Phenotypic and functional characterization of intraepithelial lymphocytes in a bovine ligated intestinal loop model of enterohaemorrhagic Escherichia coli infection

Christian Menge,1 Ivonne Stamm,1 Pauline M. van Diemen,2 Paul Sopp,3 Georg Baljer,1 Timothy S. Wallis2 and Mark P. Stevens2

1Institute for Hygiene and Infectious Diseases of Animals, Justus-Liebig-University, D-35392 Giessen, Germany
2,3Division of Microbiology2 and Division of Immunology & Pathology3, Institute for Animal Health, Compton Laboratory, Compton, Berkshire RG20 7NN, UK

Ruminants are a major reservoir of enterohaemorrhagic Escherichia coli (EHEC), which cause acute gastroenteritis in humans with potentially life-threatening sequelae. The mechanisms underlying EHEC persistence in ruminant hosts are poorly understood. EHEC produce several cytotoxins that inhibit the proliferation of bovine lymphocytes in vitro and influence EHEC persistence in calves, suggesting that bacterial suppression of mucosal inflammation may be important in vivo. In order to address this hypothesis, intraepithelial lymphocytes (IEL) obtained from ligated intestinal loops of five 9–14 day old calves were characterized 12 h after inoculation with E. coli strains. Loops were inoculated with an EHEC O103 : H2 strain, an isogenic Δstx1 mutant incapable of producing Shiga toxin 1 (Stx1) and a porcine non-pathogenic E. coli strain. The IEL mainly comprised activated CD2+ CD3+ CD6+ CD8+ T cells and resembled IEL obtained from the intestinal mucosa of orally challenged calves. Forty per cent of all IEL were potentially sensitive to Stx1 in that they expressed the receptor for Stx1. Nevertheless, analysis of IEL from inoculated loops failed to detect a significant effect of the different E. coli strains on proliferative capacity, natural killer cell activity or the cytokine mRNA profile. However, the EHEC wild-type strain reduced the percentage of CD8+ T cells in the ileal mucosa compared with loops inoculated with the Δstx1 mutant. This shift in IEL composition was not associated with inhibition of IEL proliferation in situ, since the majority of the IEL from all loops were in the G0/G1 phase of the cell cycle. These studies indicate that the ligated ileal loop model will be a useful tool to dissect the mechanisms underlying suppression of mucosal inflammation by EHEC in the reservoir host.

INTRODUCTION

Enterohaemorrhagic Escherichia coli (EHEC) infections in humans are often acquired by direct or indirect contact with ruminant faeces and can have life-threatening consequences (Paton & Paton, 1998; Roe & Gally, 2000). Strategies to lower the incidence of EHEC in cattle and sheep are expected to reduce the incidence of human infections (Stevens et al., 2002b); however, the mechanisms underlying EHEC persistence in ruminants are poorly understood. Previous studies indicated that the locus of enterocyte effacement (LEE)-encoded type III secretion apparatus mediates intestinal colonization in animal models of attaching and effacing E. coli infection (Abe et al., 1998; Mundy et al., 2003), and our laboratory has recently shown that the LEE is required for colonization of the bovine intestine by EHEC serotypes O157 : H7 and O26 : H− (P. M. van Diemen, F. Dziva, M. P. Stevens and T. S. Wallis, unpublished observations). Several of the LEE-encoded effector proteins do not influence adherence per se, indicating that they may affect colonization by subverting or inhibiting the activity of host cells (Elliott et al., 2001; McNamara et al., 2001; Tu et al., 2003). Indeed, EspB or a protein(s) dependent on EspB for secretion was recently reported to suppress activation of the nuclear transcription factor NF-κB and the synthesis of proinflammatory cytokines in vitro (Hauf & Chakraborty, 2003).

EHEC also produce several cytotoxins including Shiga toxin(s) (Stx1 and/or Stx2) and lymphostatin, the latter of
which influences intestinal colonization of calves (Stevens et al., 2002c). Both Stx1 and lymphostatin inhibit the proliferation of bovine peripheral blood lymphocytes in vitro (Menge et al., 1999; Ferens & Hovde, 2000; Stevens et al., 2002c). In addition, lymphostatin can block the proliferation of human and murine intestinal lymphocytes in vitro (Klapproth et al., 1996; Malstrom & James, 1998). Bovine intestinal intraepithelial lymphocytes (IEL) express functional Stx1 receptors, and Stx1 blocks proliferation and affects the expression of cytokines in these cells (Stamm et al., 2002; Menge et al., 2004). Studies are required to confirm that such immunomodulatory strategies are relevant in the complex environment of the intestine in the target animal species (Smith et al., 2002; Hein & Griebel, 2003).

