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Cellular responses to attaching and effacing bacteria: activation and implication of the innate immune system

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Summary

During the last decade, research on attaching-effacing (A/E) bacteria/host cell interactions has revealed much of the molecular basis of colonization and lesion formation. The colonic mucosa represents the first line of defense against these pathogens, and its integrity is required to avoid translocation of bacteria or bacterial soluble factors into the infected host. Therefore, the cellular immune response to A/E pathogens plays an important role in bacterial pathogenesis since it can clear the bacteria or modulate the inflammatory processes. Data obtained from infected patients demonstrate a correlation between the production of pro-inflammatory cytokines and the severity of the disease. *In vitro* studies of infected epithelial cells have clearly elucidated A/E bacteria-induced host signal transduction events. However, the identification of the bacterial factors responsible for cellular activation remains a subject of controversy. Experimental studies with knock-out mice infected with *Citrobacter rodentium*, a rodent A/E pathogen, indicate that innate immunity is an essential component of pathogenesis. This review summarizes *in vivo* and *in vitro* evidence for the induction and potential role of the innate immune system during infection with A/E bacteria.

Key words: EHEC • EPEC • inflammation • epithelial cells • macrophage • cytokine • nitric oxide

Abbreviations: EHEC – enterohaemorrhagic *E. coli*, EPEC – enteropathogenic *E. coli*, A/E – attaching-effacing, HC – haemorrhagic colitis, HUS – haemolytic-uremic syndrome, Stx – Shiga-toxin, Gb3 – globotriaosylceramide 3, LEE – locus of enterocyte effacement, TTSS – type III secretion system, TLR – Toll-like receptor, NO – nitric oxide, IL – interleukin, TNF- α – tumor necrosis factor alpha, PKC – protein kinase C, NF- κ B – nuclear factor kappa B, AP-1 – activator protein-1, MAPK – mitogen-activated protein kinase, ERK – extracellular signal-regulated protein kinase, JNK – c-jun N-terminal kinase, IFN- γ – interferon gamma, Erg-1 – early growth response-1, TNFRp55 – TNF receptor type I p55, NOS – NO synthase.

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INTRODUCTION

An emerging theme in the field of bacterial pathogenesis is the molecular cross-talk that takes place between the host and the pathogen and determines the progression of the infectious process. In this regard, enterohaemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC), two non-invasive enteropathogens, received particular attention this last decade. EPEC and certain EHEC are attaching-effacing (A/E) pathogens that exploit host cell signaling pathways to allow colonization of their host⁵⁰.

EPEC is a serious and widespread cause of infantile diarrhea, particularly in developing countries. Infants younger than 2 years are primarily affected, although several outbreaks of diarrhea have been reported in adults, presumably due to ingestion of large inoculums. Strains harboring the EPEC adherence factor plasmid, encoding the type IV bundle-forming pilus, are always associated with diarrhea⁵⁰.

EHEC is a food-borne pathogen causing human diseases ranging from uncomplicated diarrhea to haemorrhagic colitis (HC) and life-threatening complications, such as haemolytic-uremic syndrome (HUS) or thrombotic thrombocytopenic purpura. HUS most commonly presents as acute renal failure accompanied by the swelling and detachment of glomerular endothelial cells from the basement membrane, the deposition of fibrin microthrombi within glomerular capillaries, thrombocytopenia, and haemolytic anemia⁵⁴. HUS develops in about 5% of EHEC-infected patients, essentially children under 5 years, and accounts for the major morbidity and mortality. The main EHEC virulence factors associated with severe human disease are the two Shiga-toxins (Stx), Stx1 and Stx2. EHEC-derived Stx is produced in the lower intestine, translocates across intestinal epithelium, and induces necrosis or apoptosis of vascular endothelial cells by inhibiting protein synthesis. Endothelial cells from colon, brain and kidney express high levels of Stx receptor, the glycolipid globotriaosylceramide 3 (Gb3, also identified as CD77), thus explaining the symptomatology of HC, thrombotic thrombocytopenic purpura, or HUS⁵². The O157:H7 serotype is the major EHEC serotype associated with large outbreaks and the most frequently found in sporadic cases of HC and HUS; however, numerous other pathogenic strains have been isolated from various serotypes, including O26:H11, O103:H2, O113:H21, and O111:NM⁵⁰.

