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16. A cDNA library of human hepatoblastoma HepG2 cells was constructed in a λZAPII phage expression vector (Stratagene). For isolation of cDNA for X, about 5×10^5 plaques were screened by hybridization with a cDNA fragment that had been synthesized by PCR and labeled with [α-^{32}P]deoxycytidine triphosphate. After plaque hybridization, the pBluescript plasmid was excised and directly sequenced by a double-strand strategy in an automatic DNA sequencer (Pharmacia LKB Biotechnology Inc.). For use as PCR primers, we selected parts of the sequences of two of the proteolytic fragments, YDLEVEQAYD and GGAVNLYHV, respectively, of subunit X (*5*). The following oligonucleotides corresponding to the protein sequences were synthesized: Forward primer, 5′-TATGATTTTGAGGTTGAGCAGGCTTATGA-3′; reverse primer, 5′-ACGTGGTATAGGTTTACTGCTCCTCC-3′. With these primers, a cDNA fragment of approximately 100 base pairs (bp) was synthesized by PCR with first strand cDNA complementary to mRNA from human hepatoblastoma HepG2 cells as a template. For isolation of cDNA for Y, a cDNA fragment of the δ subunit was synthesized by PCR on the basis of the reported sequence (*7*) and used as a probe, because we recently found that Y is identical to the δ subunit (*5*).
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22. Samples of 5 to 10 μg of total RNA were separated by electrophoresis in agarose gel containing formaldehyde, transferred to a Hybond-N$^+$ nylon membrane (Amersham), and hybridized with ^{32}P-labeled probes as described previously (*4*). For Northern (RNA) blot analysis of proteasomes, cDNAs for subunits HC7-I, HC10-II, and HN3 (*14*) of human proteasomes and the cDNA of β-actin (Oncor Inc.) were used. Approximately 300-bp cDNAs of LMP2 and LMP7 of human proteasomes were synthesized by PCR on the basis of reported sequences (*4*). These probes were labeled with a commercial kit for multiprime DNA labeling (Takara Shuzo, Kyoto). The membranes were washed and autoradiographed with Kodak XAR-5 film at −70°C with the use of an intensifying screen.
23. Supported in part by grants from the Ministry of Education, Science, and Culture of Japan. We are grateful to K. Kuma (Kyoto University) for valuable advice on the statistical analysis of subunit similarity.

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MHC Class I Expression in Mice Lacking the Proteasome Subunit LMP-7

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Proteasomes degrade endogenous proteins. Two subunits, LMP-2 and LMP-7, are encoded in a region of the major histocompatibility complex (MHC) that is critical for class I–restricted antigen presentation. Mice with a targeted deletion of the gene encoding LMP-7 have reduced levels of MHC class I cell-surface expression and present the endogenous antigen HY inefficiently; addition of peptides to splenocytes deficient in LMP-7 restores wild-type class I expression levels. This demonstrates the involvement of LMP-7 in the MHC class I presentation pathway and suggests that LMP-7 functions as an integral part of the peptide supply machinery.

MHC class I molecules present peptides derived from endogenously synthesized proteins to CD8$^+$ T cells. These peptides are thought to be generated in the cytosol and transported into the endoplasmic reticulum (ER), where they associate with newly assembling class I molecules (*1*). None of the proteases involved in this process have been identified so far, but one candidate is the proteasome, a 700-kD complex that is abundantly present in the cytosol and nucleus of all cell types investigated. This large complex is composed of at least 13 to 15 distinct subunits, has several discrete proteolytic activities, and appears to constitute the major factor in nonlysosomal protein degradation of normal cellular protein turnover (*2*).

