Human Intestinal Epithelial Cell–induced CD8$^+$ T Cell Activation Is Mediated Through CD8 and the Activation of CD8-associated p56^{lck}

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Summary

The activation of CD8$^+$ suppressor T cells by normal intestinal epithelial cells in antigen-specific or allogeneic mixed cell culture systems has significant implications for the regulation of mucosal immune responses. In this study, we found that the capacity of epithelial cells to induce CD8$^+$ suppressor T cell activation appeared to be linked to the binding of CD8 molecules on the T cell surface. This appears to be mediated by a non–class I molecule expressed on the epithelial cell surface, which binds to CD8 and results in the activation of the CD8-associated src-like tyrosine kinase, p56^{lck}. Epithelial cell–stimulated p56^{lck} activation is an early event (in contrast to monocytes) and is essential for T cell activation, since proliferation could be completely abrogated by pretreatment of T cells with genestein or herbamycin, both of which are protein tyrosine kinase inhibitors. Pretreatment of T cells with anti-CD8 or of intestinal epithelial cells with an anti–epithelial cell mAb B9 inhibited p56^{lck} activation and further confirmed that CD8 on the T cell and a CD8 ligand on the epithelial cell were involved in this T cell activation event. The specificity of this reaction was confirmed in experiments in which murine transfectants 3G4 and 3G8, expressing CD4 or CD8, respectively, were used. Coculture of 3G8 with epithelial cells but not with monocytes activated p56^{lck} in this cell line, whereas p56^{lck} was preferentially activated in 3G4 cells when monocytes were used as the stimulator cells. Although stimulation through CD8- and CD8-associated p56^{lck} was important for epithelial cell–induced T cell activation, T cell proliferation could not be induced by cross-linking CD8 alone with monoclonal antibody anti-CD8. These data suggest that a second signal, possibly through the T cell antigen receptor since activation of the T cell receptor–associated kinase fyn was also seen, is required for epithelial cell–driven T cell proliferation.

Over the past several years, it has become increasingly apparent that the rules governing mucosal immune responses differ from those of the peripheral immune system. First, antigen priming via the oral route most frequently results in the development of immunologic suppression or oral tolerance rather than in an active immune response. Second, the cell types in the gastrointestinal tract are not typical of what one would find systemically. Intraepithelial lymphocytes are predominantly CD8$^+$ T cells that are, in general, anergic despite exposure to inordinate numbers of disparate foreign antigens in the gut lumen (1–3). In contrast, lamina propria T cells are activated memory cells, a rich source of cytokines, but with limited proliferative capacity to specific antigen (4, 5). An understanding of the underlying mechanisms responsible for these phenomena has been slow to evolve, partly because of our limited knowledge of how the primary trigger of an immune response, antigen, is handled by the gastrointestinal mucosa. Our laboratory as well as others has focused on the role of the intestinal epithelial cell (IEC)[1] as an APC in this system. We and others have demonstrated that IEC can take up, process, and present soluble antigens to primed T cells (6, 7). However, unlike conventional APCs, normal IEC appear to selectively activate CD8$^+$ T cells. These activated CD8$^+$ cells express suppressor function without any evident cytolytic activity. In this study, we define a mechanism whereby normal IEC can selectively activate CD8$^+$ T cells. By binding and cross-linking CD8 on T cells by a non–class I ligand expressed on normal IEC, CD8 α chain–associated p56^{lck} is activated. This activation is necessary but not sufficient for CD8$^+$ T cell proliferation.

This manuscript is in partial fulfillment of a Ph.D. thesis (Yin Li and Xian Yang Yio).

[1] *Abbreviations used in this paper:* CMF-HBSS, calcium/magnesium-free HBSS; IEC, intestinal epithelial cell; PAS, protein A–Sepharose; PKA, protein kinase A; PKC, protein kinase C.

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Materials and Methods

Isolation of Peripheral Blood T Cells and Adherent Cells. Heparinized venous blood was collected from normal donors, diluted 1:3 with sterile PBS, layered on a Ficoll-Hypaque (Pharmacia Biotech Inc., Piscataway, NJ) density gradient, and centrifuged for 30 min at 500 *g* (8). The mononuclear cells were collected from the interface and washed three times with PBS. Cells were resuspended in RPMI 1640 (GIBCO BRL, Gaithersburg, MD), and the cell density was adjusted to 5×10^6 cells/ml. T cells were separated using a rosetting technique with neuraminidase-treated sheep red blood cells and Ficoll-Hypaque density gradient centrifugation. Non–T cells from the interface were collected, washed three times with PBS, and resuspended in culture medium (RPMI 1640, 10% FCS, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine; all from GIBCO BRL). Adherent cells were obtained by incubating non–T cells in tissue culture dishes (25 million cells per dish in 10 ml culture medium for 45 min at 37°C in a humidified 5% CO_2 incubator). B cell–enriched nonadherent cells were then aspirated, and dishes were washed vigorously three times with PBS. Adherent cells were harvested by scraping cells off using a rubber policeman. Rosetted T cells were treated with 0.75% ammonium chloride on ice for 5–10 min to lyse sheep red blood cells. The T cell suspension was then washed three times with PBS and was finally resuspended in culture medium. Small numbers of contaminating adherent cells in the T cell suspension were removed by the adherence procedure described above. The purity of isolated T cell preparations was assessed by staining. Typical preparations were >95% $CD3^+$, <1% $CD14^+$, and <1% sIg^+.

Preparation of Human IEC. The procedure used for the isolation of IEC is a modification of the method used by D. M. Bull and M. A. Bookman for the isolation of lamina propria and intraepithelial lymphocytes (9). Resected tissue specimens removed from patients with colon cancer at least 10 cm away from the carcinoma (histologically normal) were washed extensively with HBSS (GIBCO BRL) containing 50 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO), and 2.5 μg/ml amphotericin B (Flow Laboratories, Inc., McLean, VA). The surface mucosa was stripped off from the underlying submucosa and minced into tiny pieces. These were placed in calcium/magnesium-free HBSS (CMF-HBSS) containing antibiotics and 1 mM dithiothreitol (Sigma Chemical Co.) and incubated in a 37°C water bath for 5 min to remove adherent mucus. The tissue pieces were washed again in CMF-HBSS and incubated in RPMI 1640 containing 3 mg/ml dispase (Boehringer Mannheim Corp., Indianapolis, IN) in a 37°C water bath for 30 min, vortexing every 5 min. During this treatment, epithelial cells and intraepithelial lymphocytes were released from the tissue. Cells that remained loosely adherent to the tissue were recovered using a rubber policeman. Dispase treatment was repeated once. Isolated epithelial cells were washed twice with CMF-HBSS and then applied to Ficoll-Hypaque and Percoll density gradients to improve the viability and purity of the preparation. The purified epithelial cells were >95% viable, <0.1% esterase positive, <0.1% $OKM1^+$, <0.1% $OKT6^+$, <1% surface Ig^+, and 1–2% $CD3^+$. Cells were usually irradiated 3,000 rad and kept in RPMI 1640 containing 5% human agammaglobulinemic serum.

