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Cell lines and *Salmonella*

An *in vitro* model for studying dose-response relations.

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Abstract

In human gastrointestinal disease caused by *Salmonella*, transepithelial migration of neutrophils follows the attachment of bacteria to epithelial tissue. This migration of neutrophils is stimulated by the release of chemokines, including interleukin-8 (Il -8), from the epithelial cells. We have developed an *in vitro* model system (human epithelial monolayers, among which Caco-2 cells grown on microtiter multiwell plates) for studying host-pathogen interactions.

After infection with different pathogens we measured Il-8 production during time. Results showed that Il-8 release was time related and varied with the pathogen. *Salmonella enteritidis* (*Se*) did induce the highest response. Subsequently, three doses of this *Se* strain were used and the Il-8 response was measured at different time points. Caco-2 cells remained intact over a period of 24h, the production of Il-8 increased in time and was found to be *Se* dose-dependent. Other tested epithelial monolayers, such as HT29 colon cancer cells, gave similar results.

Preface

This report describes an *in vitro* model for studying dose-response relations as expressed in cytokine production by human intestinal cell lines as response to exposure *to Salmonella enteritidis*. The goal of the *in vitro* model is to study the influence of environmental factors, such as food ingredients, on the virulence of gastro-enteritis causing foodborne bacteria i.e. the dose-response relation.

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Contents

Samenvatting

Infectie met *Salmonella* kan gepaard gaan met de invasie van darmepitheelcellen. De aan de invasie voorafgaande aanhechting leidt reeds tot de transmigratie van witte bloedcellen (neutrofielen) vanuit de bloedbaan naar het epitheelweefsel. De migratie wordt gestimuleerd door de productie van chemokines, waaronder interleukine-8 (Il-8) door epitheelcellen. Wij hebben een *in vitro* model systeem ontwikkeld (humaan epitheelweefsel gekweekt in microtiterplaten) waarin gastheer – pathogeen interacties kunnen worden bestudeerd. Epitheelcellen zijn gedurende een uur blootgesteld aan verschillende pathogene micro-organismen, waarna de Il-8 response is gemeten. Als controle zijn meegenomen een *Escherichia coli* stam zonder lipopolysacchride (LPS) en een probiotische *Lactobacillus*. De resultaten laten zien dat de IL-8 productie per pathogeen varieert, waarbij *Salmonella enteritidis* de hoogste respons geeft. In vervolgexperimenten zijn drie concentraties *S. enteritidis* gebruikt, waarna de respons gedurende 24 uur is gemeten. De gebruikte cellijn bleek na 24 uur nog intact, de Il-8 productie correleerde met de doses, en nam toe in de tijd. Hieruit kan geconcludeerd worden dat het door ons ontwikkelde model gebruikt kan worden voor het bestuderen van factoren die van invloed zijn op dosis-respons relaties. De respons betreft dan prikkeling van het immuunsysteem ten gevolge van adhesie en invasie van darmepitheelcellen door salmonellae.
Tenslotte wordt een benaderingwijze voorgesteld om resultaten van *in vitro* dosis-respons experimenten te vertalen naar de mens.

Summary

In human gastrointestinal disease induced by *Salmonella*, transepithelial migration of neutrophils follows the attachment of bacteria to epithelial tissue. This migration of neutrophils is stimulated by the release of chemokines, including interleukin-8 (IL-8), from the epithelial cells. We have developed an *in vitro* model system (human epithelial monolayers, among which Caco-2 cells grown on microtiter multiwell plates) for studying host-pathogen interactions.

Epithelial cells were incubated for one hour with varying pathogens. Two strains were used as negative controls, an *Escherichia (E.) coli* strain lacking lipopolysaccharide (LPS), and a probiotic *Lactobacillus* sp. Results showed that IL-8 release varied with the used pathogen, *Salmonella enteritidis* (*Se*) giving the highest response. Subsequently, three concentrations of this *Se* strain were used and the IL-8 response was measured in time. Caco-2 cells remained intact over a period of 24h, and the production of IL-8 increased in time and showed to be dose-dependent. From these results it is concluded that this model can be used for quantitative dose-response studies as far as the response is related to induction of the immune system by attachment of the organism to or to invasion of the epithelial tissue.