The A/E phenotype shared by EPEC, EHEC, and by a natural mouse pathogen, *Citrobacter rodentium*, is

characterized by intimate adherence of bacteria to intestinal epithelial cells and by effacement of microvilli. Most of the genes involved in the A/E phenotype are encoded on a pathogenicity island called LEE (for locus of enterocyte effacement). The LEE encodes a type III secretion system (TTSS) that allows bacteria to directly inject effector proteins into the underlying cells, inducing the accumulation of polymerized actin beneath the adherent bacteria, formation of a pedestal-like structure on which bacteria adhere intimately, and effacement of microvilli⁵⁰. In addition to the Esc proteins involved in the assembly of the TTSS, the LEE encodes 3 regulators (Ler, GrlA, GrlR), an adhesin (intimin), and a number of type III secreted translocator (EspA, EspB, EspD) and effector (EspG, EspF, EspH, Map, Tir) proteins¹². These translocated proteins interact with host cell components and alter signal transduction pathways, inducing the host to self-inflict damage by cytoskeletal rearrangement and formation of pedestals. Expression of the LEE genes is regulated by quorum sensing, a bacterial cell-to-cell signaling system^{67, 70}. Several lines of evidence indicate that the signal molecules activating LEE expression are small compounds produced by the gut commensal flora and host intestinal hormones (epinephrine, norepinephrine)⁷¹.

How A/E bacteria interact with cells of the colonic mucosa is less well known. Pathogen-associated molecular patterns, including lipopolysaccharide, flagellin, or microbial lipopeptides, are recognized by mammalian Toll-like receptors (TLRs; see recent review by Underhill)⁷⁷ and may thus initiate signal transduction. Activated mucosal intestinal cells produce numerous effectors of the innate immune system such as cytokines or nitric oxide (NO), which are involved in the defense of the organism against pathogens and/or in the inflammatory process. Therefore, the capacity of A/E bacteria to elicit an innate immune response in the infected colon may be a critical step in pathogenesis. This review summarizes the progress in the understanding of the A/E bacteria-host relationship and the mechanisms by which A/E pathogens may promote the activation of the innate immune system in cells of the colonic mucosa.

IN VIVO EVIDENCE FOR THE ACTIVATION OF THE INNATE IMMUNE RESPONSE DURING EHEC INFECTION

Several reports indicate that leukocytosis is an indicator for severe course in HUS due to *E. coli* O157:H7. A retrospective study of 278 children with *E. coli* O157:H7 infection showed that patients with

high counts of total white blood cells have a 7-fold increased risk of developing HUS². Prospective studies report that blood leukocytosis is associated with increased detection of EHEC in stool cultures²² and with the development of HUS^{2, 4, 22, 55, 84}. Accordingly, initial leukocytosis has been described as a predictor for the development of HUS³⁰. A putative role of leukocytes could be the transport of Stx towards the endothelial cells of target organs (gut, brain, kidneys). Indeed Stx binds to leukocytes that express Gb3, such as neutrophils, and is transferred onto Gb3-expressing endothelial cells⁷⁴. Furthermore, Stx has been detected on the surface of circulating leukocytes of patients with HUS⁷⁴, and kidney biopsies or necropsies of children with HUS show neutrophil recruitment within glomeruli^{37, 81}.

Numerous investigations describe an increase in pro-inflammatory cytokine interleukin (IL)-8 and IL-6 in children with HUS in comparison with uninfected children, or with children suffering from gastroenteritis due to virus, *E. coli* O157:H7, or other bacteria^{20, 38, 49, 56, 81, 82}. Moreover, the highest IL-8 levels, which correlate with enhanced circulating neutrophil counts, are found in HUS children who died in the acute phase of the disease²⁰. Conversely, Litalien et al.⁴⁴ and Westerholt et al.⁸² do not find any significant difference in IL-8 and IL-6 levels between HUS children and control children. Furthermore, other chemokines (GRO- α , MIP-1 β , and MCP-1) are produced at the early stage of *E. coli* O157:H7 enteritis, whether or not HC or HUS develops^{49, 55, 56}; elevated circulating G-CSF and low ENA-78 concentrations are nonetheless correlated with the severity of illness^{49, 55}.

Regarding tumor necrosis factor alpha (TNF- α) production, dissimilar results have been obtained. Data show that TNF- α concentration is increased in the blood of children with HUS or HC^{45, 49}, is augmented in only a limited number of patients^{20, 38}, or does not vary significantly during EHEC infection and HUS⁷⁹. Interestingly, Lopez et al.⁴⁵ have observed that TNF- α production is an early event that occurs in the first 10 days after the onset of bloody diarrhea; therefore it is possible to speculate that TNF- α expression has been missed in some studies, as suggested by Inward et al.³⁸.