Murine Transfectants 3G4 and 3G8. The murine transfectants 3G4 and 3G8, expressing human CD4 and CD8 antigens, respectively, were kind gifts from Dr. S. J. Burakoff (Dana-Farber Institute, Boston, MA). The construction of these hybridoma transfectants has been previously described. For construction of the 3G4 cell line (10), the T cell hybridoma BY155.16 was transfected with the defective retrovirus MNST4 carrying human CD4 cDNA, and transfectants expressing human CD4 antigen were selected by sorting. The establishment of the 3G8 cell line was similar, transfecting the T cell hybridoma BY155.16 with the retroviral expression vector MNCT8 carrying human CD8α cDNA (11).

mAbs. OKT8 (anti-CD8) and W6/32 (anti–HLA class I) were obtained from American Type Culture Collection (Rockville, MD). Both have previously been shown to inhibit human CTL activity. VG2 (anti–class II monomorphic determinant) was the kind gift of Dr. Shu Man Fu (University of Virginia, Charlottesville, VA). FFB 2.3 (anti-CD4) was a gift from Dr. David Posnett (New York Hospital, New York), and mAb 446 (anti-CD3) has been previously defined in this laboratory (12). mAbs B9 and L12 against human IEC membrane antigens were generated in our laboratory as previously described (13). These two mAbs were chosen for their ability to specifically inhibit IEC-driven $CD8^+$ T cell proliferation.

Immunoprecipitation and In Vitro Tyrosine Kinase Assay. Isolated peripheral blood T cells were incubated in an Eppendorf tube with either normal IEC, peripheral adherent cells (10 million T cells:10 million stimulator cells), or antibodies cross-linking CD4 or CD8 in a 37°C water bath for varying time periods. At specific time points, the tube was transferred to ice and cold 2× NP-40 lysis buffer (2% NP-40, 40 mM Tris base, pH 8.0, 300 mM NaCl, 400 μM EDTA, 20 mM sodium pyrophosphate, 200 mM sodium fluoride, 1 mM PMSF, 5 mM iodoacetamide, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 200 μM Na_3VO_4) (Sigma Chemical Co.) was added for 30 min, vortexing every 5 min. The lysate was centrifuged at 14,000 rpm for 10 min in a microfuge to remove cell debris. The lysate was then precleared with protein A–Sepharose (PAS; Pharmacia Biotech, Inc., Piscataway, NJ) (30 μl of a 50% PAS bead suspension per sample) for 30 min at 4°C. The clarified lysate was incubated with 4G10 (antiphosphotyrosine mAb) (Upstate Biotechnology, Inc., Lake Placid, NY) (5 μg per sample), anti-lck sera (gift of Dr. C. E. Rudd, Dana Farber, Boston, MA), or anti-fyn (Upstate Biotechnology, Inc.) for 1 h at 4°C in the presence of PAS (50 μl of a 50% PAS bead suspension per sample) and incubated for 2 h at 4°C. The immunoprecipitates were washed once with PBS, twice with 0.5 M LiCl (Sigma Chemical Co.) in 20 mM Tris, pH 8.0, and once with kinase buffer (10 mM $MnCl_2$, 50 mM Tris, pH 7.4). The immunoprecipitates were finally resuspended in kinase buffer (30 μl per sample) and mixed with γ-[^{32}P]ATP (Amersham Corp., Arlington Heights, IL) (10 μCi per sample). After an incubation of 30 min at 25°C, the reaction mixture was subjected to 10% SDS-PAGE and autoradiography.

For mAb blocking studies, we incubated T cells with OKT8 (Ortho Diagnostic Systems, Inc., Raritan, NJ) (10 μg/ml) or normal IEC with the anti–epithelial cell mAbs B9 (10 μg/ml) or L12 (10 μg/ml) at 4°C for 30 min. Unbound antibodies were removed by washing. Treated or untreated T cells were then incubated with treated or untreated normal epithelial cells at 37°C for 2 min. Cells were lysed, and $p56^{lck}$ enzyme activity was measured as described above.

Western Blot with Antiphosphotyrosine mAbs. Lysates were resolved on 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane at 15 V overnight in transfer buffer (20% methanol, 150 mM glycine, 25 mM Tris, pH 8.3). The transfer sandwich was prewetted in transfer buffer and set up in the following order: (*a*) a porous polyethylene foam sheet; (*b*) one sheet of 3-M filter paper; (*c*) the gel; (*d*) a nitrocellulose membrane sheet (0.2 μm) (Schleicher & Schuell, Inc., Keene, NH); (*e*) another sheet of

3-M filter paper; and (*f*) another sheet of porous polyethylene foam. The transfer sandwich was inserted into a transfer electrophoresis unit with the nitrocellulose membrane at the anode. After transfer, the nitrocellulose sheet was blocked by 50 ml of 5% nonfat milk in PBS. The nitrocellulose sheet was washed once with PBS and transferred to another plastic bag containing the antiphosphotyrosine antibody (4G10; Upstate Biotechnology Inc.) (1 μg/ml) in a 0.5% nonfat milk–PBS solution. The plastic bag was then sealed, and the sheet in the bag was incubated at room temperature for 2 h or 4°C overnight. The sheet was washed five times with washing buffer (0.05% Tween 20 in PBS), for 5 min each time, and then transferred to a new plastic bag containing the secondary antibody (goat anti–mouse IgG), which had been conjugated with horseradish peroxidase (1–2 μg/ml). The incubation was continued at room temperature for 1–2 h. The sheet was washed five times with washing buffer, 5–10 min each time, and incubated with 12 ml of chemiluminescence reagent (DuPont, Wilmington, DE) at room temperature for 1 min. A sheet of plastic wrap was placed onto the nitrocellulose sheet, and the sheet was exposed and developed (XAR-5 film; Eastman Kodak Co., Rochester, NY).

