Old Herborn University Seminar Monograph 5:
Effective and ineffective defence mechanisms in the gastrointestinal tract.
Editors: Hans Cottier, Peter J. Heidt, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 150-157 (1994).

THE USE OF FLOW CYTOMETRY IN THE ANALYSIS OF LIPOPOLYSACCHARIDE EPITOPE EXPRESSION IN *ESCHERICHIA COLI* O26:B6 VARIANTS

MARTIN E. EVANS

Division of Infectious Diseases, Department of Medicine, Uniformed Services University of the Health Sciences, F. Edward Hébert School of Medicine, Bethesda, Maryland, USA

SUMMARY

Gram-negative bacteraemia and septic shock due to bacterial lipopolysaccharide (LPS) are important causes of morbidity and mortality in spite of the use of potent bactericidal antibiotics. Adjunctive therapy with antibodies directed against LPS may be of value. In the evaluation of binding of LPS core- and lipid A-specific monoclonal antibodies (MAbs) to smooth bacteria using an indirect immunofluorescence technique and epifluorescence microscopy, a small proportion of *E. coli* O26:B6 were observed to bind the core-specific MAb J8-4C10. To examine *E. coli* O26:B6 further, the cells were simultaneously stained with FITC-labelled O-antigen specific MAb E5-3A5, and biotinylated MAb J8-4C10 followed by a streptavidin R-phyco-erythrin conjugate and analysed by dual parameter flow cytometry. Sixty-four percent of bacteria bound O-antigen specific MAb, 21% bound core-specific MAb, and 13 % bound both MAbs consistent smooth, rough, and semi-rough LPS phenotypes respectively. Bacteria from the stock culture were stained with MAb J8-4C10 and smooth and rough/semi-rough bacteria isolated using a fluorescence-activated cell sorter. When the sorted cells were re-analysed, 96% of the smooth bacteria stained with MAb E5-3A5, and 93% of the rough/semi-rough bacteria stained with MAb J8-4C10 confirming that the bacteria had been sorted with high efficiency. When purified LPS from the isolates was compared by SDS-PAGE and immunoblot, few high molecular weight O-antigen bearing bands were seen in LPS from bacteria that had stained with MAb J8-4C10 confirming the rough/semi-rough phenotype of these bacteria. Cultures of smooth bacteria may contain subpopulations with rough or semi-rough LPS phenotype. Flow cytometry technology facilitates identification and isolation of these subpopulations for further analysis.

INTRODUCTION

Lipopolysaccharide (LPS), or endotoxin, is a major antigenic structure on the surface of Gram-negative bacteria, and a principal mediator of septic shock. This tripartite macromolecule consists of lipid A, which is inserted into the phospholipid bilayer of the bacterial outer membrane, the covalently attached core oligosaccharide, and, in the case of smooth LPS, the distally placed O-polysaccharide.

Antibodies to LPS may be useful

adjuvants in the therapy of septic shock if they neutralise endotoxin, or opsonise bacteria and facilitate the clearance of pathogens by phagocytes. In the evaluation of the binding of LPS core- and lipid A-specific monoclonal antibodies (MAbs) to bacteria using an indirect immunofluorescence antibody staining technique and epifluorescence microscopy, a subpopulation of purportedly smooth *E. coli* O26:B6 was observed that stained with the core-specific MAb J8-4C10. This suggested that a small proportion of cells lacked masking O-side chains and were rough or semi-rough.

In this study, cells in a stock culture of *E. coli* O26:B6 were simultaneously stained with MAbs directed against the LPS O-antigen and core oligosaccharide and analysed by dual parameter flow cytometry. Subpopulations of bacteria that bound O-antigen specific MAb (smooth), core-specific MAb (rough), or both MAbs (semi-rough) were observed. Smooth and rough/semi-rough bacteria were isolated by cell sorting, and their purified LPS compared by SDS-PAGE and immunoblot. Few O-antigen bearing, high molecular weight LPS moieties were seen in the LPS of bacteria that had bound MAb J8-4C10 consistent with the rough/ semi-rough LPS phenotype predicted by flow cytometry.