Levels of anti-inflammatory mediators have been also investigated. Serum concentrations of IL-10^{44, 82, 85} and of IL-1 receptor antagonist⁴⁴ are significantly higher in EHEC-derived HUS children than in uninfected children or children with O157:H7-induced colitis, suggesting that an immunomodulation could be observed in HUS patients. However, circulating

IL-10 levels are lower in EHEC O157:H7-infected children in comparison with children suffering from viral or non-EHEC bacterial gastroenteritis^{49, 82}.

Despite some conflicting results, the data show that host inflammatory response is modulated during EHEC infection. Instead of studying absolute concentrations of each cytokine, which can vary differently in individuals, analysis of the ratio between pro- and anti-inflammatory cytokines appears to be a reliable marker of the severity of the disease^{44, 82}. A critical component of future investigations should be to determine whether the altered immune response is just a marker of the severity of the infection or whether it is a preexisting characteristic that predisposes the patients to a systemic disease such as HUS⁴. In this view, *ex vivo* lipopolysaccharide stimulation of blood cells from patients who experienced a HUS episode have been investigated by Westerholt et al.^{44, 82}. The ratios IL-1 β /IL-10 and TNF- α /IL-10 are higher in children who previously had HUS than in control individuals⁸³. These results support the contention that a specific immune status predisposes individuals to HUS when EHEC infection occurs. Clear confirmation of this hypothesis requires further analysis.

DISSECTING THE A/E BACTERIA-INDUCED SIGNALS IN EPITHELIAL CELLS

The interaction of A/E bacteria with polarized epithelial cells causes an alteration of numerous signaling pathways. Data from the last decade strongly suggest that distinct signaling events are implicated in cellular integrity and in innate immune response^{9, 10, 64}. Thus, multiple signals initiated by the activation of phospholipase C- γ 1 or phosphoinositide 3-kinase converge to induce rearrangements of the actin cytoskeleton and formation of A/E lesions^{14, 41}. EHEC and EPEC also stimulate conventional protein kinase C (PKC)^{1, 53} that is involved in the alteration of epithelial barrier functions⁵³ and probably diarrhea⁴².

A plethora of reports has highlighted our understanding of the activation of the innate cellular response to pathogenic bacteria. As for intracellular pathogens from the *Shigella*²³, *Salmonella*, or *Escherichia* genera¹⁷, there is clear evidence that the transcriptional factor nuclear factor kappa B (NF- κ B) plays a key role in the expression and production of proinflammatory cytokines by human colonic epithelial cells in response to A/E pathogens. Savkovic et al.⁶² reported for the first time that EPEC infection of the T84 cell line induces NF- κ B activation and binding to the IL-8 promoter, allowing IL-8 mRNA

expression and IL-8 synthesis. Interestingly, these authors also show that NF- κ B is not induced by non-pathogenic *E. coli*⁶², suggesting that a specific molecular dialogue between A/E pathogens and epithelial cells is necessary for an innate pro-inflammatory response. More recently it has been described that the transcription factor activator protein 1 (AP-1) is activated in T84 cells stimulated with the EHEC strain EDL 931¹⁰. Although both NF- κ B and AP-1 DNA-binding activities are required for the maximum IL-8 promoter activity in epithelial cells³⁶, the effect of A/E bacteria-induced AP-1 on pro-inflammatory gene mRNA expression remains unproven.

Signaling cascades upstream from NF- κ B activation have also been investigated. An interesting global analysis of protein tyrosine phosphorylation in T84 cells stimulated by EPEC demonstrates the activation of mitogen-activated protein kinases (MAPKs)-dependent signaling pathways⁹; the three MAPKs, extracellular signal-regulated protein kinases (ERK) 1 and 2, the c-jun N-terminal kinase (JNK), and p38 are activated upon EPEC stimulation of human epithelial cells⁹. Moreover, specific inhibitions of MAPK kinase-1 (also identified as MEK-1) or p38 in cocultures of EPEC with T84 cells^{9, 64} or with HeLa cells¹¹ lead to a decrease in IL-8 gene expression and in cytokine secretion. Similarly, p38 kinase activity is involved in EPEC-stimulated IL-8 synthesis⁹. Similar findings have been reproduced by two other groups using different strains of EHEC^{3, 10}. Additionally, EPEC-induced ERK1/2 degrades I κ B α ⁶⁴, thus demonstrating the MAPK-dependent activation of NF- κ B.