Mixed Cell Culture Responses in the Presence of Blocking mAbs. An allogeneic mixed cell culture reaction was performed as previously described (6) using 10^5 irradiated IEC as stimulators and 10^5 allogeneic isolated T cells in the presence of mAb anti-CD4, anti-CD8, anti–class I, anti–class II, or an irrelevant isotype–matched Ab control (25 μg/ml for each mAb) in culture medium. All cultures were performed in triplicate in 96-well round-bottomed microwell plates for 120 h at 37°C in a 5% CO_2 humidified incubator. During the last 18 h of culture, 1 μCi [^{3}H]thymidine (ICN Biomedicals, Inc., Costa Mesa, CA) was added, and cells were harvested onto glass fiber filter mats for counting. Counts were obtained and averaged by a scintillation counter (model LS3801; Beckman Instruments, Inc., Somerset, NJ).

Inhibition of T Cell Proliferation in the Presence of Kinase Inhibitors. 10^5 adherent cell–depleted T cells used as responder cells were cultured with 10^5 irradiated (3,000 rad) human IECs or adherent cells as stimulators in 200 μl culture medium, in the presence or absence of 200 μM genestein (Calbiochem-Novabiochem Corp., La Jolla, CA) added at time 0 or 2 h after the onset of culture. In all experiments, one set of T cells was pretreated with genestein (200 μM) for 2 h followed by removal of genestein by washing. The treated T cells were then added to cultures containing irradiated IECs or monocytes. In other experiments, inhibition by genestein was compared with either herbamycin (a specific tyrosine kinase inhibitor; 0.45–1.8 μM), staurosporine (a protein kinase C [PKC] inhibitor; 5 nM), and H-8 (a protein kinase A [PKA] inhibitor; 5 μM). All cultures were performed in triplicate in 96-well round-bottomed microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ) for 120 h at 37°C in a 5% CO_2 humidified incubator, and proliferation was assessed as described above.

Results

Binding to CD8 on T Cells Is Required for Normal IEC-induced CD8$^+$ Suppressor T Cell Proliferation. We have previously demonstrated that freshly isolated normal human IECs were able to trigger CD8$^+$ suppressor T cell proliferation in a mixed cell culture system (6). However, the mechanism of the generation of these antigen-nonspecific

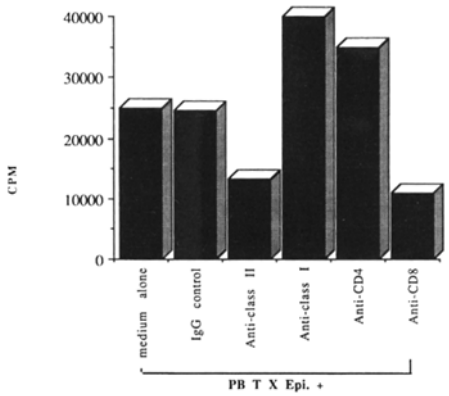

Figure 1. Inhibition of epithelial cell–stimulated peripheral blood T cell proliferation by specific mAbs. Freshly isolated IECs (10^5/well) were cultured with peripheral blood T cells at a ratio of 1:1 in triplicate cultures in 96-well U-bottomed plates in the presence or absence of either mAb anti-CD4, anti-CD8, anti–class I, anti–class II, or an isotype-matched control mAb at 25 μg/ml for 120 h. [^{3}H]Thymidine (1 μCi) was added 18 h before harvest, and thymidine incorporation was measured. This experiment is representative of at least 10 experiments. The anti-CD8 mAb OKT8 failed to inhibit PHA-induced proliferation of these same peripheral blood T cells (86,143 vs 85,459 cpm).

CD8$^+$ suppressor T cells was not addressed. To examine the role of specific surface molecules involved in this T cell activation event, we cultured T cells with varying concentrations of either anti-CD4, anti-CD8, anti–class I MHC, or anti–class II MHC mAbs. As previously reported, mAbs against CD4 have the capacity to inhibit CD4$^+$ T cell activation and proliferation (14). Thus, by adding anti-CD4 mAbs, we would be able to highlight any T–T interaction. As seen in Fig. 1, antibodies to CD4 failed to inhibit CD8$^+$ T cell proliferation induced by IECs at any concentration. However, CD8$^+$ T cell proliferation could be inhibited by anti-CD8 mAbs, suggesting that the CD8 molecule itself may be involved directly in this process. Of note was the finding that the same anti-CD8 mAb failed to inhibit the proliferation of isolated CD8$^+$ T cells to PHA (see Fig. 1 legend), suggesting that the effects seen with this mAb were directed at the interaction of epithelial cells and T

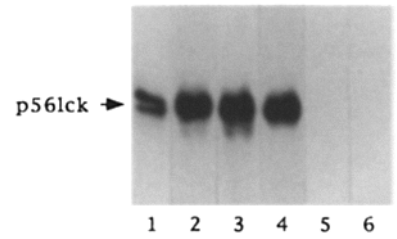

Figure 2. Determination of p56^{lck} enzyme activity in activated peripheral blood T cells using an in vitro kinase assay. Peripheral blood T cells (10×10^6 cells each) stimulated with either anti-CD4 mAb (cross-linked with rabbit anti–mouse IgG for 1 min), normal human IECs, or monocytes (for 2 min) were lysed, immunoprecipitated with an anti-lck antibody, and autophosphorylated in the presence of γ-[^{32}P]ATP. Total reactants were analyzed on 10% SDS-PAGE and exposed on film. Lane *1*, unstimulated T cells; lane *2*, cross-linked anti-CD4–activated T cells; lane *3*, enterocyte-activated T cells; lane *4*, monocyte-activated T cells; lane *5*, enterocytes only; and lane *6*, monocytes only.