MATERIALS AND METHODS

Bacteria

E. coli O26:B6 was obtained from List Biological Laboratories (Campbell, CA) and maintained as a stock culture at -70°C. Smooth *E. coli* O26S and rough/semi-rough *E. coli* O26SR were isolated from this culture using a fluorescence activated cell sorter (see below).

Reagents and media

Paraformaldehyde, bovine serum albumin, and biotin N-hydroxysuccinimide ester (NHS) were purchased from Sigma Chemical Co. (St. Louis, MO). An affinity purified, FITC-labelled, goat anti-mouse Ig was purchased from Organon Teknika (West Chester, PA). Tryptic soy agar and broth were purchased from Difco (Detroit, MI). Fluorescein isothiocyanate (FITC) on celite 10% was purchased from Calbiochem (La Jolla, CA), and a streptavidin R-phyco-erythrin conjugate was obtained from Molecular Probes, Inc. (Eugene, OR).

LPS-specific monoclonal antibodies (Table 1)

Murine MAbs reactive with different structural elements of *E. coli* LPS were prepared employing previously described immunisation, fusion, screening, and cloning procedures (*Pollack* et al., 1989). MAbs E5-3A5 (IgG2a) and E5-3G12 (IgG2a) were specific for the O-antigen of *E. coli* O26:B6 based on reactivity with purified homologous LPS, staining of high molecular weight LPS moieties on immunoblot, and lack of reactivity with heterologous rough or smooth LPS or whole bacteria (unpublished data). MAb J8-4C10 (IgG2a) was specific for 3-deoxy-D-manno-octulosonic acid (KDO) based on reactivity with the incomplete core oligosaccharide of purified *E. coli* D31m4 (Re) LPS, loss of this reactivity when KDO was cleaved from lipid A by mild acid hydrolysis, and inhibition of reactivity in ELISA by purified KDO. MAb 13-17 (IgG2a), specific for an unrelated protein on the surface of *Para-*

Table 1: Lipopolysaccharide-specific monoclonal antibodies employed in flow cytometric analyses of *E. coli* O26:B6 variants.

MAb	Isotype	Specificity*
E5-3A5	IgG2a	O26:B6 O-Antigen
E5-3G12	IgG2a	O26:B6 O-Antigen
J8-4C10	IgG2a	KDO-disaccharide
13-17	IgG2a	Negative control

*Determined in ELISA and immunoblot

mecium multimicronucleatum, was employed as a negative control (*Pollack* et al., 1989).

MAbs were purified with a membrane affinity separation system (Nygene Corp., Yonkers, NY), and conjugated with FITC by a modification of a previously described procedure (*Rinderknecht*, 1962). Briefly, 10 mg MAb in bicarbonate buffer (pH 9.5) was mixed with 2 mg FITC on celite and incubated at room temperature for 4 hours. After removal of the celite by centrifugation, the mixture was passed over a Sephadex G-25 column to separate labelled MAb from unconjugated FITC. The protein content and amount of fluorescein bound to each antibody molecule in the final preparation were measured spectrophotometrically (*Goding*, 1983).

Purified MAbs were biotinylated by mixing 1 mg MAb in bicarbonate buffer (pH 8.3) with biotin NHS and incubating for two hours at 25°C (*Goding*, 1983). The mixture was then dialysed to remove excess unconjugated biotin.

Flow cytometric assays of MAb binding to viable bacteria

Bacteria were grown, harvested and then stained using a direct, or indirect immunofluorescence technique (*Evans* et al., 1990). In the former, the cells were mixed with FITC-conjugated MAb diluted in normal saline containing 1% (wt/vol) bovine serum albumin, and incubated at 4°C for 30 minutes before being washed and resuspended in 1% paraformaldehyde. In the indirect immunofluorescence assay, bacteria were stained with either unlabelled MAb followed by a FITC-labelled goat anti-mouse Ig antibody or biotinylated MAb followed by a streptavidin R-phycoerythrin conjugate. The bacteria were inspected under an epifluorescence microscope prior to flow cytometric analysis to determine adequacy of staining and the degree of cell aggregation. Aggregates were disrupted by passing the bacteria repeatedly through a 28-gauge hypodermic needle.