It has recently become clear that at least two proteasome subunits, LMP-2 and LMP-7, are encoded in the MHC class II region. These proteasome genes are closely linked to the genes encoding the putative peptide transporters TAP-1 and TAP-2 (*3*). This finding, supported by several indirect observations, has fueled the hypothesis that the MHC-encoded proteasome subunits may be components of the proteolytic machinery that provides antigenic peptides for presentation on class I molecules. Although in vitro studies with purified proteasomes and synthetic model substrates have lent some support to this hypothesis (*4*), definitive experimental evidence substantiating a role for LMP-2 or LMP-7 in antigen presentation is still lacking.

In order to better understand the physiological role of the MHC-encoded proteasome subunits, we generated a strain of mice lacking LMP-7 by means of homologous recombination in embryonic stem (ES) cells. To ensure complete inactivation of the gene encoding LMP-7, we used a deletion-type targeting vector (Fig. 1A). Upon homologous recombination, this construct was expected to eliminate exons 1 to 5, encoding the first 247 of the 276 amino acids of the LMP-7 protein. Of 72 G418 and gancyclovir double-resistant ES clones analyzed by Southern (DNA) blotting, 19 had undergone homologous recombination. Two independent clones, representing two discrete targeting events, were used to generate chimeric males that were mated with C57BL/6 or 129/Ola females. Chimeric founders from both ES clones transmitted the mutation through the germline, and heterozygous mice from each line were intercrossed to generate animals homozygous for the targeted mutation (Fig. 1B). The absence of exons 1 to 5 of the gene encoding LMP-7 was confirmed by Southern blotting with several probes spanning the deleted region (Fig. 1B). To examine whether the mutation in the LMP-7 locus might impair expression of the closely linked transporter genes, which in turn could influence the phenotype, we quantitated RNA coding for TAP-1 and TAP-2 by Northern (RNA) blotting. There was absolutely no difference in either TAP-1 or TAP-2 expression levels between mutant and wild-type mice (Fig. 1C), which excludes such a possibility.

Mice homozygous for the LMP-7 mutation bred well and appeared healthy in a conventional animal facility. They were indistinguishable from heterozygous or wild-type littermates on gross physical inspection

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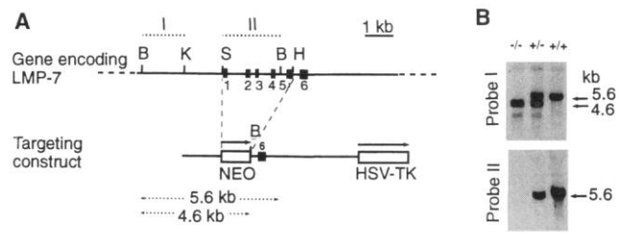

Fig. 1. Disruption by homologous recombination of the murine gene encoding LMP-7. (**A**) Genomic organization of the gene encoding LMP-7 (*10*) and the structure of the targeting vector. The LMP-7 gene was cloned from a 129/Ola-derived genomic library. The targeting vector was constructed by replacement of approximately 2.6 kb of the LMP-7 sequence between the Sal I site 30 base pairs (bp) upstream of the translational start codon and the Hinc II site at the 3′ end of exon 5 with a 1.2-kb XhoI-Hinc II fragment of pMC1neopA (Stratagene) carrying the neomycin resistance gene (*neo*). The gene encoding herpes simplex virus thymidine kinase (HSV-TK) was appended to allow selection against random integration. The isogenic targeting construct was electroporated into E14.1 embryonic stem cells as described (*11*). The ES colonies surviving G418 and gancyclovir selection were analyzed by Southern blotting. Two of several probes used for screening and verification of ES cell clones and mice are indicated (I and II), together with the expected size of hybridizing restriction fragments in wild-type and mutant LMP-7 alleles [see (B)]. Exons are depicted by black boxes. Abbreviations for restriction sites: B = Bam HI; K = Kpn I; S = Sal I; H = Hinc II. (**B**) Southern blot analysis of Bam HI–digested genomic tail DNA of offspring from a heterozygous intercross. Probe I: a 1.4-kb Bam HI–Kpn I fragment derived from the 5′ flanking region of the gene encoding LMP-7. Probe II: a 2.1-kb Sal I–Bam HI fragment containing exons 1 to 4. (**C**) Northern blot analysis of TAP-1 and TAP-2 expression in the thymus and spleen of LMP-7 knockout mice (−/−) and of wild-type littermate controls (+/+). Thirty micrograms of total RNA (thymus) or 5 μg of polyadenylate [poly(A)] RNA (spleen) were separated on a 1.5% agarose-formaldehyde gel, blotted onto nylon membrane, and probed with ^{32}P-labeled PCR fragments of TAP-1 or TAP-2 cDNA (*12*). The lower panel shows the same blots rehybridized with a ^{32}P-labeled β-actin probe to correct for the amount of RNA loaded per slot. Exposure times: 24 hours (TAP-1 and TAP-2) and 30 min (β-actin).