More recently, a careful study has investigated the role of the calcium-independent atypical PKC- ζ during EPEC infection of T84 and Caco-2 cells⁶³. Following bacterial stimulation, PKC- ζ translocates from the cytoplasm to the cell membrane and its activity is increased. Activated PKC- ζ enhances the activity of I κ B kinase β , which releases NF- κ B after I κ B phosphorylation; surprisingly, these events are MAPK-independent. This work modifies our simplistic view of the activation of an innate immune system response to A/E pathogens and supports the contention that A/E bacteria may activate several signaling pathways, thus ensuring the pro-inflammatory response (Fig. 1).

One note of caution regarding the activation of the innate response of epithelial cells is the discovery of the A/E bacteria-dependent downregulation of the immune response. In this regard, an elegant study was designed by Ceponis et al.⁶. Interferon gamma (IFN- γ) is widely produced during A/E bacteria infec-

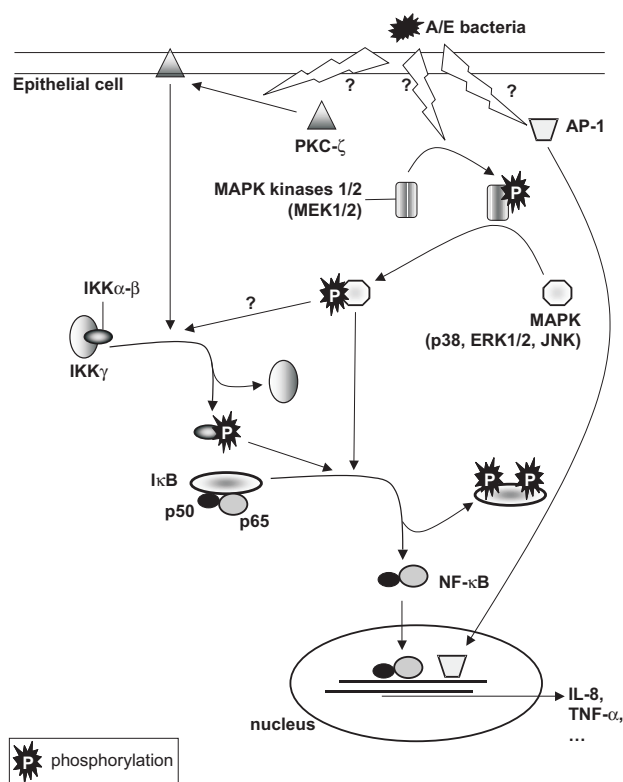


Figure 1. Possible scenarios for the activation of the innate immune system in epithelial cells in response to A/E bacteria. Host immune response is induced by interactions of bacterial components with an undefined epithelial cell receptor. Subsequently, signaling cascades through protein kinase C zeta (PKC- ζ) or mitogen-activated protein kinase (MAPK) pathways are stimulated. This leads to the activation of the nuclear factor kappa B (NF- κ B), directly or indirectly through the I κ B kinase (IKK) pathway. Alternatively, the transcriptional factor activator protein-1 (AP-1) is also induced by bacteria. NF- κ B and/or AP-1 drive the expression of cytokines and chemokines.

tions. It has been reported that IFN- γ stimulates epithelial cells *in vitro* by a Stat-1-dependent signal transduction. When IFN- γ -treated T84 cells are infected with EHEC O157:H7, but not with EPEC, Stat-1 is not phosphorylated, does not bind DNA, and cannot recruit transcription factors such as IFN regulatory factor 1. The bacterial factor responsible for this effect is not intimin, lipopolysaccharide, Stx1 or 2, a TTSS-secreted protein, and is not encoded by the plasmid pO157⁶. It is, therefore, tempting to speculate that A/E bacteria interfere with host cells not only by activating signal transduction, but also by inhibiting other signaling pathways in order to escape the host innate response.

FINDING FACTORS

The LEE or not the LEE?

The EPEC strain E2348/69 activates IL-8 secretion in T84 cells by a MAPK-dependent pathway; interest-

ingly, activation of the ERK1/2, JNK, or p38 is not observed when $\Delta eaeA$ (which does not possess intimin) and $\Delta escN$ (lacking a functional TTSS) mutant strains have been used to stimulate the cells⁹. Similarly, a wild-type strain of EPEC stimulates mRNA expression of the transcription factor early growth response 1 (Erg-1) and of IL-8 in HeLa cells, whereas a mutant strain deficient in the gene *espB* (EspB is a constituent of the translocation machinery inserted in the host cell membrane) fails to induce IL-8 and Erg-1¹¹; furthermore, an $\Delta escC$ mutant (that lack a functional TTSS) induces lower levels of IL-8 and Erg-1 mRNAs than the parental strain¹¹, suggesting that an intact EPEC LEE is necessary to stimulate epithelial cells.

