells in the PLN and spleen. Despite the presence of large numbers of antigen-reactive CD8$^+$ T cells in the epithelium, tissue destruction or any signs of disease were not observed (see below).

Transfer of OT-I cells into 232-6 mice yielded similar but less robust responses (Figure 2). Thus, at day 4 after transfer, OT-I cells made up $\sim$40% of PP CD8$^+$ cells and $\sim$10% of MLN CD8$^+$ cells. Only minor populations of OT-I cells were detected in PLN, LP, or IEL populations. By day 5, the responses in the PP and MLN had declined, while the OT-I population in the IEL and LP had increased to $\sim$10% of the CD8$^+$ populations in those sites. Minor populations of OT-I cells were found in spleen in PLN. These results correlated with the lower OVA expression levels detected in the distal jejunum and ileum of 232-4 mice, whereas the highest expression levels were detected in the proximal and distal jejunum of 232-6 mice with $\sim$2-fold less mRNA in the ileum (not obvious for 232-4 in the overexposure shown necessitated by the low level of mRNA in 232-6 mice). Lower levels of OVA mRNA were present in the duodenum of 232-4 mice ($\sim$7-fold lower than that of distal jejunum), and OVA mRNA was barely detectable in the duodenum of 232-6 mice. This difference in mRNA levels was not related to transgene copy number as both lines contained $\sim$5 copies of the transgene (data not shown). Attempts at detection of OVA by Western blotting or immunohistochemistry were unsuccessful perhaps due to rapid protein degradation by intestinal epithelial cells or due to very low protein expression.

Figure 2. Adoptively Transferred Ova-Specific CD8 T Cells Preferentially Expand in Mucosal Tissues

Ly5.2$^+$ OT-I cells ($5 \times 10^5$) were transferred to 232-4 or 232-6 mice, and tissues were analyzed for the presence of CD8$^+$ Ly5.2$^+$ cells by fluorescence flow cytometry at 4 and 5 days after transfer. The values represent the means and standard errors of three determinations for spleen and at least six determinations for all other tissues.
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to the IFABP-tOVA mice. The mice were monitored for weight changes during the course of the experiment and for general signs of disease. Infected mice from line 232-4 exhibited severe malaise and profound weight loss (Figure 3A), resulting in death in most cases by day 6 postinfection. In contrast, mice from the 232-6 line suffered only a small and transient weight loss with no mortality (Figure 3B). These results correlated well with the demonstration that mice from line 232-4 expressed higher levels of OVA mRNA than did 232-6 mice and indicated that antigen dose may be critical for the outcome of the response.

Antigen-Specific Tissue Destruction Is Dependent upon Inflammatory Mediators and Antigen Load

After OT-I transfer and VSV-ova infection, gross pathology in the form of substantial fluid buildup and severe inflammation was observed only in the small intestine in both lines of transgenic mice. To determine the underlying cause of the disease, histological analysis was performed on intestinal tissue following OT-I cell transfer with or without VSV-ova infection (Figure 4). No effect of OT-I cell transfer and VSV-ova infection was observed in nontransgenic B6 mice or in either 232-4 or 232-6 mice that received only OT-I cells without infection. The latter result was perhaps surprising given the fact that the intestinal epithelium was heavily infiltrated with antigen-specific OT-I cells at this point (Figure 2 and see Figure 3. Virus Infection Induces Disease after Transfer of OT-I Cells below).

Virus Infection Triggers CD8 T Cell-Mediated Intestinal Disease

Since the encounter of transferred OT-I cells with endogenous OVA resulted in activation followed by deletion, we wished to test whether inclusion of inflammatory signals generated via virus infection would alter the outcome of the response. To this end, mice were infected with VSV-ova following transfer of $5 \times 10^5$ OT-I cells to the IFABP-tOVA mice. The mice were weighed every other day beginning on the day of infection (day 0). Each symbol represents an individual animal.

mRNA levels detected in the small intestine of 232-6 mice as compared to the 232-4 mouse and also demonstrated the sequential nature of the response—i.e., primary activation in PP and MLN followed by migration and expansion of OT-I cells in the LP and the epithelium. Results of analysis of CFSE-stained OT-I cells after transfer correlated with these findings (data not shown). By day 17 in both transgenic strains, transferred cells were not detectable, indicating that deletion had occurred (data not shown). Although it is possible that OVA may be produced in tissues other than the gut and we were unable to detect the mRNA, the primary sites of OT-I cell activation (PP and MLN) and the correlation of mRNA levels in the intestinal epithelium with the level of OT-I cell activation in 232-4 versus 232-6 mice suggested that the source of the antigen was the intestinal epithelial cell.