The fluorescence emissions of 5,000 stained bacteria were quantified in each sample using a FACScan® flow cytometer (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA). Live gates and forward scatter threshold were optimised to exclude large bacterial aggregates and cell debris from the analyses. Fluorescence data were collected, displayed, and analysed in log format.

Cell Sorting

The *E. coli* O26:B6 LPS phenotypic variants were sorted, as previously described (*Evans* et al., 1990), with a FACS II® cell sorter (Becton-Dickinson) on the basis of differential staining with the core-specific MAb, J8-4C10. Bacteria were sorted into cold tryptic soy broth and then subcultured on agar.

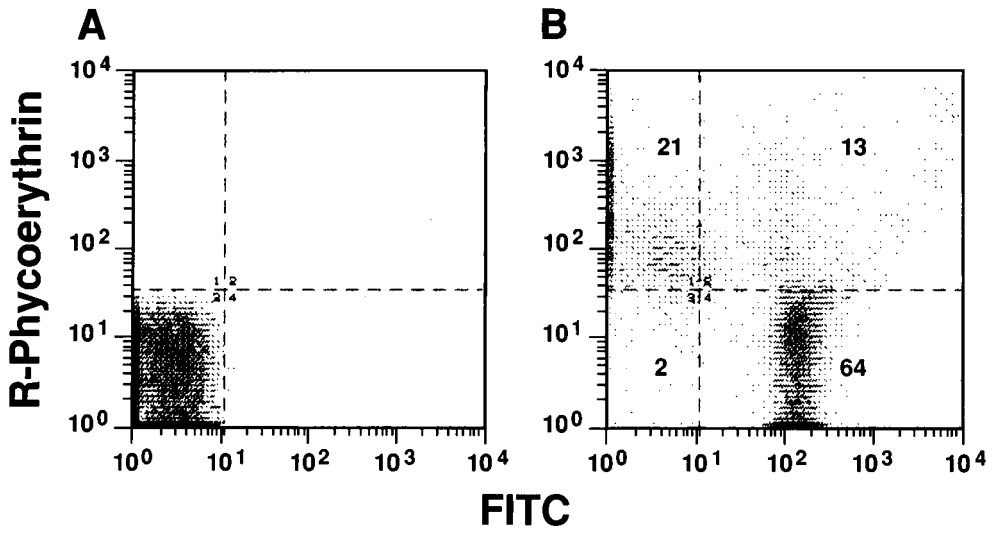

Figure 1: Dot plots of dual parameter flow cytometric analyses of cells from a stock culture of *E. coli* O26:B6 stained with A) FITC-labelled negative control MAb and streptavidin R-phyco-erythrin conjugate, or B) FITC labelled LPS O-antigen-specific MAb and biotinylated core-specific MAb followed by a streptavidin R-phyco-erythrin conjugate. Numbers refer to the percent of cells in each quadrant.

SDS-PAGE and immunoblot

LPS was prepared from proteinase K digests of whole cell lysates, and resolved by SDS-PAGE using a 4% stacking gel and a 14% separating gel according to the method of *Tsai* and *Frash* (1982). For immunoblots, LPS was transferred onto nitro-cellulose and exposed to MAbs as described previously (*Evans* et al., 1990).

RESULTS

Identification of smooth, rough and semi-rough sub-populations within a stock culture of *E. coli* O26:B6: A stock culture of *E. coli* O26:B6 was stained with the FITC-labelled, O-antigen-specific MAb, E5-3A5, and the biotinylated, core specific MAb, J8-4C10, followed by a streptavidin R-phyco-erythrin conjugate. As negative control, cells were stained with the FITC-labelled MAb 13-17, and the streptavidin R-phyco-erythrin conjugate. The bacteria were then analysed by dual parameter flow cytometry. Fully 99.8% of cells exposed to the negative control reagents fell into quadrant 3 (Figure 1A). Only 2% of the bacteria staining with MAb E5-3A5 and MAb J8-4C10 fell into this quadrant (Figure 1B). The majority of cells (64%) stained exclusively with MAb E5-3A5 suggesting that these bacteria maintained the smooth *E. coli* O26:B6 phenotype. Conversely, 21% of the cells stained only with the core-specific MAb J8-4C10 indicating that these cells had lost O-antigen and were rough. A third subpopulation (13%) stained with both

Table 2: Comparison of LPS phenotype of *E. coli* O26:B6 and sorted variants *E. coli* O26S and *E. coli* O26SR using dual parameter flow cytometric analyses.