and had normal numbers of T and B cells in their peripheral lymphoid organs and thymuses of typical size and constitution. Cells from these organs were analyzed by a panel of monoclonal antibodies (mAbs) recognizing CD4, CD8, TCRαβ, CD3ε, Thy-1, CD45R (B220), and heat-stable antigen (HSA). No obvious peculiarities were apparent with any of these reagents.

Expression of MHC antigens was analyzed with antibodies specific for H-2K, H-2D, and I-A. Whereas class II staining did not reveal any abnormalities, there was a clear reduction of MHC class I cell-surface expression in LMP-7–deficient mice (Fig. 2). This reduction was evident on all major lymphoid subpopulations and on large peritoneal cells (probably macrophages) for both K^b and D^b expression. Comparison of logs of mean fluorescence intensities in multiple independent staining experiments (n = 25) revealed a typical decrease in class I staining of 25 to 45%, with H-2K and H-2D molecules being similarly affected. In three of these mice, the reduction was less obvious and reached only about 10%.

It was of interest to see whether the decrease in class I cell-surface expression observed in mice lacking LMP-7 would have any functional consequences for the presentation of class I–restricted antigens. For this purpose, we compared the ability of wild-type and LMP-7–negative splenic antigen presenting cells (APCs) to process and present the male self-antigen HY in vitro. As responder cells, we used $CD8^+$-enriched lymph node cells from mice bearing a transgenic T cell receptor specific for HY presented on D^b (Fig. 3A). HY-specific responder cells proliferated vigorously when cultured with splenocytes from male control mice; the response was down by 50 to 70% when splenocytes from LMP-7–deficient animals were used as presenters. Equivalent results were obtained with lymph node–derived APCs. Thus, mutant APCs are still able to process and present the endogenous antigen HY, albeit with a drastically reduced efficiency. Further experiments are needed to clarify whether this striking reduction in the ability to present HY is due solely to reduced class I cell-surface expression or, alternatively or additionally, it