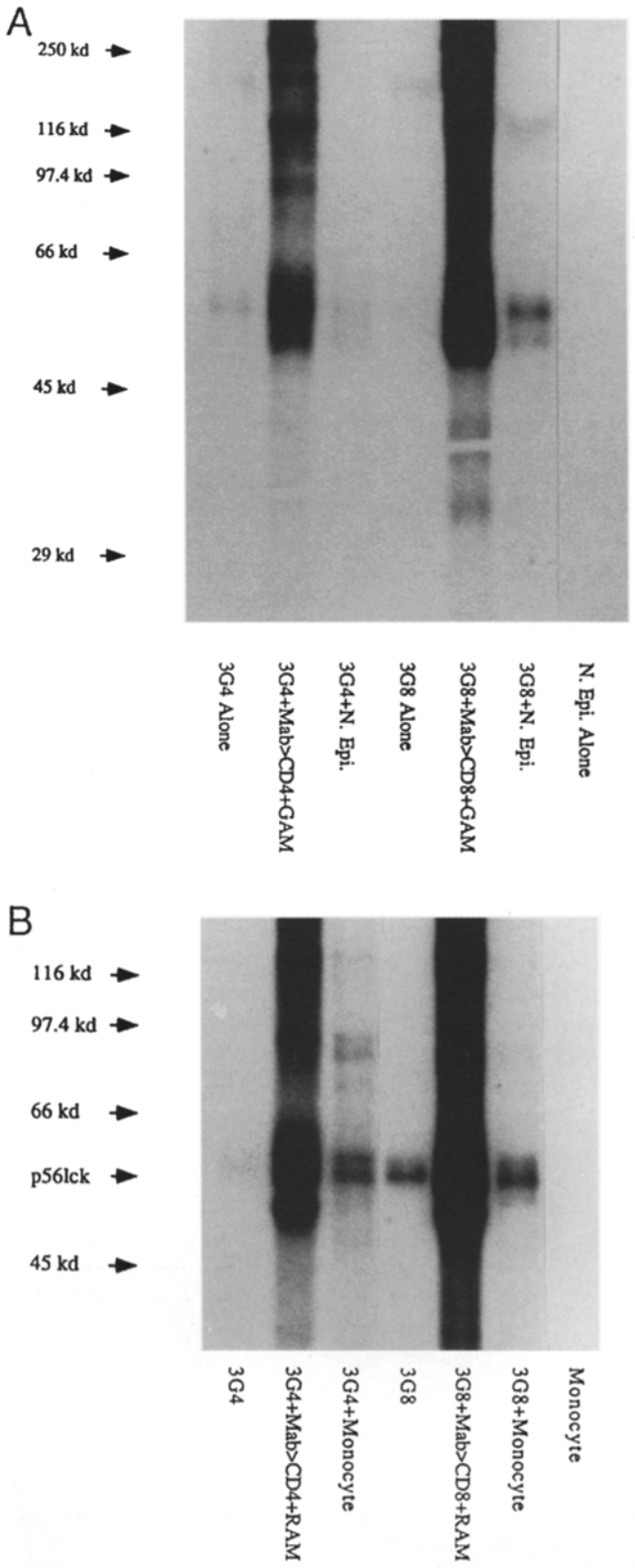

Figure 3. Analysis of T cell surface molecules responsible for $p56^{lck}$ activation induced by IECs. (*A*) Murine T cell hybridomas transfected with human CD4 or CD8 cDNA (3G4 and 3G8, respectively) were used as responder cells in coculture with human IECs or with anti-CD4 or anti-CD8 mAbs as a positive control. The level of $p56^{lck}$ enzyme activity was measured in an in vitro protein tyrosine kinase assay. The position of $p56^{lck}$ is depicted by the arrow. (*B*) Murine transfectants 3G4 and 3G8 were stimulated by monocytes or with the appropriate mAb (anti-CD4 or anti-CD8, respectively) as positive controls, and the activation of $p56^{lck}$ was determined.

cells and were not due to nonspecific suppression of $CD8^+$ T cells. Furthermore, this anti-CD8 mAb (OKT8) has been previously shown to inhibit CTL activity (15). Interestingly, an mAb against a framework determinant of class I (W6/32), the conventional ligand for CD8, failed to inhibit T cell proliferation. In fact, the pattern of proliferation was comparable to that seen with anti-CD4. Similar to anti-CD8, W6/32 has been shown to inhibit CTL activity (16).

In contrast, the anti–class II mAb, VG2, was capable of inhibiting T cell proliferation comparable to that seen with anti-CD8. Since T cell activation can be inhibited by anti–class II Abs present in the culture wells (17), we pretreated the epithelial cells with the anti–class II mAb VG2 for 30 min at 4°C, washed them free of unbound antibody, and used them as stimulators in MLR cultures with isolated $CD4^+$ or $CD8^+$ T cells as responder cells. Pretreatment with class II mAbs resulted in the inhibition of $CD4^+$ but not $CD8^+$ T cell proliferation (data not shown). These data suggest that our initial findings relating to inhibition of proliferation in allogeneic IEC:T cell cultures by a polyclonal anti–class II antibody (6) were due to direct inhibition of T cell activation by this antibody. Furthermore, the finding that IEC-driven $CD8^+$ T cell proliferation persists even in the presence of anti-CD4, anti–class I, and anti–class II mAbs provides support for an alternate pathway, potentially a novel ligand for CD8, that may be activating these cells. The increase in ^3H incorporation seen in the anti-CD4 and anti–class I mAb–treated cultures was reproducible in all experiments and may represent freer access of CD8 to a novel ligand.

Both Normal IECs and Monocytes Induce the Activation of $p56^{lck}$. Recent findings indicate that CD4 and CD8 molecules serve not only to enhance the weaker binding of the T cell receptor to the MHC–Ag complex on APCs but also to transduce signals themselves to T cells through binding to their respective ligands. Although CD4 and CD8 intrinsically do not possess kinase activity, their intracytoplasmic tails are physically associated with the src-like protein tyrosine kinase, $p56^{lck}$ (18, 19). Cross-linking CD4 or CD8 by monoclonal antibodies has been shown to induce $p56^{lck}$ activation. In a representative experiment shown in Fig. 2, we investigated the activation of $p56^{lck}$ protein tyrosine kinase activity after culturing T cells with normal IECs or monocytes for 2 min. Under reducing conditions, the unstimulated T cell lane shows the basal level of $p56^{lck}$ activity. Cross-linking CD4 with anti-CD4 mAb, culture with normal IECs, or monocytes results in a marked increase in $p56^{lck}$ enzyme activity. No detectable $p56^{lck}$ activity was seen in the stimulator cells alone, i.e., normal epithelial cells or monocytes. These studies imply that, during cocul-

ture of T cells with normal epithelial cells or monocytes, $p56^{lck}$ is triggered and activated as an early event.

