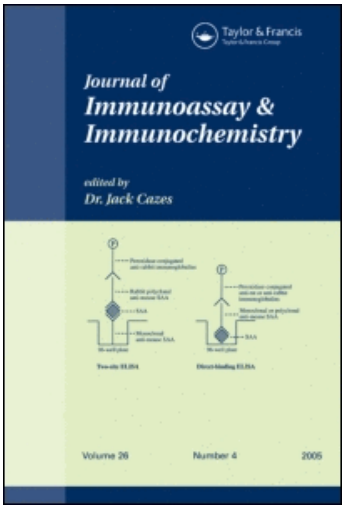

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Krakauer, Teresa(1996) 'Detection of Adhesion of Superantigen-Activated T Lymphocytes to Human Endothelial Cells by Elisa', Journal of Immunoassay and Immunochemistry, 17: 1, 1 — 12
To link to this Article: DOI: 10.1080/01971529608005775
URL: http://dx.doi.org/10.1080/01971529608005775

JOURNAL OF IMMUNOASSAY, 17(1), 1-12 (1996)

DETECTION OF ADHESION OF SUPERANTIGEN-ACTIVATED T LYMPHOCYTES TO HUMAN ENDOTHELIAL CELLS BY ELISA

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ABSTRACT

A sensitive ELISA using monoclonal antibodies (mAb) reactive with surface molecules specific for various leukocytes was devised to measure the adhesion of these cells to cultured monolayers of human umbilical vein endothelial cells. Superantigens, staphylococcal enterotoxin B or toxic shock syndrome toxin 1 were used to activate human peripheral blood mononuclear cells. The extent of adhesion of these cells to endothelial cells was assayed by measuring the optical density produced by a complex of peroxidase-labeled streptavidin, biotin-conjugated $F(ab')_2$ antimouse Ig and monoclonal antibody specific for leukocytes on fixed leukocytic cells that had adhered to endothelial cells. This method was fast and sensitive, and because the detection is by a specific marker on the cell of interest, it can be used in preparations of unseparated mixtures of cells. An increase in adhesion of superantigen-activated $CD4^+$ and $CD8^+$ T lymphocytes to endothelial cells may contribute to the pathologic mechanism of superantigens.

(KEY WORDS: Leukocyte adhesion, endothelial cell, ELISA, superantigen)

INTRODUCTION

The migration of leukocytes from blood to tissue is one of the most important cellular responses to tissue injury and infection. Adhesion of leukocytes to endothelial cells (EC) precedes extravasation and is a critical step in the establishment of an inflammatory response. Numerous studies indicate that cell-

surface molecules on both the leukocytes and endothelial cells mediate specific cell adhesion (as reviewed in 1-3). Thus the binding of ß2 integrins on leukocytes to ICAM-1 on EC contributes significantly to the adhesion of lymphocytes to EC. Endothelial VCAM-1 supports the adhesion of lymphocytes and monocytes through the interaction with the integrin VLA4 on these cells (4). Increased adhesion occurs through quantitative changes in the expression of adhesion molecules as well as the activation of these molecules to a higher affinity state for their ligands (1-3). In addition, the expression of adhesion molecules (ICAM, ELAM and VCAM) on EC are upregulated by the cytokines, IL-1 and TNFα (1,2).

The superantigens, staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin 1 (TSST-1), bind to MHC class II on antigen-presenting cells and stimulate T cells via specific Vß region of the T cell receptor (5). These bacterial proteins are potent stimulators of T cells and their toxicity is mediated by the excessive production of proinflammatory cytokines (6-8). In this report, a sensitive ELISA was used to study the changes in adhesion of superantigen-activated human peripheral blood T lymphocytes to EC.

MATERIALS AND METHODS

Monoclonal antibodies and other reagents

Monoclonal anti-CD4 and anti-CD8 were obtained from AMAC (Camarillo, CA). OKM1 was kindly provided by Dr. John Ortaldo (Laboratory of Experimental Immunology, BRMP, National Cancer Institute, Frederick, MD). Human rIL-1ß was a gift from the Biological Response Modifier Program, National Cancer Institute.