Finally, an approach is suggested for translation of *in vitro* results to the *in vivo* situation.

1. Introduction

Risk assessment is the science of understanding hazards, how likely they are to occur and the consequences if they do occur. Microbiological risk assessment of foodborne pathogens consists of four steps:

- identification of a pathogen or toxin in a specific product (hazard identification);
- exposure assessment (quantification of the likely intake of the pathogen);
- hazard characterisation (quantitative evaluation of the adverse effects associated with infection of the pathogen), including dose-response assessment; and
- risk characterisation, which can be considered as an estimation of public health effects based on hazard identification, exposure assessment and hazard characterisation (1).

Salmonella spp. are found on many food products and are causative agents of gastrointestinal disease (GID). Interaction between such pathogens and the human body starts by attachment to the epithelial tissue of the small intestine. Studies by McCormick et al (2, 3, 4) showed that the attachment of pathogenic *Salmonella typhimurium* was rapidly followed by the transepithelial translocation of neutrophils. Neutrophils were attracted by interleukin-8 (Il-8), the neutrophil chemotactic peptide synthesised in epithelial cells.

Results from a study by Jung et al (5) revealed that infection of human epithelial monolayers with invasive bacteria not only resulted in the expression of Il-8, but also in the expression of Il-6, Il-1 and tumour necrosis factor-α (TNF-α). Expression of Il-8, Il-6 and TNF-α was also detected in freshly isolated human intestinal epithelial cells.

This study was aimed to develop an *in vitro* model-system for dose-response assessment. We measured Il-8, Il-6, Il-1β, and TNF-α production as response on exposure of various human cell-lines to various doses of pathogens, among which *Salmonella enteritidis*. The ultimate goal of the *in vitro* model-system is to study the effect of environmental factors, such as food ingredients, on the dose-response relation.

2. Materials & Methods

2.1 Bacterial strains

Species	Reference
Listeria (L.) monocytogenes	RIVM (strain LIS1149800050, serotype 4b)
Lactobacillus (Lb.) casei	RIVM
Escherichia (E.) coli	6 (strain WG5)
E. coli	University of Utrecht (serotype O33)
Salmonella (S.) enteritidis	RIVM (strain 97-198)
S. enteritidis	University of Utrecht (strain 857)

2.2 Bacterial culture media and growth conditions

Stationary phase cultures of bacteria were stored at –70^{o}C in Brain Heart Infusion (BHI) broth plus 50% glycerol. *Lb. casei* was stored in medium according to De Man, Rogosa and Sharpe (MRS, Merck) plus 50% glycerol.
Inocula were prepared by resuscitating –70^{o}C stocks in 10ml of growth medium for 8 h. at 35^{o}C, after which cells were inoculated into 60ml of fresh medium. *Lb. casei* was grown on MRS; all other strains were grown on Listeria Enrichmont Broth (Oxoid).
Stationary phase was reached after 10h at 35^{o}C (*Lb. casei*: 14h) and checked by OD_{600} measurement. Colony forming units (cfu) were counted (after serial dilution in peptone physiological salt solution) on growth medium plus 1.5% agar.
Of each stationary phase culture 40ml was centrifuged at 5000 x g for 10 min. at 5^{o}C and washed in 40ml cold (5^{o}C) tissue culture medium (Dulbecco's Modified Eagle Medium (DMEM)) without foetal calf serum (FCS). This suspension was spun for again 10 min. at 5^{o}C and 5000 x g, after which the pellet was resuspended into 4ml of DMEM without FCS. This concentrated suspension was diluted decimally in DCEM without FCS and dilutions were kept on ice for at most 1h.

2.3 Cell lines

Human cell line	reference
Caco-2	University of Utrecht
HT29-12	7
HT29-21	7

2.4 Culture media and growth conditions

Cells were stored in 10 % DMEM, plus 10 % (v/v) DMSO, plus 100 μg ml^{-1} gentamycin in liquid nitrogen. For each experiment, after thawing, cells were grown confluently in DMEM (Flow Laboratories Amstelstad, Amsterdam, The Netherlands) with 20% FCS (v/v) in culture flasks according to Koninkx *et al* (8). After trypsin treatment, aliquots were seeded at 40.000 cells per cm^2 in 12 well tissue culture plates (Costar, Corning Costar Europe, Badhoevedorp, The Netherlands). Cells were grown confluently in 19 days in plates with or without filter inserts (Greiner, The Netherlands). After 19 days wells contained approximately 5 x 10^5 cells/cm^2.