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The antigen-dependent destruction of the epithelium was clearly evident in the tissue sections, since the intestinal crypts were intact, and the IFABP promoter is not active in immature crypt enterocytes (Sweetser et al., 1988; Green et al., 1992). There was also an accompanying cellular infiltration of the lamina propria and muscle layer, extreme crypt hyperplasia, and atrophy of the villi. Tissue damage was not observed in other organs. These results indicated that the amount of OVA produced by IEC significantly influenced the outcome of the immune response and thereby affected the balance of factors maintaining homeostasis. The addition of inflammatory mediators in the form of virus along with antigen recognition was apparently the decisive element in induction of tissue destruction and resulting disease.

IFABP-tOVA Mice Exhibit Increased Expansion of Donor OT-I Cells in the Periphery and the Mucosa

Since an obvious physiological effect occurred as a consequence of virus infection, we wished to determine whether intestine-specific effects were evident with respect to the transferred OT-I T cells. After adoptive transfer of $5 \times 10^5$ OT-I cells into B6 mice without virus
Figure 4. Virus Infection and OT-I Transfer Induces Destruction of the Intestinal Epithelium

OT-I cells ($5 \times 10^5$) were transferred to B6, 232-4, or 232-6 mice, and 1 day later the mice were infected with VSV-ova or were not infected. Four days later tissue sections from duodenum, jejunum, and ileum from B6 and IFABP-tOVA transgenic animals were stained with H&E. All images are magnified 200x.

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infection, 0.1% of PLN cells were of donor origin 5 days after transfer and these were CD44^lo^ (Figure 5A and data not shown). However, as shown here and in Figure 2, an increase in transferred OT-I cells occurred in naive 232-4 and 232-6 mice, which was accompanied by upregulation of the activation antigen CD44 (data not shown). At day 4 after VSV-ova infection, an increase in LN OT-I cells was evident in B6 mice (up to 3.6% of total), and this increase was significantly accentuated in the IFABP-tOVA transgenic mice to 30% and 40% of total lymphocytes in 232-4 and 232-6 mice, respectively (Figure 5A). This result indicated that the presence of endogenous OVA resulted in substantial potentiation of the OT-I cell response.

In the intestinal mucosa of uninfected B6 mice, OT-I cells were not detected (Figure 5B), in keeping with our previous results demonstrating that naive OT-I cells do not enter the mucosa (Kim et al., 1998). In contrast, in naive 232-4 and 232-6 mice, significant mucosal populations of OT-I cells were found on day 5 after transfer, and as with the LN, a larger population was present in 232-4 mice. The IEL compartment of infected B6 mice contained 11% OT-I cells, again showing the necessity of activation of these cells in the periphery before migration to the mucosa. In stark contrast, infection of 232-4 and 232-6 mice resulted in a massive expansion of OT-I cells in the epithelium, with OT-I cells comprising 98% and 85% of the IEL in 232-4 and 232-6 mice, respectively. Since such an impressive expansion was not observed in immunized B6 mice, the increase in OT-I cells was likely due to the increased expansion in the periphery because of the presence of endogenous OVA and the subsequent trafficking of increased numbers of cells into the epithelium, where recognition of IEC-expressed OVA induced further proliferation.

We also sought to visualize the expansion and infiltration of the mucosa with antigen-specific CD8^+^ T cells. Therefore, cryostat sections of intestine from OT-I-transferred 232-4 mice on days 2, 3, and 4 after VSV-ova infection or 5 days after transfer without infection were stained with anti-Ly5.2 mAb (donor specific). Two days after infection, Ly5.2^+^ cells could not be detected (Figure 6A), while on day 3 a small number of donor cells were found (Figure 6B). At day 3 most OT-I cells were found in the epithelial layer rather than the LP (Figure 6B). By day 4 after infection, a massive expansion/infiltration of OT-I cells had occurred (Figure 6C). The epithelium was severely damaged or absent at this time point. OT-I cells were generally not evident in the crypts but were present in the surrounding LP tissue and could be seen in the muscle layer. When 232-4 mice were not infected after OT-I cell transfer, the epithelium was extensively infiltrated by OT-I cells according to flow cytometric data (Figure 5B). As shown in Figure 6D, the
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Figure 5. Transgenic Ova Expression Amplifies the OT-I Response to VSV-Ova Infection