Intestinal loop models have been used in ruminants to study enteropathogenic responses to bacterial pathogens. EHEC elicit enteropathogenic responses in such loops and adhere to the epithelium, forming attaching and effacing lesions (Sandhu & Gyles, 2002; Stevens et al., 2002a). Recently, Gerds et al. (2001) established that intestinal loops are a valuable model for the analysis of mucosal immune responses. We therefore assessed the phenotype and function of IEL in a bovine ligated intestinal loop model of EHEC infection in order to identify bacterial and host factors that modulate inflammatory responses during EHEC infection of cattle.

METHODS

Bacterial strains, toxin and anti-toxin. The bacterial strains used were PMK5 (wild-type EHEC O103 : H2 oae subtype- e stx1); Mariani-Kurkdjian et al., 1993), an isogenic PMK5Δstx1 mutant (Stevens et al., 2002a) and NADCC5738 (Dean-Nystrom et al., 1997), which is a nalidixic acid-resistant derivative of the porcine non-pathogenic E. coli O43 : H28 strain 123 (Moon et al., 1968). Bacterial strains were cultured in brain heart infusion (BHI) broth for 18 h at 37°C. Intraepithelial lymphocytes (IEL) were purified by anion-exchange chromatography as described by Stamm et al., 2002 and NADC5738 (Dean-Nystrom, 2002a) and NADC5738 (Dean-Nystrom et al., 1998). Bovine intestinal intraepithelial lymphocytes (IEL) express functional Stx1 receptors, and Stx1 blocks proliferation and affects the expression of cytokines in these cells (Stamm et al., 2002; Menge et al., 2004). Studies are required to confirm that such immunomodulatory strategies are relevant in the complex environment of the intestine in the target animal species (Smith et al., 2002; Hein & Griebel, 2003).

Animals. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 (UK) and were approved by the local Ethical Review Committee. Conventional Friesian bull calves were fed on milk replacer twice daily with free access to water and were maintained at 37°C by the use of heated mats. Four mid-ileal loops per animal approx. 40 cm in length with 10 cm spacers were ligated with surgical silk and inoculated with 40 ml bacterial culture (PMK5, 4.58 ± 0.70 × 10^10 cfu. per loop; PMK5Δstx4.02 ± 0.67 × 10^10 cfu. per loop; NADC5738 5.84 ± 1.37 × 10^10 cfu. per loop) or sterile medium (BHI) as a negative control. Each strain was tested once per animal and the experiment was repeated in a total of five calves.

Isolation of IEL. Twelve hours after loop inoculation and immediately after the administration of an overdose of anaesthetic, the infected mucosa was collected into ice-cold PBS. IEL (approx. 2 × 10^7 cells per loop) were isolated from the recovered mucosa after treatment with 1-A-DTT (1 mM in PBS, 15–25 min at 37°C with shaking) by incubation with an EDTA solution containing an inhibitory concentration of antibiotics (2 mM EDTA in PBS, 100 U penicillin ml^−1, 100 μg streptomycin ml^−1, 2.5 μg gentamicin ml^−1; 20 min at 37°C with shaking) and mechanical detachment (vortexing). The cells were resuspended in Percoll at a density of 1.05 g ml^−1, layered onto Percoll at a density of 1.0816 g ml^{-1} and separated by centrifugation at 677 g for 20 min (Menge et al., 2004).