Conversely, intimin of EHEC O157:H7 is not required for stimulation of Caco-2 cells, since the *eae* isogenic mutant or the parental strain induce similar levels of MAPK and NF- κ B activation and of IL-8 production³. These data have been reinforced by the finding that LEE-positive and LEE-negative virulent strains of EHEC of various serotypes, such as O157:H7, O113:H21, O91:H21, O48:H21, or O91:H7, stimulate Hct-8 cells⁵⁹. Remarkably, chemokine mRNA expression levels and chemokine production are 2- to 100-fold greater when T84 cells³⁵ or Hct-8 cells⁵⁹ are stimulated with LEE-negative EHEC strains in comparison with stimulation by LEE-positive strains. Rogers et al.⁵⁹ question whether LEE-negative strains might compensate for the inability to induce the A/E lesions by increasing the induction of the innate immune response. Another explanation could emerge from an intriguing work by Hauf and Chakraborty³¹. These authors demonstrate that NF- κ B is transiently activated in HeLa cells by an O26:H⁻ EHEC strain; nonetheless, cells stimulated with an EspB-deficient strain exhibit increased and prolonged DNA-binding activity of NF- κ B, suggesting an inhibition of the cellular response by LEE-encoded EspB; this inhibition could occur through a direct effect of EspB or indirectly by the disruption of the translocon. The inhibitory effect of EHEC- and EPEC-derived EspB is also observed in TNF- α -challenged HeLa cells. The biological consequence is that cells stimulated with a $\Delta espB$ mutant express more IL-8, IL-6, and IL-1 β mRNAs and produce more cytokines than cells stimulated with the parental strain. Although the results were obtained with HeLa cells instead of colonic epithelial cells, it is conceptually interesting to consider that the LEE might be involved in the inhibition of the innate response. Therefore, an EspB-dependent inhibition of NF- κ B activation could explain the fact that LEE-positive STEC strains induce a lower pro-inflammatory response in

Hct-8 cells than do LEE-negative strains^{35, 59}. The study of the effect of LEE-positive and LEE-negative strains on NF- κ B activation in human colonic epithelial cells is warranted to reveal the global impact of the LEE on the innate response that occurs upon A/E bacteria stimulation (Fig. 2).

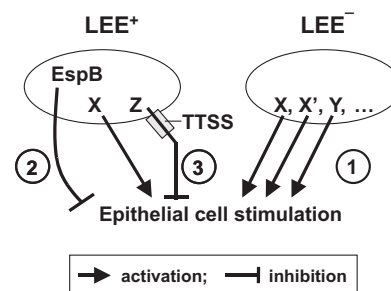


Figure 2. Potential activation of epithelial cells by LEE-positive and LEE-negative EHEC. Several reports have outlined that EHEC strains that harbor the LEE induce less cytokine production than LEE-negative strains. 1 – LEE-negative EHEC could express modified (X') or different (Y) inducing factors than LEE-positive strains. 2 – LEE-encoded proteins, such as EspB, could inhibit epithelial cell stimulation. 3 – Proteins encoded by genes outside the LEE (Z), but translocated in eukaryotic cells by a functional LEE-encoded type III secretion system (TTSS), could inhibit cellular activation.

In conclusion, data obtained with EPEC strains differ from those resulting from experiments performed with EHEC. Investigations have reported that LEE-encoded effectors are different in EPEC and EHEC⁵; thus, the transfer of the EPEC LEE⁴⁷, but not of the EHEC LEE¹⁸, to a commensal *E. coli* converts the strain into an A/E lesion-forming bacteria. Nonetheless, T84 cells have been infected with EPEC, whereas Caco-2 and Hct-8 cells have been used for EHEC experiments; it should be interesting to study the impact of the LEE from EPEC and EHEC on the same epithelial cell line. In addition, some experiments^{3, 9} have been done in conditions in which the LEE is poorly or not expressed, e.g. bacteria in stationary phase in Luria Broth^{15, 60}; thus, monitoring the expression of the LEE during the *in vitro* experimental procedures is an essential parameter that should account for a clear interpretation of the results.