Bacterium	% Population Binding MAb			
	E5-3A5*	E5-3A5 + J8-4C10	J8-4C10	Unstained
O26:B6	68	22	9	1
O26S	96	2	2	0
O26SR	0	93	0	7

*E5-3A5 specific for LPS O-antigen, J8-4C10 specific for core epitope

MAbs suggesting that these cells produced some O-antigen, but not enough to mask underlying core epitopes and were semi-rough.

Isolation of *E. coli* O26:B6 subpopulations binding O-antigen- or core-specific MAbs

To further examine the apparently smooth and rough/semi-rough subpopulations identified in dual parameter flow cytometric assays bacteria were stained with core-specific MAb J8-4C10, analysed, and sorted (Figure 2). Approximately 15% of the unsorted cells from the stock culture stained with the MAb (Figure 2A). Re-analysis of the sorted bacteria showed that the unstained (designated *E. coli* O26S) and stained (designated *E. coli* O26SR) cells were 97% and 92% "pure" respectively (Figure 2B and 2C). These bacteria were subcultured on agar and single colonies picked and regrown.

Sorted and unsorted bacteria were then stained with both FITC-labelled MAb E5-3A5 and R-phyco-erythrin conjugated MAb J8-4C10 and examined by dual parameter flow cytometry. The unsorted cells were comprised of four subpopulations as observed before (Table 2). Fully 96% of *E. coli* O26S stained with the O-antigen-specific MAb, and 93% of *E. coli* O26SR stained with the core-specific MAb. Bacteria staining with both MAbs were not seen.

SDS-PAGE and immunoblot analyses of *E. coli* O26S and O26SR LPS

Data from flow cytometric analyses suggested that the stock culture of *E. coli* O26:B6 consisted of smooth, rough, and semi-rough bacteria. Additional MAb binding studies of isolated bacteria suggested that *E. coli* O26S was smooth and *E. coli* O26SR was rough. Purified LPS from these bacteria was examined by SDS-PAGE and immunoblot to confirm the LPS phenotypes of these bacteria predicted by flow cytometry analyses. The LPS from both *E. coli* O26S and *E. coli* O26SR stained equally well with the core-specific MAb J8-4C10 on immunoblot (data not shown). Few high molecular weight bands staining with the O-antigen specific MAb E5-3G12 were apparent in the LPS of *E. coli* O26SR compared to that of *E. coli* O26S (Figure 3).

DISCUSSION

Flow cytometry has been an indispensable tool for the examination of immune mechanisms and pathophysiological events among eukaryotic cells (*Loken* et