2.5 Exposure

Exposure was performed according to McCormick *et al* (2). Shortly, just before infection, cells were washed three times with 1ml of serum and antibiotic free DMEM. At t = 0h, 40μl of the concentrated or diluted bacterial suspension was used for exposure. After a one hour exposure, bacterial suspensions were removed and cell lines were washed three times using serum free DMEM to which 50μg ml^{-1} gentamycin was added.

2.6 Detection of cytokines

Samples, taken at indicated time intervals from the supernatants of the exposed cell lines as well as from the baso-lateral side of the filter inserts, were stored at –70^oC to be analysed later. Cytokine concentrations were determined by ELISA, according to Garssen *et al* (9).

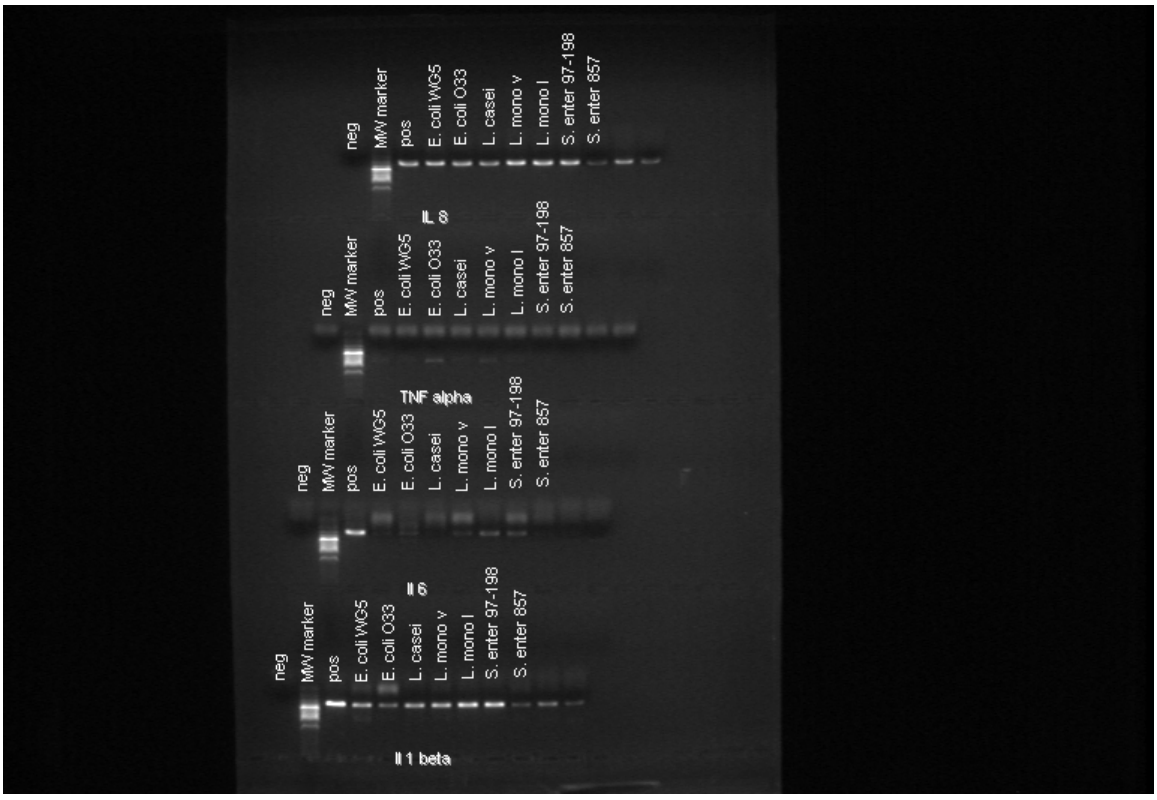
2.7 RNA isolation, cDNA synthesis and PCR

Isolation of RNA encoding TNF-α, Il-1β, Il-6, Il-8, RNA transcription into cDNA, and amplification of cDNA were performed as described in Garssen *et al* (9).