The Pathway of Activation of $p56^{lck}$ Induced by Normal Epithelial Cells and Monocytes is through CD8 and CD4 Molecules, Respectively. Although stimulation through either CD4 or CD8 molecules can activate $p56^{lck}$, other surface molecules (e.g., IL-2R, CD2) have been reported to have the capacity to activate, or to be associated with the activation of, $p56^{lck}$ (20–21). To assess the specific molecules involved in a $p56^{lck}$ activation by normal epithelial cells and monocytes, we used two transfected murine T cell hybridomas expressing either human CD4 (3G4) or CD8 (3G8) molecules. 3G4 and 3G8 cell lines were cocultured with normal epithelial cells or monocytes, and $p56^{lck}$ enzyme activity was measured. From Fig. 3 *A*, one can clearly see that normal epithelial cells activate $p56^{lck}$ in the 3G8 cell line but not in the 3G4 cell line. In contrast, monocytes activate $p56^{lck}$ in 3G4 cells (Fig. 3 *B*). While 3G8 appears to be more vigorous in its response to any stimulus (mAb or IEC), probably both because of the greater expression of CD8 on 3G8 than of CD4 on 3G4 and the formation of CD8α/α homodimers, the difference in using monocytes vs IEC as stimulator cells is clear. In the case of epithelial cell stimulation, there was no detectable up-regulation of $p56^{lck}$ enzyme activity in 3G4 cells when compared with the unstimulated background control, whereas a >10-fold increase in $p56^{lck}$ enzyme activity was seen in 3G8 cells. Monocytes stimulated $p56^{lck}$ enzyme activity in 3G4 cells to a much greater extent than in 3G8 cells, especially since the background $p56^{lck}$ activation in the 3G8 cells was comparable to the monocyte-stimulated activity. Since the only human surface molecules expressed on these transfectants were either CD4 or CD8, these data support the concept that IEC express a molecule (non–class I) that binds to CD8 to activate $p56^{lck}$.

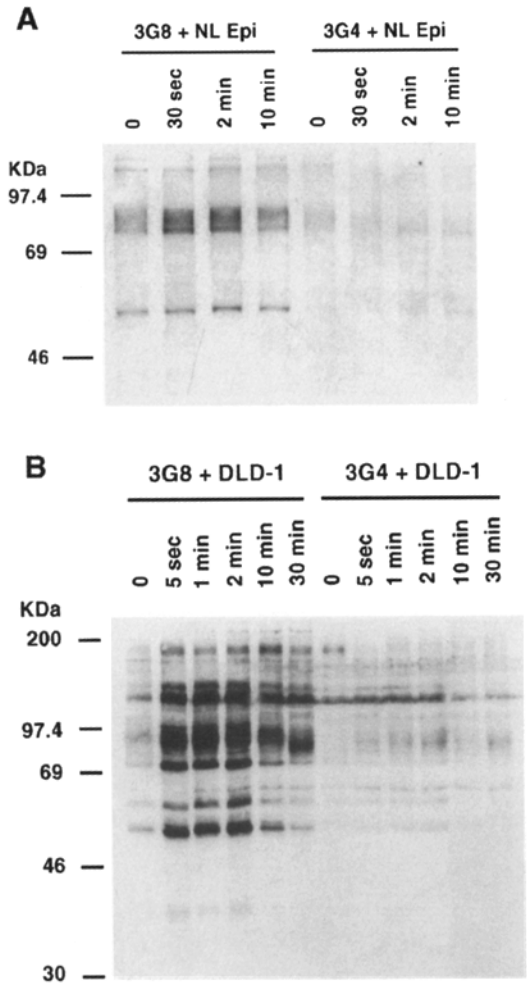

Figure 4. Antiphosphotyrosine Western blot of lysates from (*A*) normal IEC:3G4/3G8 or (*B*) the epithelial cell line DLD-1:3G4/3G8 cocultures. 3G4 or 3G8 cells were cocultured with equivalent numbers of freshly isolated normal IEC (*A*) or DLD-1 cells for varying time periods, lysed, and resolved on 7.5% SDS-PAGE. The gel was transferred onto nitrocellulose and subjected to Western blot analysis using the antiphosphotyrosine mAb 4G10 as described in Materials and Methods. These blots are representative of at least five experiments.

To determine whether activation of $p56^{lck}$ was selective or part of a global activation of intracellular kinases, we performed similar cocultures (IEC and 3G4 or 3G8 cells) but analyzed tyrosine phosphorylation by Western blot using the antiphosphotyrosine mAb 4G10. Increased phosphorylation of a 56-kD band was seen at 30 s in the 3G8 but not in the 3G4 cells as described above, peaked at 2 min, and returned to baseline at 10 min (Fig. 4 *A*). Concomitantly, there was tyrosine phosphorylation of substrates at 72 and 86 kD with similar kinetics. Given the autophosphorylation of $p56^{lck}$ seen in the kinase assays, these higher molecular weight bands appear to be lck substrates. No other phosphorylated proteins were seen in the 3G4 cells. Similar results were obtained by coculturing the malignant IEC line DLD-1 with 3G4 and 3G8 cells, although a greater number of phosphorylated bands were detected (Fig. 4 *B*). However, some of these substrates appear to be derived from the epithelial cell line itself, as many were seen at the onset of culture (time 0).

Activation of $p56^{lck}$ Occurs Early and Is Critical for $CD8^+$ T Cell Proliferation. To determine the kinetics of $p56^{lck}$ activation, normal IECs were incubated with T cells for variable time periods and lysed, and $p56^{lck}$ activity was measured. In Fig. 5 *A*, epithelial cell–stimulated $p56^{lck}$ activation could be seen maximally at 1 min, persisted for 5 min, and declined at 10 min. After 30 min, the enzyme activity of $p56^{lck}$ was nearly baseline. In contrast to epithelial cells, monocyte-stimulated T cell–associated $p56^{lck}$ activity increased gradually, reaching a maximum at ~30 min and maintaining the activated state for up to 2 h (Fig. 5 *B*). These differences in the kinetics of $p56^{lck}$ enzyme activity induced by normal IECs versus monocytes may reflect the different functional requirements of $p56^{lck}$ in the activation of different T cell populations.