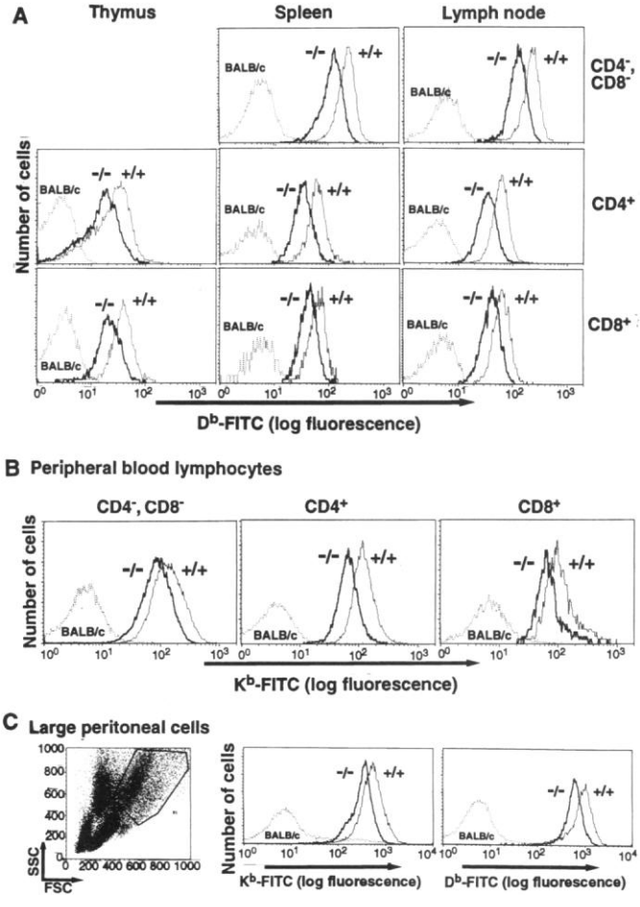

Fig. 2. Three-color cytofluorimetric analysis of MHC class I cell-surface expression in LMP-7–deficient mice (−/−) and in wild-type littermate controls (+/+). (**A**) H-2D^b expression on thymocytes, splenocytes, and lymph node cells. (**B**) H-2K^b expression on peripheral blood lymphocytes. (**C**) H-2K^b and H-2D^b expression on large peritoneal exudate cells. Cell suspensions were prepared from thymuses, spleens, pooled inguinal, mesenteric, and axillary lymph nodes, and peripheral blood. Splenocytes and peripheral blood cells were depleted of erythrocytes by ammonium chloride treatment. Peritoneal cells were obtained by flushing the peritoneal cavity of unmanipulated mice with medium. Staining was done in 96-well plates (2.5×10^5 cells per well) in 100 μl of phosphate-buffered saline plus 2% fetal bovine serum (FBS) with mAbs at optimal dilution as determined before. The mAbs used were: anti-CD4–phycoerythrin (PE) conjugate (H129.19; Gibco), anti-CD8–R613 conjugate (53-6.7; Gibco), anti-H-2D^b–fluorescein isothiocyanate (FITC) conjugate (KH95; Pharmingen, San Diego, California), and anti-H-2K^b–FITC conjugate (AF6-88.5; Pharmingen). Stained cells were analyzed on a FACScan cytometer (Becton Dickinson). D^b- and K^b-specific green fluorescence (FITC) was measured on populations selected by gating on three parameters: light scatter, and orange (CD4) and red (CD8) fluorescence. Equivalent results were obtained with FITC-conjugated mAbs B22-249 (anti-D^b) and K9-178 (K^b). The line in the dot blot in (C) demarcates the area used for electronic gating of large peritoneal cells.

is caused by some specific defect in HY processing.

Recent work with mutant cell lines (5) and knockout mice (6) has established that peptides form an integral part of MHC class I molecules with heavy chain and β2-microglobulin. Peptide in the ER is essential for correct folding, assembly, transport, and stable cell-surface expression of MHC class I molecules. Reduced expression of K^b and D^b in LMP-7–deficient mice may be due to a lack of sufficient amounts of correctly processed peptide. If so, it should be possible to restore wild-type expression levels with exogenously added peptides. To test this hypothesis, we incubated splenocytes from LMP-7–negative mice and control littermates in vitro with two synthetic peptides: NP366-374, an influenza nucleoprotein–derived epitope that has been found as a natural ligand on D^b molecules, and VSV52-59, which has been eluted from K^b and is an epitope of the vesicular stomatitis virus nucleoprotein. Both peptides were able to induce higher levels of class I surface expression in a strictly isotype-specific manner on wild-type splenocytes, which suggests that also on normal cells is class I expression limited by the availability of appropriate peptide ligands. Incubation of splenocytes deficient in LMP-7 with each peptide resulted in a stronger relative increase in cell-surface expression of the respective class I molecule to levels of K^b and D^b that were equivalent to those on peptide-induced wild-type cells (Fig. 4). Again, the effect was isotype-specific. These findings demonstrate that addition of appropriate peptides can restore wild-type class I expression levels and thus correct the defect in class I surface expression observed in LMP-7–deficient mice.