Flagellin

Flagella are essential for motility and adherence of pathogenic bacteria, as recently reviewed by Ramos et al.⁵⁸. In addition, monomers of flagellin exhibit features of microbe-associated molecular patterns and are detected by TLR-5³². Thus, an innate immune response can be activated by a TLR-5-dependent sig-

nal transduction that leads to NF- κ B activation³². Low concentrations (~ 10 ng/ml) of purified flagellin from the serogroups H6, H7, and H21 stimulate IL-8 secretion by human colonic epithelial cells^{3, 59, 88} by p38- and ERK1/2-dependent pathways⁸⁸. Isogenic *fliC* mutants of EPEC or EHEC that lack flagellin H6⁸⁸ or H7³ and H21⁵⁹ completely fail to elicit IL-8 synthesis by human epithelial cells. Consistent with the basolateral localization of TLR-5 in polarized epithelial cells, more IL-8 is produced by cells stimulated at the basolateral surface by purified flagellin from EHEC and EPEC in comparison with activation of the apical surface^{3, 88}. Importantly, Zhou et al.⁸⁸ raise an important technical feature in their manuscript: EPEC grown in Luria Broth express *fliC*, synthesize flagellin H6, and stimulate epithelial cells, whereas bacteria in DMEM do not express flagella and do not induce IL-8 production by T84 cells.

The *Stx* paradox

EHEC differs from other A/E bacteria by the presence of the genes *stx1* and/or *stx2* that encode for the Stxs. Members of the Stx family are AB₅ holotoxins composed of a 32-kDa A subunit in noncovalent association with a pentameric ring of the B subunit (~ 7.7 -kDa each). The B subunits are recognized by Gb3 and allow the internalization of the toxin. The A subunit is the enzymatic component of the toxins that alters ribosomal function by an *N*-glycosidase activity on the 28S RNA of the 60S subunit¹⁹, leading to cell death. Stx-dependent epithelial cell apoptosis has also been described (see review by Cherla et al.)⁷. Although known as an inhibitor of protein synthesis, several studies support the notion that Stx might stimulate the innate immune response.

Concentrations lower than 100 ng/ml of purified Stx1 or Stx2 induce mRNA expression and production of cytokines and chemokines (TNF- α , IL-8, MCP-1) in butyrate-treated Caco-2 cells⁸⁶ expressing high levels of Gb3⁴⁰, and in Hct-8 cells^{75, 76}, in which the Gb3 status remains unknown. However, induction of chemokines such as ENA-78 or GRO- α in Hct-8 cells requires a stimulation with more than 10 μ g/ml of Stx1⁷⁶. Yamasaki et al.⁸⁷ demonstrated that the cytokine-inducing activity of Stx is independent of Gb3 but requires the intracellular translocation of the toxin, since protein synthesis was inhibited during the experiment. Supporting this concept, a Gb3-independent retrograde transport for Stx was recently reported in T84 cells⁶⁵. Additionally, cytokine mRNA stability is increased when cells are stimulated with Stx1⁷⁶. In Hct-8 cells, Stx-mediated epithelial cell activation is MAPK dependent and involves p38 and the JNK/stress-activated protein kinases complex⁶⁸.

⁷⁵. Interestingly, blocking Stx1-induced p38 and JNK activation prevents HCT-8 apoptosis⁶⁸. While the B subunit is not involved in the stimulation of cytokine expression in epithelial cells, recombinant Stx1 mutated in the A subunit does not induce cytokine expression, cell toxicity, or inhibition of translation⁸⁶. Accordingly, ricin and modeccin, two compounds that share *N*-glycosidase activity with Stx, inhibit epithelial cell protein synthesis and increase IL-8 production^{75, 87}. These data support the general concept that the inhibition of protein synthesis by Stx is responsible for the induction of cytokine production. Similarity to the “ribotoxic stress response”³⁹ has been demonstrated by Smith et al.⁶⁸.

In contrast, O157:H7 *E. coli*-induced IL-8 production by Gb3-expressing Caco-2 cells⁴⁰ is independent of the production of Stx³. Similar phosphorylation of ERK1/2 and p38, IL-8 mRNA expression, and IL-8 production are observed when cells are stimulated with the EHEC O157:H7 strain 86-24 or with an isogenic *stx2* mutant. In addition, Caco-2 cells do not synthesize IL-8 when activated by 10 μ g/ml of purified Stx2³. This finding differs from Yamasaki's results⁸⁶, and should be due to the Gb3 status due to the effect of butyrate. More recently, Paton's group has reported that IL-8 concentration in culture supernatants of Hct-8 cells stimulated with 100 ng/ml of recombinant flagellin is ~ 100 -fold greater than in the supernatants of cells stimulated with the same amount of purified Stx1 or Stx2⁵⁹. Similar chemokine production was observed when cells were stimulated with the strain 98NK2 (an O113:H21 that possesses Stx2 only) or with the isogenic mutant Δ *stx2*⁵⁹. Thus, the conflicting data could result from differences in the nature of the epithelial cells and bacteria, in the culture conditions, in the time of stimulation, or in the amounts of Stx. The real impact of cellular Gb3 status deserves further deeper investigations.