3. Results

Initially we exposed Caco-2 cells to *S. enteritidis* 97-198 and *L. monocytogenes*. Using 12-well plates with filter inserts we were unable to detect cytokines by ELISA at the basolateral side of the cells 8 h. after infection. However, results of rtPCR showed transcription of the genes encoding Il-1β, Il-6, and Il-8 (Figure 1). We failed to detect TNF-α, both in samples and in the positive control.

Figure 1. Detection of mRNA encoding Il-1β, Il-6, Il-8 and TNF-α.

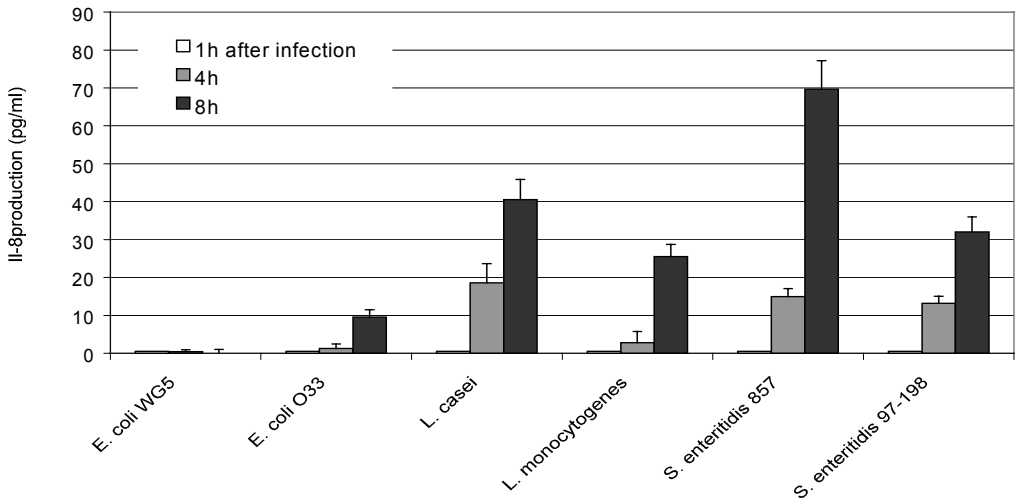


Abbrevations: MW marker: molecular weight marker; neg: negative control; pos: positive control; L. mono: *L. monocytogenes*; v: early log phase; l: late log phase; S. enter: *S. enteritidis*.

In subsequent experiments we used 12-well plates without filter insert to measure Il-8 production. Caco-2 cells were exposed to *S. enteritidis* 857 and 97-198, *L. monocytogenes* and *E. coli* O33. All pathogens induced production of Il-8, measured 4 and 8h after infection. The amount of Il-8 produced increased in time and varied between pathogens (Figure 2). After 4 and 8 h *E. coli* O33 gave the lowest response, *S. enteritidis* 857 and 97-198 gave the highest. Il-8 was not detected in non-exposed wells and in wells without

epithelial cells (not shown). The non-pathogenic considered *Lb. casei* also induced Il-8 production, whereas the non-pathogenic *E. coli* WG5 did not.

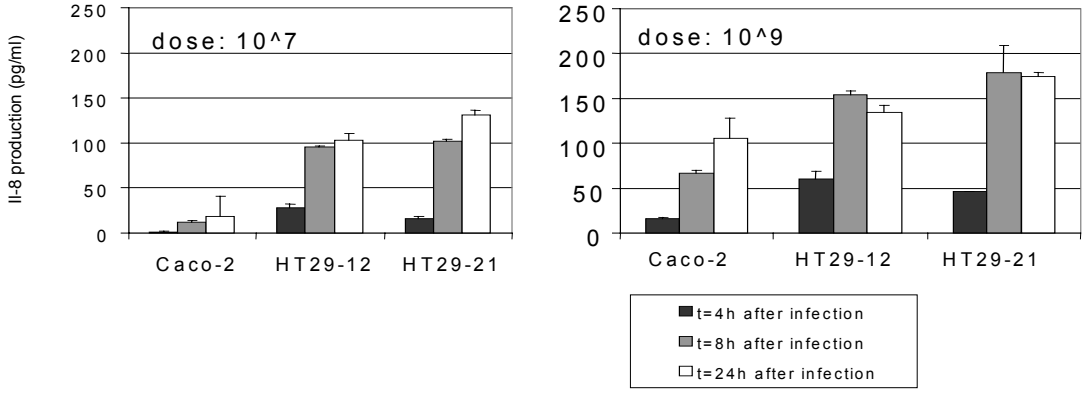
Figure 2. Il-8 Production by Caco-2 cells in response to exposure to varying pathogens.