That LMP proteins might play a part in antigen processing was suggested as long as 8 years ago (7). Until now, however, only limited experimental data supporting such a function have existed. On the contrary, a serious challenge to this hypothesis has come from experiments with a human lymphoblastoid cell line that has a large homozygous deletion in the MHC class II region encompassing both TAPs and LMPs. In this mutant cell line, expression of stably assembled class I molecules and apparently normal antigen processing were restored by transfecting TAP-1 and TAP-2 alone, thus in the absence of LMP-2 and LMP-7 (8, 9). These findings do not contradict our results but need to be reinterpreted. In these earlier experiments, it was not possible to correctly compare class I expression levels, partly because a genuine positive control cell line supertransfected with LMP-2 or LMP-7 or both was not available, partly because the transfectants were selected (8) or even sorted (9) for high levels of class I expression before analysis. This manipulation probably masked the influence of the LMPs on class I expression levels. For instance, variant transfectants might have been selected in which LMP deficiency was offset by some adaptation; for example, the overexpression of the transfected genes encoding the TAP-1 and TAP-2 transporters or the compensatory expression of other, homologous proteasome subunits with a similar function.

Taken together, our results provide strong evidence for a role for LMP-7 in the class I presentation pathway. Although the exact function of LMP-7 still remains unclear, the efficient rescue of normal class I expression levels by antigenic peptides indicates a specific role in the peptide supply machinery. Whether LMP-7 acts simply as a docking molecule that physically links the proteasome and the TAP transporter system to increase the efficiency of peptide translocation, or whether LMP-7 actively participates in the generation of antigenic peptides, for example, by altering the specificity of the proteasome, awaits further experimentation.

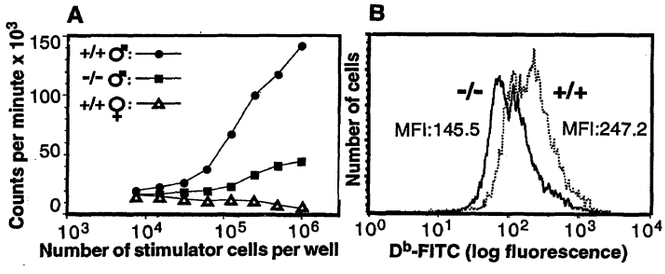

Fig. 3. (**A**) Proliferative response of HY-specific, D^b-restricted splenic T cells to stimulator splenocytes derived from a wild-type male (filled circle), an LMP-7–negative male littermate (filled square), or a wild-type female (open triangle) as negative control. (**B**) Anti D^b-FITC staining of the wild-type (+/+) and LMP-7–deficient (−/−) male splenocytes used as stimulators in (A). Responder cells were prepared from the spleen or lymph nodes of mice expressing a transgenic T cell receptor specific for HY and D^b (13). Responders were depleted of B cells and $CD4^+$ T cells with magnetic Dynabeads (Milan Analytica) and plated at 3×10^4 cells per well in flat-bottomed 96-well plates in IMDM medium containing 10% FBS, supplemented with exogenous interleukin-2 (partially purified supernatant of concanavalin A–stimulated rat splenocytes). Stimulator cells (erythrocyte-depleted splenocytes) were irradiated (22 gray) and plated at increasing densities as indicated. Cultures were assayed for proliferation after 3 days. Uptake of [^{3}H]thymidine, which was added for the last 12 hours, was measured by liquid scintillation counting. The counts per minute shown represent the arithmetic mean of triplicate cultures. An aliquot of stimulator cells was stained with an antibody to (KH95; Pharmingen) just before irradiation. Class I expression was analyzed on FACScan (Becton Dickinson) gating by light scatter for live cells. MFI is mean fluorescence intensity (arbitrary units). Equivalent results were obtained in four independent experiments.