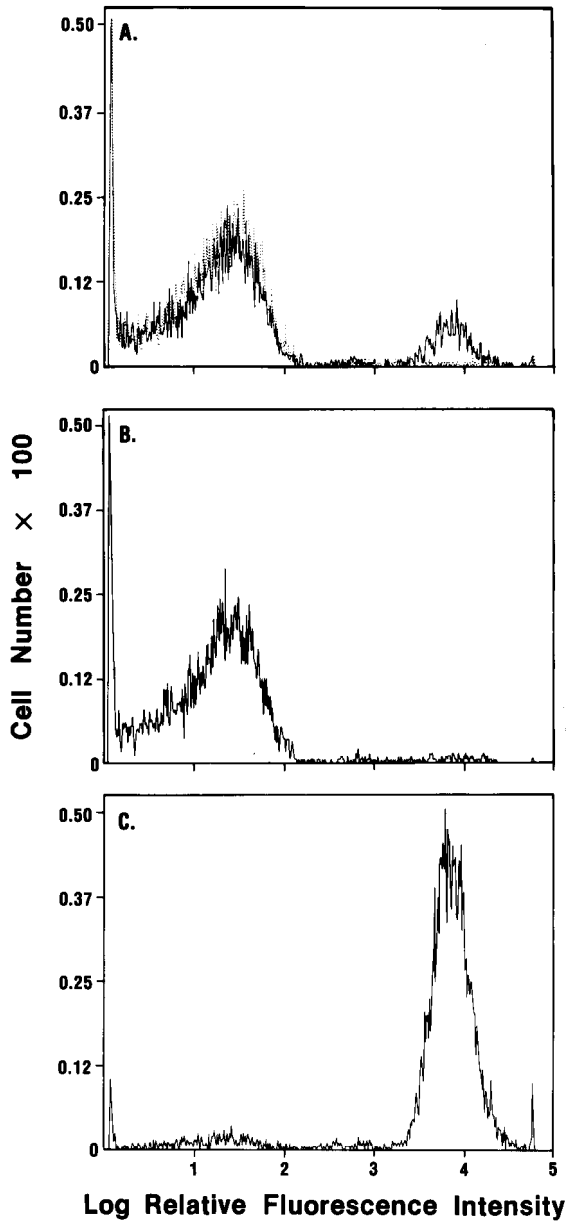

Figure 2: Subpopulations of *E. coli* O26:B6 identified and sorted with a FACS II® on the basis of differential staining by an LPS core-reactive MAb. Presort histograms (A) of nonstaining and staining cells. The fluorescence signals of cells stained with negative control MAb are shown as a dotted line overlying the larger histogram. Postsort histograms of nonstaining bacteria (B) with presumably smooth LPS, and staining cells (C) with presumably rough/semirough LPS.

al., 1982; *Herzenberg* et al., 1976; *Kruth*, 1982). With this technology, characteristics of individual cells can be measured at a rate of approximately 3,000 cells per second. Flow cytometry has rarely been applied to the study of bacteria and their interactions with host cells in infectious diseases (*Steen*,

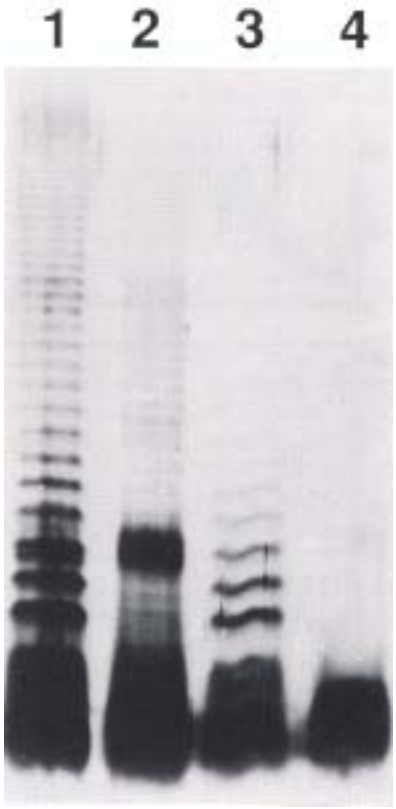

Figure 3: Immunoblots of bacteria sorted from a stock culture of *E. coli* O26:B6. LPS from proteinase K digests of whole cell lysates were separated on SDS-PAGE (*E. coli* O26S lanes 1, 3 and *E. coli* O26SR lanes 2, 4), transferred to nitro-cellulose, and stained with LPS O-antigen specific MAb. Lanes 1, 2 contained 10 μg LPS and lanes 3, 4 1 μg LPS.

1990; *Boye* et al., 1990). Instead, analyses in microbiology often rely upon measurements of mean values of large numbers of cells, as in antibiotic susceptibility testing, or the examination of a few select cells, as in studies employing electron microscopy. These analyses assume that variation among cells will centre around a single mean, and that subpopulations with markedly different phenotype do not exist.