Immunophenotyping. Freshly isolated IEL were transferred to microtitre plates for the staining of cell differentiation and activation markers for flow cytometry as described (Menge et al., 1999; Stamm et al., 2002). IEL were incubated with antibodies/rStxB1 in the dark for 20 min. Detected antigens and the respective antibodies used were: CD2 (IL-A 43), CD4 (IL-A 11), CD6 (IL-A 57), CD8α (IL-A 103), CD211 (IL-A 65), a macrophage/granulocyte differentiation antigen (IL-A 24), WC1 (yeb T cells, IL-A 29), CD25 (IL-A 111), CD71 (IL-A 77), MHC-II (J11) (all antibodies provided by J. Naessens, International Livestock Research Institute, Nairobi, Kenya), CD3 (MM1A), CD8β (BAT82A), ACT-2 (CACT26A), TcR1-N7 (CACTB81A), TcR1-N6 (CACTB6A), TcR1-N12 (CACT61A) (all antibodies purchased from VMRD, Pullman, WA, USA) and CD77 (clone 38.13; Beckman Coulter). Binding of rStxB1 (30 μg ml^{-1}) was detected with anti-StxB1 (45 μg ml^{-1}). Visualization was carried out with FITC-labelled secondary antibodies. Cells were analysed with a FACSCalibur flow cytometer acquiring 5000 events for each sample.

Natural killer (NK) cell activity assay. A bovine lymphoma cell line (BL-3, ECACC 86962401) was used as target cells. Target cells (T) prestained with 3,3′-diodotacycloloxacyclobacarnoyl perchlorate (DIO) were added to IEL as effector cells (E) in different ratios (E : T 100 : 1, 33 : 1 and 11 : 1) and incubated for 18 h at 37°C. Affected target cells were detected by flow cytometry according to their altered morphology (i.e. increase in granularity).

Cell cycle determination/DNA analysis. IEL were fixed with ethanol (70%, v/v) immediately after isolation. DNA was stained with propidium iodide (PI) after RNase A digestion and proportions of cells in the different cell cycle compartments were assessed by flow cytometry analysis according to their PI signal (Noguchi, 1991).

Lymphocyte stimulation-proliferation assay. To examine proliferative capacity after mitogen stimulation, IEL were cultured for 3 days at 37°C in medium supplemented with rhuIL2 (200 U ml^{-1}) and either phytohaemagglutinin (PHA-P, 2.5 μg ml^{-1}) or phorbol-12-myristate-13-acetate (PMA, 5 ng ml^{-1}) and ionomycin (500 ng ml^{-1}). The proportion of viable cells transformed to blast cells and non-transformed non-blast cells relative to an unstimulated control was determined by flow cytometry according to the light scatter characteristics of cells with PI exclusion of dead cells.
Cytokine mRNA profile. After incubation of IEL in medium supplemented with 2.5 μg PHA ml⁻¹ and 200 μg rhuIL2 ml⁻¹ for 30 min, RNA was isolated from cells using an RNeasy MiniKit (Qiagen), treated with DNase I and reverse transcribed to cDNA. Cytokine-specific PCRs for il2, il4, il8, il10 and ifn-γ were carried out following standard procedures. Previously published cytokine primers (Gohin et al., 1997; Morsey et al., 1996) were used with minor modifications: il2 (sense, 5′-TTTGCATTGCAACTACTT-3′; antisense, 5′-GCT TTGACAAAAGGTAATCC-3′), il4 (sense, 5′-GCCACCCGGTTCACCT GGAAC-3′; antisense, 5′-TCCCAAGAATTCTCGAGC-3′), il8 (sense, 5′-GCAATTTGCTAAAGGAAAGGA-3′; antisense, 5′-GAGTCT TGCTCTCAGGTC-3′), il10 (sense, 5′-GTGTGCTGTCTCTGTCTG- 3′; antisense, 5′-TCTCCTCAAGCCGTTCACAC-3′), ifn-γ (sense, 5′-GCT TTACTGCTCTGTTGCTGCT-3′; antisense, 5′-GACTTCTCCTGCGGT TTCTG-3′) and gapdh (sense, 5′-ATCTGTTGCCACCCAG-3′; antisense, 5′-GATTGCAAGGATGAGG-3′). The GAPDH gene was used as a control for constitutive gene expression. The amplification reaction was carried out for a total of 35 cycles as follows: 94°C for 90 s, with a precycle of 94°C for 15 s and final extension at 72°C for 30 s. Cytokine signals were evaluated after gel electrophoresis from densitometry measurements and values were normalized to a GAPDH signal.

Statistical analysis. Data were analysed statistically using BMDP (Statistical Software) and SigmaStat software (SPSS). P values were calculated by Student–Newman–Keuls test and one-way repeated measures ANOVA and considered significant when P < 0.05.