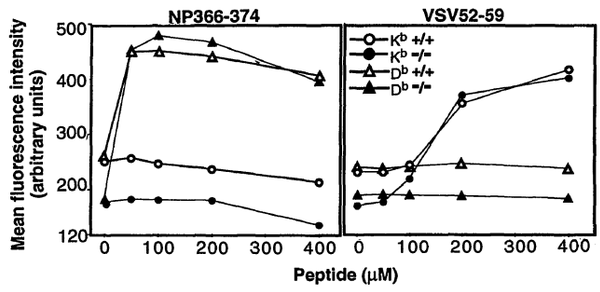

Fig. 4. Induction of wild-type class I expression levels on LMP-7–deficient $CD4^-$, $CD8^-$ splenocytes with a D^b- (left panel) or K^b-specific (right panel) peptide. Spleen cells prepared from a knockout mouse (filled symbols) or from a wild-type littermate (open symbols) were depleted of erythrocytes by ammonium chloride treatment and cultured at 37°C (5% CO_2) in IMDM medium with 10% FBS in 96-well, flat-bottomed plates with the indicated amounts of the D^b-specific peptide NP366-374 [ASNENMETM (*14, 15*)] or the K^b-specific peptide VSV52-59 [RGYVYQGL (*14, 16*)]. After 12 hours, cells were harvested and triple-stained with CD4-PE, CD8-R613, and D^b-FITC (triangles) or K^b-FITC (circles). Class I expression was measured on the CD4-CD8 double-negative population. The mAbs used were as in Fig. 2. Peptides were synthesized on an AMS422 Multiple Peptide Synthesizer (Abimed) by an F-Moc strategy. Similar results were obtained in six independent experiments.

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CTCTACCAT (3′); TAP-2 primers: ATGGCGCTGTCCTACCTGAGGCCCT (5′) and TCCTTGTAGTGGCTGACTTCCTGCT (3′).
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17. We thank A. Berns for a 129/Ola-derived genomic library, D. Mathis and C. Benoist for many helpful suggestions, and A. Livingstone and A. Lanzavecchia for critically reading the manuscript. The Basel Institute for Immunology was founded and is supported by Hoffmann-La Roche Ltd., Basel, Switzerland.

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Regulatory T Cell Clones Induced by Oral Tolerance: Suppression of Autoimmune Encephalomyelitis

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Experimental autoimmune encephalomyelitis (EAE) is a cell-mediated autoimmune disease that serves as an animal model for multiple sclerosis. Oral administration of myelin basic protein (MBP) suppresses EAE by inducing peripheral tolerance. T cell clones were isolated from the mesenteric lymph nodes of SJL mice that had been orally tolerized to MBP. These clones were CD4$^+$ and were structurally identical to T helper cell type 1 (T_H1) encephalitogenic CD4$^+$ clones in T cell receptor usage, major histocompatibility complex restriction, and epitope recognition. However, they produced transforming growth factor–β with various amounts of interleukin-4 and interleukin-10 and suppressed EAE induced with either MBP or proteolipid protein. Thus, mucosally derived T_H2-like clones induced by oral antigen can actively regulate immune responses in vivo and may represent a different subset of T cells.

One of the primary features of the immune system is the discrimination of self from nonself (*1*). Immunologic tolerance provides the immune system with self-nonself discrimination and is crucial to prevent pathogenic reactivities against self antigens. Several mechanisms may contribute to immune tolerance, with the primary one being clonal deletion of autoreactive cells in the thymus (*1–3*). However, not all autoreactive cells are deleted (*3*, *4*), and therefore, mechanisms must exist to regulate autoreactive T cells in the peripheral immune compartment. Mechanisms of peripheral immune tolerance include anergy (*5*) and active suppression (*6*). Active cellular suppression has not been unequivocally demonstrated as a mechanism for peripheral tolerance because of difficulties in cloning and in demonstrating that cloned T cells can actively regulate the responses of other immune cells in vivo.