While the effect of Stx on cytokine production by human epithelial cells remains a subject of controversy, there is unanimity about the pro-inflammatory effect of Stx onto human macrophages. Blood-purified human monocytes, differentiated THP-1 cells, and U937 cells express low levels of Gb3^{21, 57, 80} and are not sensitive to the toxic effect of Stx1 and Stx2; the treatment of these cells by Stx elicit TNF- α and IL-1 β production^{57, 61}. Conversely, undifferentiated THP-1 cells express high levels of Gb3, are Stx-sensitive, and fail to produce cytokine when treated with sublytic doses of Stx1 or 2^{21, 57}. NF- κ B, AP-1⁶¹, and PKC²¹ are activated in differentiated THP-1 cells upon Stx1 stimulation. Nonetheless, it has not been directly established that Stx1-induced cytokine expression is NF- κ B and/or AP-1 dependent.

However, PKC inhibitors, but not PKA inhibitors, suppress Stx1-induced macrophage TNF- α production²¹. As for epithelial cells, the A subunit of Stx1 and 2, but not the B subunit, is essential for cytokine production by differentiated human macrophages, and cell treatment with ricin also elicits cytokine production²¹.

SIMPLIFYING THE INFLAMMATORY RESPONSE PICTURE: LESSONS FROM KNOCK-OUT MICE

Cytokines in C. rodentium colitis

Use of *C. rodentium* as a murine model for A/E bacterial infection has greatly expanded our knowledge of the *in vivo* role of the bacterial factors essential for colonization and for A/E lesions, e.g. intimin³³ or Tir¹³. Mice infected with *C. rodentium* also represent an excellent model to study the inflammatory response of the colonic mucosa³⁴, and genetically engineered mice are a powerful tool in investigating the role of genes from the immune response.

C. rodentium induces in mouse colon a strong Th1 cytokine response characterized by the expression and production of IFN- γ , IL-12p40, IL-1 β , and TNF- α ^{24, 33, 34}. Therefore, studies with mice deficient in one of these cytokines or their receptors provide a deeper insight into their role during bacterial infection. The role of TNF- α has been studied by using TNF receptor type I p55 (TNFRp55)-deficient C57BL/6 mice²⁹. Enhanced colonic bacterial burden has been found in TNFRp55^{-/-} mice, but wild-type and TNFRp55^{-/-} C57BL/6 exhibit similar clearance of bacteria. When compared with *C. rodentium*-infected wild-type mice, the infected knock-out mice show an increase in colonic hyperplasia and of CD4⁺ cells in the lamina propria, and enhanced IFN- γ and IL-12p40 mRNA transcripts in gut tissue. More recently, Spahn et al.⁶⁹ investigated the biological role of lymphotoxin, a TNF-family cytokine, during *C. rodentium* infection. Infected mice lacking lymphotoxin- α , lymphotoxin- β , or lymphotoxin- β receptor exhibited increased mortality, weight loss, and bacterial burden in comparison with wild-type mice.

Mice with disrupted IFN- γ or IL-12p40 genes have significantly higher numbers of *C. rodentium* in the colon and bacterial clearance is delayed compared with wild-type mice⁶⁶. The intensity of inflammation, clinical course, and immunopathological parameters are also worsened in both knock-out mice; in addition, mortality is even higher in the IL-12p40 knock-out group⁶⁶. Interestingly, the authors sought to determine the mechanism by which IFN- γ - or IL-12p40-deficient mice have increased pathology

during *C. rodentium* infection. Serum IgG and fecal IgA response patterns are identical between wild-type and knock-out mice, suggesting that the enhanced susceptibility is not due to an inability to produce anti-*C. rodentium* antibodies⁶⁶. Rather, they demonstrate that mice lacking IFN- γ or IL-12p40 express a lower level of mRNA of the β -defensin mBD-3 than wild-type mice in the late stage of infection⁶⁶, suggesting the crucial need for innate immunity for resistance to the A/E pathogen.