RESULTS AND DISCUSSION

Comparative phenotypic and functional characterization of bovine ileal and colonic IEL in orally challenged calves

Anatomical constraints and the need to recover sufficient viable IEL from the epithelial layer for functional studies led us to focus on the small intestine for ligated loop experiments to study the effect of E. coli strains on mucosal inflammation. Despite a recent report suggesting that lymphoid follicle dense epithelium in the terminal rectum is the primary site of E. coli O157 : H7 colonization in weaned calves and adult cattle (Naylor et al., 2003), colonization of the ileum, caecum and colon has been reported in calves infected with E. coli O157 : H7 (Cray & Moon, 1995; Brown et al., 1997; Dean-Nystrom et al., 1997, 1999; Grauke et al., 2002). Non-O157 EHEC apparently do not share a tropism for the terminal rectum (Naylor et al., 2003), and serotypes O5 and O111 have been observed to adhere extensively to the colonic epithelium (Stevens et al., 2002c). In order to determine whether phenotypic or functional differences were detectable between IEL from ileal and colonic sites, conventional calves were challenged orally with either EHEC strain PMK5 or non-pathogenic E. coli strain NADC5738, and intestinal mucosa was obtained 3 days after inoculation to prepare IEL. The general composition of IEL subpopulations was very similar in the ileum and colon of orally challenged calves (Fig. 1), with the exception that ileal IEL preparations contained higher percentages of T cells and of cells expressing MHC-II and ACT-2 (a tissue-specific activation marker) as compared with colonic IEL preparations. The Stx receptor Gb/CD77 was found on approximately 50 and 35 % of the IEL from ileal and colonic preparations, respectively. We previously observed that bovine lymphocytes express different isoforms of Gb/CD77 molecules that have incorporated fatty acids of varying length and display different affinities for anti-CD77 and rStxB1 (Menge et al., 2001, 2004; Stamm et al., 2002). Consistent with that, only approx. 35 % of ileal as well as colonic IEL were capable of binding rStxB1 in the present study.

IEL composition differed little between calves that were inoculated with PMK5 or NADC5738. It is noteworthy, however, that the portion of Gb/CD77⁻ and rStxB1 binding ileal IEL was reduced in the calf that received the EHEC strain. In addition, the portion of ileal and colonic intra-epithelial T cells expressing CD8α and CD8β was markedly lower in this animal. In turn, other T cell populations including CD3⁺, CD4⁺, CD6² and N12⁺ γδ T cells were enhanced at colonic sites in the PMK5-inoculated animal.

The majority of IEL from both calves were in the G0/G1 phase of the cell cycle and mitogen stimulation did not result in proliferative responses in comparison to unstimulated cells (data not shown). IEL are known to respond only poorly to mitogens and nominal antigens in vitro, despite their activated appearance and phenotype (Mowat & Viney, 1997). Nevertheless, isolated IEL were still functionally active in that they exerted an NK cell activity towards a homologous cell line, and inoculation with EHEC did not influence this activity (Fig. 2). Detection of this activity required high effector to target cell ratios and was slightly higher in colonic IEL preparations.

Immunophenotype of IEL exposed to EHEC in situ in a ligated intestinal loop model of EHEC infection