In ELISA, we had previously documented the perplexing cross-reactivity of MAb J8-4C10 with rough, Re chemotype, LPS from *E. coli* D31m4, and presumably smooth LPS from *E. coli* O26:B6 (*Pollack* et al., 1989). We speculated that this cross reactivity was due either to shared epitopes in the LPS core and O-antigen of the respective LPS, or to the presence of both smooth and rough LPS in material purified from *E. coli* O26:B6. SDS-PAGE and immunoblot showed that MAb J8-4C10 reacted only with fast-migrating core structures and not O-antigen determinants of *E. coli* O26:B6 LPS, indicating that the MAb did not recognise an epitope in the O-antigen. This was consistent with data from other assays which suggested that MAb J8-4C10 recognised determinants in the KDO-disaccharide. The presence of cells in an *E. coli* O26:B6 stock culture with variable staining in immunofluorescence microscopy assays suggested that the LPS

purified from these cells and used in ELISA may have been a mixture of smooth and rough LPS. By staining the bacteria simultaneously with core-specific- and O-side chain-specific MAb and analysing the cells with dual parameter flow cytometry, we were able to document the presence of subpopulations of smooth, rough, and semi-rough bacteria within the stock culture. Smooth (*E. coli* O26S) and rough/semi-rough (*E. coli* O26SR) bacteria were differentially stained with MAb J8-4C10 and isolated by cell sorting. Dual parameter flow cytometric analyses of the sorted variants confirmed that the isolates were smooth and rough. The LPS of the isolated bacteria was then further characterised by SDS-PAGE and immunoblot. Although both LPS's appeared to contain the ladder-like pattern produced by LPS with progressively fewer O-side chain units, this pattern was less intense in LPS from *E. coli* O26SR compared to *E. coli* O26S. The difference between the two LPS's was even more marked in lanes which contained less LPS. These data confirmed that bacteria with variant LPS phenotypes were present in the original *E. coli* O26:B6 culture.

Since many of the pathophysiological events in infectious disease are due to host cell interaction with surface structures on bacteria, analysis of these structures on single cells and elucidation of variation within bacterial populations may be of value. Flow cytometry has proven useful for the detection of bacterial surface antigens, the identification of phenotypic variants, and the isolation of these cells for further analysis.

LITERATURE

Boye, E., and Løbner-Olesen, A.: Flow cytometry: illuminating microbiology. The New Biologist. 2, 119-125 (1990).

Evans, M.E., Pollack, M., Hardegen, N.J., Koles, N.L., Guelde, G., and Chia, J.K.S.: Fluorescence-activated cell sorter analysis of binding by lipopolysaccharide-specific monoclonal antibodies to gram-negative bacteria. J. Infect. Dis. 162, 148-155 (1990).

Goding, J.W.: Immunofluorescence. In: Monoclonal antibodies: principles and practice. Academic Press, London, 208-249 (1983).

Herzenberg, L.A., Sweet, R.G., and Herzenberg, L.A.: Fluorescence-activated cell sorting. Sci. Am. 234, 108-117 (1976).

Hitchcock, P.J., and Brown, T.M.: Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154, 269-277 (1983).

Kruth, H.S.: Flow cytometry: rapid biochemical analysis of single cells. Anal. Biochem. 125, 225-242 (1982).

Loken, M., and Stall, A.M.: Flow cytometry as an analytical and preparative tool in immunology. J. Immunol. Meth. 50, R85-R112 (1982).

Pollack, M., Chia, J.K.S., Koles, N.L., Miller, M., and Guelde, G.: Specificity and cross-reactivity of monoclonal antibodies reactive with the core and lipid A regions of bacterial lipopolysaccharide. J. Infect. Dis. 159, 168-188 (1989).

Rinderknecht, H.: Ultra-rapid fluorescent labelling of proteins. Nature. 193, 167-168 (1962).

Steen, H.B.: Flow cytometric studies of microorganisms. In: Flow Cytometry and Sorting. Wiley-Liss, Inc., New York, 605-622 (1990).

Tsai, C., and Frasch, C.E.: A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115-119 (1982).