Genestein and Herbamycin, Tyrosine Kinase Inhibitors, Inhibit Both Normal Epithelial Cell– and Monocyte-stimulated Peripheral Blood T Cell Proliferation. To test whether $p56^{lck}$ activation is critical for $CD8^+$ T cell proliferation, we cul-

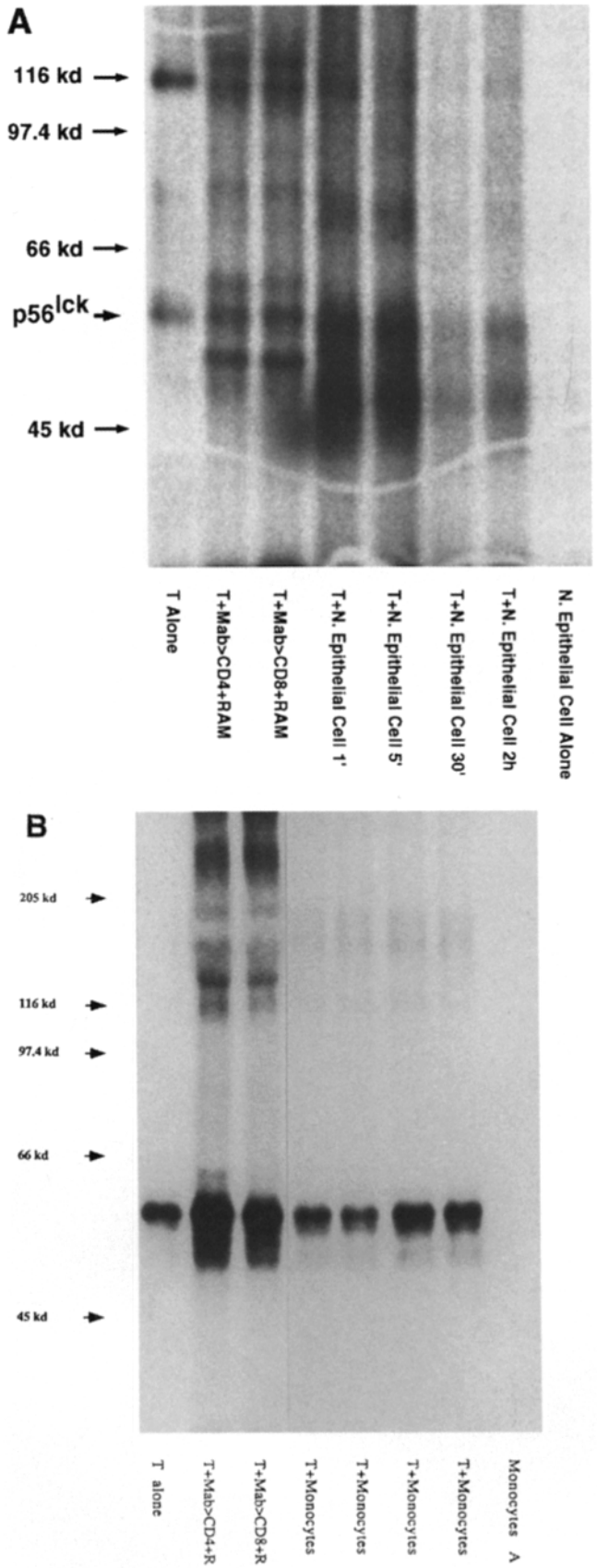

Figure 5. Time course of tyrosine autophosphorylation of $p56^{lck}$ activated by IECs (*A*) and monocytes (*B*). Peripheral blood T cells were cultured with epithelial cells (*A*) or monocytes (*B*) for varying time periods. The reaction was stopped by adding cold 2× lysis buffer. The lysate was immunoprecipitated with anti-lck antibody, and $p56^{lck}$ tyrosine kinase activity was then measured in an in vitro tyrosine kinase assay. Cross-linking with anti-CD4 or anti-CD8 served as positive controls. The arrow depicts $p56^{lck}$.

tured T cells with normal enterocytes or monocytes in the presence of varying concentrations of genestein, a tyrosine kinase inhibitor. Since the kinetics of $p56^{lck}$ activation was different depending upon the nature of the stimulator cells, we added genestein early (2 h before culture), at the onset of culture, or 2 d after culture. Preincubation of T cells with genestein for 2 h resulted in complete inhibition of T cell proliferation induced by enterocytes (Fig. 6 *A*), while genestein added at the onset of culture of T cells and epithelial cells resulted in only 50% inhibition. No inhibition of T cell proliferation was seen when genestein was added 2 d after the onset of culture, suggesting that inhibition of an early event was critical (data not shown), and genestein's effects were not mediated via nonspecific toxicity. In contrast, monocyte stimulation of T cells was inhibited regardless of when genestein was added, consistent with the kinetics data (Fig. 6 *B*). Preincubation of T cells with genestein allows for the complete inhibition of intracytoplasmic tyrosine kinase before coculture with stimulator cells. This inhibition of an early response to epithelial cell stimulation indicates that the initial tyrosine kinase activation is critical for normal epithelial cell–driven T cell proliferation. Activation of tyrosine kinases is also important in monocyte-driven T cell proliferation but appears to require both early and ongoing events. Therefore, the pathways promoting T cell proliferation used by normal epithelium and monocytes appear to be different. Since genestein may not be a specific tyrosine kinase inhibitor, we confirmed these data comparing the effects of herbamycin, a selective tyrosine kinase inhibitor, with the effects of staurosporine (a PKC inhibitor) (5 nM) or H8 (a PKA inhibitor) (5 μm). As seen in Fig. 6 *C*, herbamycin (at varying concentrations) but not staurosporine or H8 added at the onset of culture inhibited IEC-driven T cell proliferation. These findings support the concept that the activation of a tyrosine kinase is critical to the T cell proliferation induced by IEC.

The Activation of CD8/$p56^{lck}$ Is Necessary but Not Sufficient for Normal IEC-driven T Cell Proliferation. Given the findings described above, we wanted to determine whether stimulation through CD8 alone was sufficient to drive T cell proliferation. In these experiments, we cultured PBMC with mAb OKT8, 446 (anti-CD3), L12 (IgG1 here acting as a negative control), or PHA in the presence or absence of IL-2 for 2 d. mAb 446 (anti-CD3) was able to stimulate T cell proliferation in the absence of IL-2 (data not shown). In contrast, mAb OKT8 (anti-CD8) could not stimulate T cell proliferation even in the presence of IL-2 and accessory cells, despite the fact that $p56^{lck}$ activation was seen (Fig. 2). These results indicate that signals through CD8 alone are not sufficient to drive T cell proliferation, and they raise the possibility of the requirement for a second signal.