Intriguingly, in other forms of experimental murine colitis, namely the dextran sulphate sodium and 2,4,6-trinitrobenzene sulfonic acid models, blocking IL-12⁵¹ or lymphotoxin- β receptor⁷² improves colonic inflammation. It is therefore tempting to speculate that different mechanisms orchestrate A/E bacteria-induced mucosal inflammation from those in other forms of experimental colitis.

The NO question

Besides classical analysis of the effects of cytokines in infected mice, the biological role of NO, a messenger of the mucosal innate immune response, has been investigated. NO is a free radical that possesses numerous biological functions in the vascular, central nervous, digestive, and immune systems. Cellular production of NO requires the enzyme NO synthase (NOS) that metabolizes arginine as a substrate. Two constitutive isoforms, the neuronal NOS (NOS1) and the endothelial NOS (NOS3), and an inducible isoform (NOS2) have been identified in mammals. The last is expressed in numerous cells, e.g. neutrophils, macrophages, and epithelial cells, in response to cytokines⁴⁶ or bacterial components^{27, 73}. More particularly, high amounts of NOS2-derived NO are cytotoxic for pathogens^{26, 28}, including *C. rodentium*⁷⁸, induce apoptosis, or decrease the integrity of the epithelial barrier by acting on tight junctions¹⁶. Thus, NO is a major effector of the innate immune system that possesses many effects in the gastrointestinal tract⁸.

Mice infected with *C. rodentium* express a high level of NOS2 mRNA in the infected colon and show NOS2 protein in colonic epithelium and lamina propria^{24, 78}. Interestingly, NOS2 is not expressed in Tir-positive epithelial cells, but predominantly in uninfected cells⁷⁸. In addition, measurement of NO or reactive nitrogen intermediates in the serum demonstrates that infected mice produce more NO than control mice^{24, 66}. Together, these findings clearly establish that NOS2 is upregulated and functional during *C. rodentium*-induced colitis. Although infected NOS2-deficient C57BL/6 mice exhibit lower mor-

tality levels than wild-type mice^{24, 78}, studies by Simmons et al.^{66, 78} and by Vallance et al.^{66, 78} indicate that loss of NOS2 expression does not significantly affect pathology or levels of colon colonization. In contrast, a recent article by Wilson's group shows that severe *C. rodentium*-induced colitis in wild-type mice is improved with NOS2 deletion: Clinical (survival, loss of body weight, and colon weight), histopathological (mucosal thickening, inflammatory cell infiltration, mucin depletion), and immunopathological (IFN- γ , TNF- α , IL-1 β mRNA levels) parameters are significantly attenuated in NOS2-knock-out mice versus wild-type mice²⁴. In addition, they show that arginase 1 is induced in the colon of infected mice²⁴. This enzyme metabolizes the same NOS2 substrate, arginine, thus inhibiting NO production²⁵. *C. rodentium*-infected mice treated with an arginase inhibitor exhibit increased inflammation²⁴, suggesting that restoration of NO production is deleterious. Conflicting data with the previous studies could result from differences in technical features, e.g. age of mice or level of bacterial inoculum, as discussed in the manuscript by Gobert et al.²⁴.

Although the pathophysiological role of other components of the innate system, e.g. cyclooxygenase⁴⁸ or reactive oxygen intermediates⁴³, has been investigated using knock-out mice in others colitis models, their potential implications in *C. rodentium* infection remains unknown and deserves further investigation.

CONCLUSIONS

Classical *in vitro* analyses have facilitated the study of the expression of mediators from the innate immune system in response to A/E bacteria, and of the bacterial factors responsible for cellular activation. Nonetheless, according to experimental conditions, different results and conclusions have been proposed. The recognition that epithelial cells are differently stimulated by EPEC and EHEC represents a significant and intriguing advance. Dissimilar structure of the LEE and serotypes of flagellin and the presence of Stx in EHEC are possible candidates to explain the molecular basis of this discrepancy; nonetheless the precise effects of these factors remain to be clearly established. *In vivo* studies in patients infected with EHEC have revealed a correlation between the induction of a pro-inflammatory innate immune response and the development of severe diseases such as HUS. In contrast, the use of *C. rodentium* in genetically engineered mice shows the protective effect of pro-inflammatory cytokines. These findings highlight that 1) an extrapolation of experimental data from mice to humans has to be made with caution, and/or 2) other host factors than cytokines are involved. Further investigation of the interactions between A/E bacteria and host innate immunity is warranted to understand the precise pathophysiological role of host factors and to ensure the development of novel therapeutic strategies.

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