Dose: 40 µl of a suspension containing 10^9 bacteria ml^{-1} was added to a microtiter well containing 1 ml DMEM.

Caco-2, the enterocyte-like HT29-12 and mucus producing HT29-21 cells were used in dose-response experiments with *S. enteritidis* 97-198. The lowest applied dose (10^5 cells) did not result in the production of detectable levels of Il-8 in either cell type (not shown). Exposure to either 10^7 or 10^9 cells of *S. enteritidis* 97-198 ml^{-1} led to dose-dependent detectable levels of Il-8 in all three cell lines (figure 3a and 3b).

Figure 3. Il-8 Production in different cell lines after infection with two doses of S. enteritidis.



4. Discussion and conclusion

Risk assessment forms the scientific basis for risk analysis, a new approach to control food safety. Dose-response studies form an essential element of risk assessment. Epidemiological information on outbreaks with microbiological pathogens however often lack reliable data on infective doses. Incubation times vary before symptoms occur, potential sources might have been thrown away, or contaminated products might have been kept under conditions where bacteria could grow or die off. Data on dose-response relations therefore have to be studied otherwise. Especially when factors influencing the virulence of gastro-intestinal pathogens , like food ingredients, have to be studied.

Human volunteer experiments suffer from ethical restraints. Animal studies have limited value: human pathogens are not always pathogenic to animals and parts of the gastro-intestinal tract as well as the immune system of animals can differ from the human system (10, 11).

We have chosen to study dose-response relations in human intestinal epithelial cell line systems for the following reasons:

They do not suffer from ethical restraints; although mono cell systems lack a complete immune system, they might be a very useful *in vitro* model to study quantitatively some primary aspects of host-pathogen interactions like attachment, invasion, translocation and signalling for influx of neutrophils. All these events are, for example, part of the pathophysiology of localised enteritis caused by *Salmonella*.

Human colon epithelial cells express an array of cytokines in response to the presence of bacteria. Infection of Caco-2 cells, grown on filter insert tissue culture plates, with low number of pathogens induced the synthesis of mRNA encoding for Il-8 and Il-1β, whereas the synthesis of mRNA encoding for Il-6 and TNF-α varied when challenged. We were unable to detect cytokines by ELISA. In further experiments using tissue culture plates without filter inserts we were able to detect Il-8. A smaller volume of culture medium, i.e. higher concentration of cytokines, can explain this. The Il-8 response in Caco-2 cells showed to be pathogen-dependent. Exposure to *S. enteritidis* 97-198 showed an Il-8 response that was dose related and time dependent.

Il-8 is a neutrophil attractant. Data presented in this contribution show a dose-dependent increase in Il-8 production in three human epithelial cell lines. We hypothesise that *in vivo* this should result in an increase in total number of neutrophils. This hypothesis will be tested in a rat model as a first step in the validation of our model. We will infect cell lines of rat

origin as well as live rats. Since rats do not produce Il-8 but MIP-2, we will measure the Il-8 analogue MIP-2 production and neutrophil concentration respectively. Preliminary data already indicate that high *Se* doses did induce increased MIP-2 in serum of rats and strongly increase of the numbers of neutrophils (12).
Subsequently, the obtained ratio between MIP-2 production and number of neutrophils in the live rat model can be used to predict the increase in neutrophils in for instance mouse as data on Il-8 production in infected cell lines of mouse origin are available. In a next step, the prediction will be checked *in vivo*. When validated live in animal models for aspects of host-pathogen interactions like attachment, invasion, translocation and signalling for influx of neutrophils, human intestinal epithelial cell lines may offer a powerful model, which can be used to study human host-pathogen relationships in a quantitative way.

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