OT-I cells ($5 \times 10^5$) were transferred to B6, 232-4, or 232-6 mice, and 1 day later the mice were infected with VSV-ova or were not infected. Five days after cell transfer, mice were sacrificed, and PLN (A) or IEL (B) were analyzed for donor phenotype (Ly5.2<sup>+</sup>) and CD8 expression by fluorescence flow cytometry. Percentages shown are based on total lymphocytes. Means of values for two mice (B6 controls) or seven mice each (232-4 and 232-6) are shown.

Epithelium appeared normal in this situation. That is, although large numbers of OT-I cells were detected in the epithelium, no tissue damage was evident. These results indicated that some form of functional tolerance was maintained even though potentially autoreactive T cells were directly apposed to antigen-expressing enterocytes. Furthermore, the associated physical manifestations seen in animals infected with VSV-ova did not occur in uninfected mice, indicating that the presence of large numbers of antigen-specific cells in the area of antigen expression was not sufficient to break tolerance.

Induction of Split Anergy in Autoreactive OT-I Cells

Since all 232-6 mice survived following OT-I cell transfer and VSV-ova infection, we were able to examine the long-term impact of IEC antigen expression on autoreactive T cells. By days 14–21, 232-6 mice had completely recovered and the intestinal tissue appeared normal. At this time, most mice contained small numbers of OT-I cells in the epithelium, while only a subset of animals retained OT-I cells in the MLN. At later time points (>30 days), OT-I cells were either completely deleted or a small number remained, indicating the maintenance of functional tolerance.
significant population of cells remained in the IEL compartment but were not present in any other tissue examined (data not shown). The fact that OT-I cells were not found in other tissues suggested that recognition of IEC-expressed OVA was unable to induce deletion but also did not result in overt tissue damage. Since the epithelium-resident OT-I cells at these times were apparently anergic in vivo, we wished to test whether such cells could respond to TCR stimuli in vitro. To this end, IEL containing OT-I cells from VSV-ova-infected B6 or 232-6 mice were tested for IFN-γ production following 5 hr stimulation with ova-peptide in vitro. Surprisingly, while 85% of OT-I cells from B6 mice were induced to produce IFN-γ, none of the OT-I cells in 232-6 mice could be triggered to produce IFN-γ (Figure 7A). Similar results were obtained when TNFα induction was assayed (data not shown). We also assessed the lytic activity of OT-I cells in 232-6 mice (Figure 7B). In contrast to their inability to produce cytokines, these cells exhibited high levels of lytic activity toward ova-peptide-bearing target cells. The level of lytic activity on a per cell basis was similar to that obtained from OT-I cells in VSV-ova-infected B6 mice (Kim et al., 1998). These results indicated that chronic encounter of antigen by OT-I IEL in 232-6 mice resulted in a state of split anergy in which lytic activity but not cytokine production could be triggered.