Mid-ileal loops were constructed in a total of five conventional calves aged 9–14 days and inoculated with either PMK5, PMK5 Δstx1, NADC5738 or sterile medium as a control. Since it is known that Stx1 inhibits the activity of bovine lymphocytes in vitro (Stamm et al., 2002; Menge et al., 2003), the stx1 mutant was included to determine whether mucosal immunomodulatory effects due to Stx1 could be detected in vivo. Viable IEL could be isolated after 12 h from all mid-ileal loops inoculated with the different E. coli strains. Immunophenotyping of the IEL revealed their composition was very similar to IEL derived from ileal mucosa from orally inoculated calves (Figs 1 and 3). IEL from mid-ileal loops comprised mainly activated CD2⁺ CD3⁺ CD6⁺ ACT-2⁺ T cells, with approximately 60 % CD8α⁺, 50 % CD8β⁺, 10 % CD4⁺ and 25 % γδ T cells, suggesting that the integrity of the mucosal layer had been maintained (Fig. 3). Forty per cent of all IEL expressed the Stx receptor Gb/CD77 and were capable of binding rStxB1. Few changes in the phenotype of the recovered IEL could be detected between loops inoculated with EHEC and control loops filled with NADC5738 or sterile medium (Fig. 3). Student–Newman–Keuls test following one-way repeated measures ANOVA revealed that the wild-type Stx1-producing EHEC strain PMK5 significantly reduced the percentage of CD8α⁺ T cells.
by 5.52 ± 3.4% (P ≤ 0.05) compared with loops inoculated with the PMK5 Astx1 mutant or the non-pathogenic E. coli strain. A slight decrease in the portion of CD8β+ CD6+ and CD2+ IEL in PMK5-inoculated loops could also be detected, although differences reached significance only in the latter case. These findings reflect the differential expression of Stx receptors by several IEL subpopulations: in the ileum of adult cattle, the majority of Gb3/CD77 receptors by several IEL subpopulations: in the ileum of adult cattle, the majority of Gb3/CD77 receptors are expressed on CD8α+ CD6+ CD8β+ T cells, whereas CD4+ T cells and B cells express much less Gb3/CD77 (Menge et al., 2004). Inoculation of the loops with PMK5 did not influence the number of Gb3/CD77+ IEL, but slightly reduced the number of rStxB1-binding cells. In vitro, Stx1 affects bovine peripheral lymphocytes early in the activation process (Stamm et al., 2002) when the cells express an isoform of Gb3/CD77 with a high affinity for rStxB1 that is not recognized by anti-CD77 (Menge et al., 2003). Stx1 thus probably eliminated from the loop mucosa only those IEL that were not recognized as Gb3/CD77+ cells in either loop.

The finding that Stx1 specifically depletes a subset of bovine lymphocytes in the complex environment of the intestine is a significant step forward in our understanding of the modulation of mucosal immune responses by EHEC. To the best of our knowledge, the present study provides the first direct evidence that Stx1 acts as a virulence factor in cattle. Suppression of immune function in the gut through depletion of CD8α+ and probably CD8β T cells may facilitate intestinal colonization; however, there is presently a paucity of published data to support this role (reviewed by Smith et al., 2002). Stx-positive E. coli O157 : H7 have been reported to colonize the intestines of weaned calves more effectively than Stx-negative strains (Dean-Nystrom et al., 1998); however, the strains used were not isogenic and the differences could be due to traits other than Stx production.

**Cell cycle progression and proliferation of IEL exposed to EHEC in situ**

Between 88.65 ± 2.11 and 90.14 ± 2.43 % of IEL (mean ± SD of triplicate determinations of five animals) from the loops were in the G0/G1 phase of the cell cycle. The response to mitogen stimulation was low and did not differ between IEL preparations obtained from the different loops (data not shown). The effect of Stx1 on IEL composition is therefore not a consequence of inhibition of cell proliferation in situ. We recently reported that the ability of Stx1 to block the proliferation of bovine peripheral CD8α+ Gb3/CD77+ T lymphocytes is due to direct toxic action and is not mediated via perturbation of autocrine cytokine release within the lymphocyte cultures (Menge et al., 2003). Although previous reports failed to detect a cytotoxic activity of Stx1 for bovine lymphocytes (Menge et al., 1999; Ferens & Hovde, 2000), Stx1 was recognized as a potent cytotoxin in other cellular systems (Sandvig, 2001). Therefore, we cannot exclude the possibility that Stx1 eliminated sensitive IEL from the mucosa of PMK5-inoculated loops.

The IEL composition in loops inoculated with a non-pathogenic E. coli strain did not significantly differ from
represents another EHEC factor that blocks lymphocyte proliferation in vitro (Klapproth et al., 2000; Stevens et al., 2002c); however, no lymphostatin-like effects on IEL phenotype and function were detected in the present study.