Oral administration of antigens (oral tolerance) is a classic method of inducing antigen-specific peripheral immune tolerance (*7*). Orally administered antigens can induce active suppression (low antigen dose) or clonal anergy (high antigen dose) (*8*). Oral tolerance is a biologically relevant method of inducing peripheral tolerance: it involves the physiologic interaction of proteins with the gut immune system, a process that has evolved to prevent systemic immune responses to ingested proteins. In addition, the oral administration of autoantigens has become clinically relevant and is being investigated as a treatment for human autoimmune diseases (*9*).

One of the mechanisms of peripheral immune tolerance after orally administered antigens is the generation of regulatory T cells that mediate active suppression (*10*). In the Lewis rat these T cells are CD8$^+$ and act by secreting transforming growth factor–β (TGF-β) after being stimulated by the fed antigen (*11*). These cells do not produce antigen-specific suppressor factors and therefore differ from previously described suppressor T cells that have been difficult to characterize (*6*). Characterization of regulatory T cells that mediate active suppression, including epitope specificity, T cell receptor (TCR) usage, and individual cytokine secretion cannot be established from bulk-cultured cells.

To generate regulatory clones associated with oral tolerance, we fed SJL mice [which are susceptible to experimental autoimmune encephalomyelitis (EAE)] myelin basic protein (MBP) and examined cytokine patterns produced by mesenteric lymph nodes. Mesenteric lymph nodes from animals fed either MBP or hen egg lysozyme (HEL) and cultured in vitro with the relevant antigen secreted TGF-β, interleukin-4 (IL- 4), and IL-10, but minimal interferon (IFN-γ) (Table 1, groups 1 to 4). Cytokine secretion was dependent on antigen-specific stimulation in vitro with the fed antigen. If cells from animals fed MBP and then immunized intraperitoneally with MBP in complete Freund's adjuvant (CFA) were cultured in vitro with MBP, antigen-specific TGF-β secretion was enhanced (Table 1, groups 5 to 7). Thus, in addition to the secretion of T helper cell type 2 (T_H2) cytokines, immune responses in the gut preferentially involve the generation of cells that secrete TGF-β .

Cells from animals fed MBP and immunized with MBP in CFA were studied in bulk culture to determine which cells secreted the respective cytokines (Fig. 1). Both CD4$^+$ and CD8$^+$ cells secreted TGF-

Table 1. Cytokine production by mesenteric lymph node cells from orally fed SJL mice. Seven groups of SJL/J mice (*27*), four mice each, were fed with MBP or HEL (*24*). Two days after the last feeding, mice in groups 5 to 7 received an intraperitoneal injection of 100 μg of MBP in CFA (*24*). Mesenteric lymph node cells from all groups of mice were prepared 14 days after immunization (or 16 days after the last feeding) and were cultured in serum-free medium containing MBP or HEL (50 μg/ml) as indicated. Culture supernatants were collected at 40 hours for IFN-γ and IL-4 and at 72 hours for TGF-β and IL-10. Cytokine concentrations were determined by ELISA (*28*) and are expressed as picograms per milliliter. Results represent pooled data from three independent experiments.

Group	Antigen used for			TGF-β	IFN-γ	IL-4	IL-10
	Feeding	Immunization	In vitro stimulation				
1	MBP	None	HEL	180 ± 23	<75	<75	<100
2	MBP	None	MBP	429 ± 34	77 ± 36	580 ± 38	350 ± 47
3	HEL	None	HEL	1050 ± 120	88 ± 17	570 ± 108	261 ± 22
4	HEL	None	MBP	230 ± 38	<75	<75	<100
5	MBP	MBP	HEL	320 ± 19	83 ± 41	340 ± 29	246 ± 18
6	MBP	MBP	MBP	6020 ± 233	252 ± 39	965 ± 201	869 ± 31
7	HEL	MBP	MBP	415 ± 49	813 ± 71	520 ± 84	410 ± 50

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