Discussion

The data presented introduced a novel model for the study of T cell reactivity to intestine-specific autoantigen. Since it has been difficult to identify self-proteins that may play a role in the etiology or chronicity of IBD, it has not been possible to assess the impact antigen specificity has in these situations. These results demonstrate that recognition of an IEC-specific antigen by cytotoxic T cells in vivo can result in tissue damage, although it remains unclear whether direct recognition of self-antigen occurs in IBD. Prior studies of CD8 T cell-IEC interactions suggested that the outcome of such an encounter might be downmodulation of immunity (Li et al., 1995), rather than pathology. Indeed, the results of our adoptive transfer experiments in the absence of VSV-ova infection (Figure 2) and the induction of partial anergy following the destructive episode (Figure 7) support this hypothesis. Our findings also make it apparent...
that IEC can have APC-like qualities in vivo, which may function to potentiate destruction or tolerance at this site, depending on the initial stimuli. Moreover, the location and the amount of antigen present can greatly influence the magnitude and the eventual outcome of the immunological response. Thus, although lower IEC antigen levels were sufficient to induce substantial expansion of antigen-reactive CD8 T cells, reduced tissue damage and tolerance induction were the endpoint of the response. In contrast, higher antigen levels resulted in sustained CD8 T cell activation and irreversible tissue pathology in most cases. Recently, a report showing intestinal pathology caused by hsp60-specific CD8 cells indicated that antigen-specific cells can participate in IBD-like disease (Steinhoff et al., 1999). This model has various characteristics that differ from ours. We were able to follow the progression of the cellular response from initiation to the cytotoxic end, whereas the hsp60 model transfers in vitro activated cells, hence losing any impact of in vivo priming. Also, the proactivated cells will migrate to all tissues in the body (Wack et al., 1997), and since they already secrete TNF_{α} and IFN_{γ}, these cytokines could cause the initial cell damage in the liver and mucosa, since it is known that IEC are extremely sensitive to TNF_{α} (Neurath et al., 1997; Mackay et al., 1998; Piguet et al., 1998). This may cause an increase in hsp60 and so propagate ensuing damage. Moreover, the pathology associated with this model only occurs in immunocompromised animals (scid and TCR{β}^{−/−} mice) and is not organ specific.

What factors are involved in CTL-mediated IEC destruction? CD8 cells are known to use several mechanisms for destruction of antigen-bearing targets. Perforin is one of the major CTL mediators of cellular lysis (Kagi et al., 1996), and CD8 T cells can also produce other mediators of tissue damage, including TNF and lymphotoxin (LT) (Ware et al., 1995; Krakauer et al., 1999). CD8 cells can also cause cell death via FASL–FAS interactions (Kagi et al., 1995). Various cytokines have been implicated in animal models of IBD, such as TNF (Neurath et al., 1997; Guy-Grand et al., 1998; Mackay et al., 1998), IFN_{γ} (von Herrath and Oldstone, 1997; Guy-Grand et al., 1998), and IL-1 (Probert et al., 1995). TNF inhibition has also been shown to be effective in treatment of IBD in some patients (Rutgeerts, 1998). Our demonstration of lytic activity in OT-I cells from the epithelium at a time when tissue destruction was not evident implied that direct lytic activity, perhaps perforin mediated, may not have a major role in IEC damage, although this remains to be tested. Furthermore, the downregulation of cytokine inducibility in long-lived OT-I IEL may suggest the involvement of soluble mediators in tissue damage. Additional experimentation will be required to determine the relative resistance or sensitivity of IEC to CD8-derived instruments of cell death.

The finding that apparently IEC-derived antigen was once encountered antigen, destroyed tissue, and subse-
quent deletion in our system occurred in the lymphoid system (Huang et al., 2000). Our finding that virus infection was required to induce tissue damage is in keeping with recent data indicating that inflammatory signals are essential for productive immune responses in vivo (Bennett et al., 1998; Khoruts et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). In addition, a recent report indicated that adjuvants in the form of viral or bacterial infections could break tolerance to a widely dispersed self-protein (Ehl et al., 1998), although our system presents a more fitting physiological assessment of segregated antigen, since sequestered protein would need to be cross-presented.