**Cytokine mRNA synthesis and NK cell activity of IEL exposed to EHEC in situ**

Several EHEC virulence factors inhibit the synthesis of cytokines by mitogen-activated mucosal lymphocytes in vitro, including lymphostatin (Klapproth et al., 1996; Malstrom & James, 1998) and Stx1 (Menge et al., 2004). We therefore assessed the effect of exposure of bovine IEL in situ to different E. coli strains on the mitogen-activated transcription of il2, il4, il8, il10 and ifn-γ. Upon mitogenic stimulation of recovered IEL for 30 min in vitro, suitable amounts of intact RNA could be recovered and subjected to semi-quantitative RT-PCR. IEL preparations obtained from different donor animals varied in their cytokine gene expression (Fig. 4). While inoculation of loops with PMK5 increased the IL2 signal in four out of five animals in comparison with loops inoculated with PMK5 Δstx1, the

loops inoculated with PMK5 Δstx1. The latter strain, but not NADC5738, contains the gene for lymphostatin (lifA), which influences colonization of the bovine intestine by EHEC serotypes O5 and O111 (Stevens et al., 2002c). Lymphostatin

**Fig. 3.** Phenotype analysis of ileal IEL isolated from ligated loops after 12 h of inoculation with EHEC wild-type strain PMK5 (filled bars), isogenic PMK5 (stx1 mutant; open bars), non-pathogenic E. coli strain NADC5738 (striped bars) or BHI broth (hatched bars). Analysis was performed by flow cytometry and subsequent calculation of the percentage of viable lymphocytes positive for the respective antigen. Values represent means and SD from duplicate determinations of five animals. For statistical analysis, one-way repeated measures ANOVA and Student–Newman–Keuls test were performed (for details see text). Mφ, diff. ag, macrophage/granulocyte differentiation antigen.

**Fig. 4.** Cytokine gene expression of ileal IEL isolated from ligated loops after 12 h of inoculation with EHEC wild-type strain PMK5 or isogenic PMK5 (stx1 mutant). IEL were incubated in vitro for 30 min at 37°C in culture medium supplemented with 2.5 μg PHA-P ml⁻¹ and 200 U recombinant huIL2 ml⁻¹. RNA was harvested from cells and subjected to semiquantitative RT-PCR. Values are band intensities of the specific PCR product relative to the GAPDH signal obtained from the same sample and expressed relative to loops inoculated with NADC5738. Symbols represent IEL preparations from different animals.
expression of IFN-γ was reduced to different extents in all five animals. The amounts of IL4-, IL8- and IL10-specific mRNA (Fig. 4; data for IL10 not shown) varied inconsistently between the loops and the animals.

IEL preparations from all the inoculated loops exhibited a higher NK cell activity than ileal IEL obtained from the orally challenged calves, but no significant differences between IEL exposed to the different Escherichia coli strains were detectable (Fig. 5).

Gut-associated lymphoid tissues trap antigen at sites of infection and present it to migratory lymphocytes, leading ultimately to the development of antigen-specific mucosal immunity. It may be speculated that EHEC cytotoxins suppress these events once the bacteria are intimately associated with the epithelium in order to prevent clearance. Accordingly, Stx1 had been shown to hinder peripheral lymphocyte functions in vitro (Menge et al., 1999), and presumably in vivo (Hoffman et al., 1997). However, the fact that humoral and mucosal immune responses against EHEC antigens can be readily detected after experimental and natural infection of cattle (Johnson et al., 1996; Pirro et al., 1995) argues against a general immune suppression. Moreover, IEL differ strikingly functionally from peripheral lymphocytes (Mowat & Viney, 1997). IEL do exhibit cytotoxicity against virus-infected cells (Müller et al., 2000), but, in the first place, these cells form an indispensable part of the mucosal regulatory network that maintains intestinal homeostasis (Fiocchi, 1997). By releasing soluble factors, IEL control the migration and activation of inflammatory cells as well as a number of epithelial cell functions including proliferation (Mowat & Viney, 1997). It is thus tempting to assume that, by modulating IEL functions, EHEC prevent the onset of mucosal inflammatory responses that would otherwise follow bacterial adhesion to the mucosal surface (Zhou et al., 2003). In addition to directly affecting epithelial cell functions (Hoey et al., 2002, 2003), EHEC would be able, by this mechanism, to influence epithelial cell turnover indirectly, which correlates with the duration of EHEC shedding in calves (Magnuson et al., 2000). Using Stx1 as a prototype for EHEC factors with immunomodulatory activity, the bovine ligated intestinal loop model proved suitable to dissect the effect of bacterial factors on different aspects of the intestinal immune response during EHEC O103:H2 infections in this reservoir host.

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Effect of EHEC on bovine IELs in situ