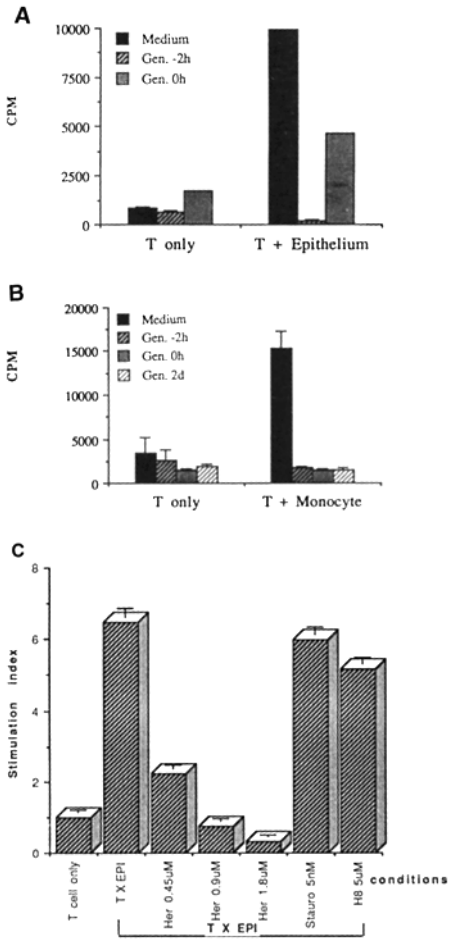

Figure 6. Inhibition of IEC-stimulated T cell proliferation in the presence of the protein tyrosine kinase inhibitors genestein and herbamycin. (*A*) T cells treated or untreated with genestein (200 μM) 2 h before or at the onset of culture were incubated with irradiated IECs for 5 d. [^{3}H]Thymidine was added 18 h before cell harvesting, and thymidine incorporation was measured in a scintillation counter. (*B*) T cells treated or untreated with genestein (200 μM) 2 h before, at time 0, or 2 d after the onset of culture were incubated with irradiated monocytes for 5 d, and [^{3}H]thymidine incorporation was measured. Viability was comparable in treated and untreated cultures on day 5 before harvesting. (*C*) T cells were cocultured with normal IEC in the presence or absence of herbamycin (0.45–1.8 μm), staurosporine (5 nM), or H8 (5 mM) for 5 d as described above. Neither herbamycin (at any concentration), staurosporine, or H8 had any inhibitory effect on T cells stimulated with PHA (T + PHA = 44,780 cpm; T + PHA + 0.45 μm herbamycin = 56,443 cpm; T + PHA + 1.8 μM herbamycin = 54,398 cpm; T + PHA + 5 nM staurosporine = 42,408 cpm; T + PHA + 5 μM H8 = 38,077 cpm). These findings are representative of two experiments.

Given the system used to induce CD8$^+$ T cell proliferation (allogeneic cells or Ag-pulsed autologous IEC), the second signal may be mediated through the T cell Ag receptor. To address this possibility more directly, we cocultured allogeneic IEC with nylon wool–purified T cells for 2 min, lysed the cells, immunoprecipitated the TCR-associated kinase fyn, and performed an in vitro kinase assay. These studies could not be performed with the murine transfectants since their Ag receptors would not be expected to recognize human alloantigens. As seen in Fig. 7

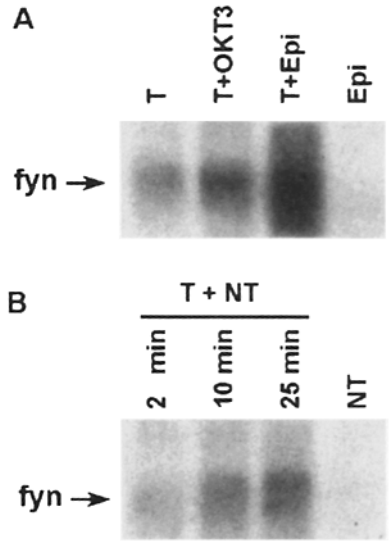

Figure 7. Activation of fyn in T cell–IEC (*A*) or T cell:non–T cell (*B*) cocultures. Cocultures were established as described in Figs. 2 and 5 except that anti-fyn was used as the immunoprecipitating mAb. For allogeneic IEC:T cell cocultures, the reaction was stopped at 2 min and compared with the response seen using the anti-CD3 mAb OKT3. In the T cell:non–T cell cocultures, the reaction was stopped at 2, 10, and 25 min. Both coculture conditions result in the phosphorylation of fyn, although the kinetics is different.

A, fyn kinase activity was increased after T cell:IEC coculture. Monocytes were also able to activate fyn kinase activity but with different kinetics (peaking at 25 min) (Fig. 7 *B*). Since p56^{lck} is not known to phosphorylate fyn, these findings suggest an independent coactivation step involving the TCR.

The Activation of p56^{lck} Induced by Normal Epithelial Cells Is Blocked by mAb Anti-CD8 and B9. Since mAb anti-CD8 could block epithelial cell–stimulated CD8$^+$ T cell proliferation while mAbs to class I, the conventional ligand for CD8, could not, we used anti–epithelial cell mAbs to define the ligand for CD8 expressed on epithelial cells. mAbs B9 and L12 were selected for their ability to inhibit normal epithelial. cell induced CD8$^+$ T cell proliferation when present in the culture (13). B9 stains intestinal epithelium from all sites (except for esophageal epithelium) with villus>crypt staining, and it fails to stain T cells, B cells, or monocytes. L12 stains intestinal epithelium more intensely than B9 and also fails to stain PBMC. To determine whether the activation of p56^{lck} in CD8$^+$ T cells induced by normal epithelial cells occurs via the binding of a novel CD8 ligand on epithelial cells to CD8 on T cells, we used mAb OKT8 and anti–epithelial cell mAbs B9 and L12 to block p56^{lck} activation. Fig. 8 shows that OKT8 and B9 can inhibit p56^{lck} activation, while L12 generally does not. These findings suggest that mAb B9 might recognize a ligand for CD8 on the epithelial cell surface or at least be situated near such a ligand. mAbs to CD1d, which we have previously demonstrated to be inhibitory for proliferation in T cell:IEC cocultures, failed inhibit activation of CD8-

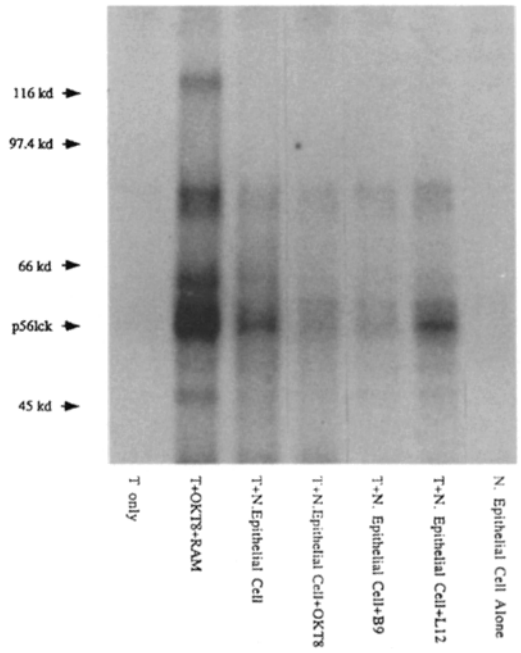

Figure 8. Inhibition of IEC-induced $p56^{lck}$ activation in peripheral blood T cells by mAbs. IECs were treated with mAb B9 or L12, or T cells were treated with mAb OKT8 (anti-CD8), for 30 min at 4°C, and unbound antibodies were removed by washing. Treated or untreated epithelial cells were cocultured with treated or untreated T cells, and the induction of $p56^{lck}$ enzymatic activity immunoprecipitated by anti-lck antibody was measured in an in vitro tyrosine kinase assay as described in Figs. 2 and 5.

associated $p56^{lck}$, suggesting that this molecule does not associate with CD8 (data not shown).