The immune response mounted to IEC-expressed antigen resulted in deletion in secondary lymphoid tissues, and this was likely a consequence of cross-tolerance due to processing and presentation of antigen derived from epithelial cells by professional APC, as has been shown for other tissues (Adler et al., 1998; Heath et al., 1998). However, these models cannot be directly compared to ours due to differences in tissue dynamics and the form of antigen expressed. Many transgenic nominal “self-antigens,” including those using ova, have been expressed as transmembrane or soluble proteins. Thus, in those systems it is conceivable that intact protein could be transferred to APC via shedding or other mechanisms, while in our model presumably only intracellular protein fragments or cell fragments would be available. The interaction of OT-I cells with intestinal epithelial cells also presented distinctions with regard to OT-I cell interaction with the pancreas and other tissues. While transfer of about 10^{6} OT-I cells into RIP-ova mice in the absence of additional inflammatory signals causes diabetes (Kurts et al., 1997), the infiltration of the intestinal epithelium with large numbers of autoreactive OT-I cells in the absence of VSV-ova infection did not cause tissue damage in our mice, suggesting anatomically distinct mechanisms of tolerance induction. Surprisingly, even after the initial destructive episode after virus infection, OT-I cells remaining in the epithelium were not overtly autoreactive. Therefore, unlike in other models, tolerance was induced at two levels in our system. First, as seen with antigens in other tissues (Adler et al., 1998; Heath et al., 1998), primary T cell activation and subsequent deletion in our system occurred in the lymphoid tissue, draining the site of antigen expression (MLN and PP). Second, following initial activation, OT-I cells migrated to the intestinal epithelium, where they once again encountered antigen, destroyed tissue, and subsequently remained in the epithelium as partially anergic cells. Thus, “split tolerance” based on anatomical location was evident in which deletion was the norm in lymphoid tissue while induction of partial anergy was the result in the mucosa. Moreover, the split anergy observed in OT-I IEL from ova-bearing hosts represents a distinct level of tolerance induction in vivo. In vitro, a
CTL clone was unable to produce IL-2 in the absence of costimulation, but lytic activity was retained (Otten and Germain, 1991). Also, long-lived male antigen-specific splenic T cells and LCMV-specific CD8 T cells in antigen-bearing hosts are refractory to cytokine induction, but whether such cells were cytotoxic was not tested (Tanchot et al., 1998; Zajac et al., 1998). Perhaps the interaction of OT-I cells with antigen presented by IEC, which do not express classical costimulators, resulted in nonresponsiveness. Overall, the tissue dynamics of the intestinal epithelium and the ability to analyze a tissue-resident population of autoreactive T cells outside of secondary lymphoid tissues provides powerful tools for delineating mechanisms of tissue damage and tolerance induction.

Experimental Procedures

Mice

C57BL/6 (Ly5.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The OT-I mouse line (Hogquist et al., 1994) was generously provided by W. R. Heath (Sid, Parkville, Australia) and F. Carbone (Monash Medical School, Prahan, Victoria, Australia) and was maintained as a C57BL/6-Ly5.2 line on a RAG2-/- background. IFABP-tOVA transgenic mice were created using a construct containing the long form of the IFABP promoter (nucleotides 1178 to +28; a generous gift of J. I. Gordon, Washington University School of Medicine, St. Louis, MO) (Sweetser et al., 1988; Green et al., 1992), truncated OVA-tOVA cDNA (encoding amino acids 138-386), which does not include the signal sequence so the protein remains cytosolic (Shastry and Gonzalez, 1993), and the human growth hormone gene (nucleotides 498-2652; a generous gift of J. I. Gordon, Washington University School of Medicine, St. Louis, MO). Mice were kept in specific pathogen-free housing and were analyzed between 8-10 weeks of age.

RNA Analysis

Total RNA from the indicated tissues was isolated by cell lysis with guanidine isothiocyanate followed by centrifugation over a cesium chloride cushion (Chirgwin et al., 1979). Purification of poly(A) RNA was accomplished using a Poly(A) Quik mRNA Isolation Kit from Promega (Madison, WI). One microgram of poly(A) RNA was dot blotted onto a nylon membrane that was then hybridized with a 32P-labeled OVA cDNA fragment. The blot was stripped and reprobed with a GAPDH-specific cDNA probe to allow for mRNA quantitation. A Molecular Dynamics (Sunnyvale, CA) PhosphorImager was used to quantitate hybridization.

Isolation of Lymphocyte Populations and Adoptive Transfer of OVA-Specific CD8 T Cells

IEL and LP cells were isolated as described previously (Goodman and Lefrancois, 1988; Laky et al., 1997). Lymph nodes (LN) and spleens were removed and single cell suspensions were prepared. Peripheral LN included brachial, axillary, and superficial inguinal nodes. The resulting preparation was filtered through Nitex and the filtrate centrifuged to pellet the cells. For adoptive transfer, 5 x 10^6 OT-I-RAG +/-LY5.2 pooled LN cells were injected i.v. into Ly5.1 B6 or transgenic hosts. Where indicated, mice were infected 24 hr later by i.v. injection of 1 x 10^6 pfu vesicular stomatitis virus encoding OVA (SVS-ova) (Kim et al., 1998).

Detection of Antigen-Specific CD8 T Cells with MHC Tetramers

Mice were infected by i.v. injection of 1 x 10^6 pfu of VSV-ova. Six days later, lymphocytes were isolated and VSV nucleoprotein (N)-specific or OVA-specific CD8 T cells were detected using H-2K^d tetramers containing the N protein-derived peptide RGYVVGGL (Van Bleek and Nathenson, 1990) or the ovalbumin-derived peptide SIINFEKL (Carbone and Bevan, 1989). Peptides were purchased from Research Genetics (Huntsville, AL). Tetramers were produced essentially as previously described (Altman et al., 1996; Murali-Krishna et al., 1998). Briefly, H-2K^d containing the BirA-dependent biotinylatable substrate sequence (the construct was generously provided by J ohn Altman, Emory University, Atlanta, GA) was folded in the presence of human i2-microglobulin and the N or OVA peptide. Biotinylation was performed with biotin-protein ligase (Avidity, Denver, CO). Tetramers were then produced from biotinylated HPLC-purified monomers by addition of streptavidin-allophycocyanin (APC, Molecular Probes, Eugene, OR).

Flow Cytometric Analysis

Lymphocytes were resuspended in PBS 0.2% BSA 0.1% NaN3, (PBS/ BSA/Na3) at a concentration of 1 x 10^6 to 1 x 10^7 cells/ml followed by incubation at 4°C for 30 min with 100 µl of properly diluted mAb. The mAbs were either directly labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), Cy5, APC, or were biotinylated. For the latter, avidin-PE-Cy7 (CalTag, Burlingame, CA) was used as a secondary reagent for detection. For tetramer staining, cells were first reacted with PE-labeled anti-CD8 (CalTag Laboratories) and FITC-labeled anti-CD11a at 4°C followed by staining for 1 hr at room temperature with APC-coupled MHC tetramers. After staining, the cells were washed twice with PBS/BSA/NaN3, and fixed in 3% paraformaldehyde in PBS. Relative fluorescence intensities were then measured with a FACScalibur (Becton-Dickinson, San Jose, CA).

Histological Analysis

Duodenum, jejunum, and ileum from experimental animals were fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA). Paraaffin-embedded tissue was sectioned and then stained with hematoxylin and eosin. For immunohistology, tissues were embedded in O. C. T. medium and snap-frozen in liquid nitrogen. Six micron cryostat sections were reacted with biotinylated anti-Ly5.2 mAb followed by detection using the TSA-amplification system according to manufacturer’s instructions (New England Nuclear, Boston, MA). All images are magnified 200x.

Measurement of Cytolytic Activity

Cytolytic activity was measured using 51Cr sodium chromate EL4 cells (an H-2b thymoma) with or without the addition of 1 x 10^6 cells/ml of the ova-derived peptide SIINFEKL. Serial dilutions of effector cells were incubated in 96-well round bottom microtiter plates with 2.5 x 10^5 target cells for 6 hr at 37°C. Percent-specific lysis was calculated as follows: 100 x [(c.p.m. released with effectors) – (c.p.m. released alone)] / [(c.p.m. released by detergent) – (c.p.m. released alone)].

Intracellular Detection of IFN-g

IEL were isolated from OT-I-transferred VSV-oza immunized B6 mice or 232-6 IFAPB-tOVA transgenic mice at the indicated times. Cells were cultured in DMEM/5% FCS/5% Nu Serum (GIBCO-BRL) with added HEPES, 2-mercaptoethanol, and antibiotics at a density of 1 x 10^6 cells/ml in a 24-well dish at 37°C. To restimulate OT-I cells, cultures were treated with 1 µg/ml of SIINFEKL peptide. Golgiplug (containing brefeldin A, PharMingen, San Diego, CA) was added to unstimulated and stimulated cultures at a dilution of 1:1 µ/ml. Cells were harvested after 5 hr and stained for cell-surface antigens as previously described. Cells were then fixed in 4% paraformalde- hyde/PBS for 20 min at 4°C, washed twice, and stored overnight at 4°C. The next day, the cells were permeabilized by incubating in Perm/Wash solution (PharMingen) for 20 min. The permeabilized cells were incubated with anti-IFN-g-FITC (XMGL2, 5 µg/ml, Phar- Mingen) or control rat IgG1-FITC (R3-34, 5 µg/ml, PharMingen) for 30 min at 4°C and washed twice in Perm/Wash solution. The fluorescence intensities were immediately measured on a FACScalibur.
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References