Discussion

The fact that oral tolerance can be mediated via the generation of T suppressor cells is well established (22, 23). The mechanism of activation of such cells is still not defined. In this study, we demonstrate that $CD8^+$ suppressor T cells activated by normal IEC require binding to the CD8 molecule itself. In this setting, cross-linking CD8 activates the src-like tyrosine kinase, $p56^{lck}$. This, in turn, may phosphorylate a number of substrates, resulting in nuclear signaling for cell proliferation. Positive activation of cells through CD8 contrasts with studies by van Seventer et al. (15), who reported that anti-CD8 mAbs could inhibit cytotoxicity of CTL clones. Although we have not found anti-CD8 mAbs to be inhibitory to $CD8^+$ T cell proliferation induced by mitogens, the results from van Seventer et al. are not incongruent with ours. First, we are analyzing a distinct phenomenon, suppressor T cell activation. Second, we are dealing with a ligand for CD8 expressed on IEC that is not class I, since anti–class I mAb treatment of IEC failed to inhibit $CD8^+$ T cell proliferation while it can inhibit CTL activity (16). In addition, class I alone does not appear to be capable of activating CD8-associated $p56^{lck}$, and coculture of 3G4 or 3G8 cells with the thyroid epithelial cell line LA-1 expressing class I at levels comparable to normal IEC (but failing to express B9 or L12) fails to induce tyrosine phosphorylation in either cell type. Third, recent studies suggest that the inhibitory effect mediated by soluble anti-CD4 or anti-CD8 interferes with coreceptor formation with the TCR–CD3 complex (24–26). Furthermore, we are not dealing with a T cell–T cell interaction (CD4–CD8), since antibodies to CD4 also fail to inhibit $CD8^+$ T cell proliferation. In fact, an interesting finding that is consistently observed is the enhancement of $CD8^+$ T cell proliferation in the presence of anti-CD4 and anti–class I mAbs. This may relate to the enhanced accessibility of CD8 molecules for a non–class I ligand (in the case of anti–class I), promoting a greater response in the $CD8^+$ T cells. In the setting of anti-CD4 treatment, there may be an increase in the ability of $CD8^+$ rather than $CD4^+$ T cells to interact with IEC. Regardless, the end result is a positive growth signal transmitted to $CD8^+$ cells.

The nature of the positive signal to $CD8^+$ T cells appears to be the activation of $p56^{lck}$. Several laboratories have described an src-like tyrosine kinase, $p56^{lck}$, associated with the intracytoplasmic tail of CD4 and the CD8α chain (18–19). Cross-linking of these molecules with antibody results in induction of kinase activity with autophosphorylation as well as phosphorylation of a number of associated substrates that modulate TCR stimulation (27, 28). Conceivably, different binding to CD4 or CD8 may alter the activation and substrate phosphorylation seen. In addition, the kinetics of $p56^{lck}$ activation is critical, since, as the multiple substrates move closer to $p56^{lck}$ at varying times, patterns of phosphorylation and signaling may induce specific cellular activation. In the studies presented here, $p56^{lck}$ is activated within 1 min of T cell:IEC coculture, which is much more rapid than the induction of $p56^{lck}$ by conventional APCs (e.g., monocytes). Interestingly, this activation is inhibited by antibodies to CD8, providing further evidence that the CD8 molecule itself plays a critical role in this process. Lastly, the activation of this tyrosine kinase is critical for the subsequent $CD8^+$ T cell proliferation, since treatment of the cells with two different tyrosine kinase inhibitors (but not a PKC or PKA inhibitor) before coculture inhibits their ability to proliferate.

These findings are especially interesting with regard to IEL. Several groups have reported that CD8 expressed on a major proportion of IEL in the mouse is of the α/α homodimeric form as opposed to the α/β heterodimeric form seen in peripheral $CD8^+$ T cells (29, 30). Since $p56^{lck}$ is associated with the CD8α chain, it is intriguing to postulate that a double dose of $p56^{lck}$ in cells living adjacent to IECs (putatively bearing an activating ligand for such CD8 molecules) may account for the activated yet relatively anergic state of IELs described.

However, cross-linking CD8 and activation of $p56^{lck}$ alone are not sufficient for $CD8^+$ T cell proliferation. In the absence of IEC, anti-CD8 mAbs in the presence or absence of cytokines failed to promote cell proliferation. Therefore, it appears that a second signal, possibly through

the TCR (since our initial studies related to either antigen-specific or alloreactive responses) must be required. Support for such a possibility is provided by the finding that fyn, a TCR-associated kinase not activated by $p56^{lck}$, is also activated in T cell:IEC cocultures (Fig. 7).

Perhaps the most compelling data underscoring the dichotomy between the peripheral and mucosal immune systems comes from the studies using the murine T cell hybridomas transfected with genes either encoding human CD4 or CD8α. Monocytes predominantly stimulate $p56^{lck}$ activation in CD4 transfectants, whereas IEC stimulate $p56^{lck}$ in CD8 transfectants. These findings suggest that, although IEC express class II molecules, these are either expressed at such a low level to prevent sufficient CD4 binding or, alternatively, as has been suggested in the mouse, they are aberrantly expressed (i.e., abnormal form) (31) and incapable of binding to CD4 effectively. In either case, such a scenario compromises the interaction of IEC with $CD4^+$ T cells and promotes the interaction of IEC with $CD8^+$T cells.

The existence of suppressor T cells has been controversial. Direct activation of such cells has been fraught with difficulties in vitro. Perhaps, given the nature of the mucosal immune system, the gut is the only environment where suppression can be reproducibly induced.

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