Anti-IL-1ß antibodies were obtained from Collaborative Research (Bedford, MA). Human rTNFα, anti-TNFα, goat anti-mouse IgG, and biotin-conjugated $F(ab')_2$ anti-mouse IgG were obtained from Boehringer-Mannheim (Indianapolis, IN). Peroxidase-labeled streptavidin was obtained from KPI (Gaithersburg, MD). SEB and TSST-1 were obtained from Toxin Technology (Sarasota, FL).

Cell preparations

Endothelial cells were obtained from Clonetics (Palo Alto, CA) and cultured with RPMI containing penicillin (100 U/ml), streptomycin (100 µg/ml), 10% heat-inactivated fetal calf serum (FCS), heparin (5 U/ml) and EC growth supplement (50 µg/ml) in tissue culture flasks until they reached confluency. They were then trypsinized (0.05% trypsin and 0.025% EDTA) and plated in 96-well microtiter trays for adhesion assays.

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood from normal donors by density gradient centrifugation over Ficoll-Hypaque. Activated T cells were obtained by treating PBMC (3×10^6 cell/ml) with either SEB or TSST-1 (100 ng/ml) in 50 ml Falcon polypropylene tubes for various times as indicated in each experiment, washed once, and resuspended to 6×10^6 cells/ml before use.

Adhesion assay

EC at $1.2\text{-}2 \times 10^5$ cells/ml were cultured in 96-well flat-bottom plates (0.1 ml/well) for 1 to 2 days. Cells were then used directly for adhesion or in some experiments, treated with 1 ng/ml of IL-1 for 5 h. Each test was performed in triplicate. Medium was then removed, and 0.1 ml/well of PBMC (6×10^6 cells/ml) were added in RPMI with 10% FCS. Cells were

allowed to adhere at 37°C for 30 to 40 min in a 5% CO_2 incubator. Nonadherent cells were removed by gentle aspiration. Plates were washed three times with PBS with 10% FCS at room temperature. After the adhesion step, cells were fixed with cold ethanol and rinsed once with wash buffer (PBS with 0.05% Tween 20). At this point, the plates can be stored semi-dry in a plastic bag with a seal up to 4 months or can be used immediately. Microscopic examination showed that cells were attached individually and not by self-aggregation. The number of adherent cells was estimated by microscopic examination of three random fields in each well and these numbers correlated well with subsequent measurements by ELISA.

Measurement of adherent cells by ELISA

A sensitive ELISA with mAb reactive with surface molecules specific for various leukocytic cells was used to measure the adherence of these cells to cultured monolayers of EC, as previously described (9). The primary mAb used to detect the adherent cells depended on what cells were used or, when a mixed population of cells was used, what cell type was to be assayed. Multiple cell types can be examined by using different detecting mAb. OKM1, anti-CD4 and anti-CD8 were used to measure the adhesion of monocytes, $CD4^+$ T cells and $CD8^+$ T cells, respectively. The concentration of mAb used was determined empirically to achieve a low background (in the absence of the adherent cells) but high absorbance for the adherent cells. This primary mAb (50 μl/well) in ELISA buffer (PBS with 10% FCS, 0.05% Tween 20 with 100 μg/ml of human IgG) was added to plates containing fixed cells (adherent cells and EC) for 40 min at 37°C. Plates were washed three times with wash buffer and

biotin-conjugated $F(ab')_2$ anti-mouse Ig (100 μl/well of 1:8000) in ELISA buffer was then added. Plates were incubated for 30 min at 37°C and then washed four times. Peroxidase-labeled streptavidin (100 μl/well of 1:500) in ELISA buffer was added. Plates were incubated for 45 min at 37°C and washed four times. Bound enzyme was detected by adding 100 μl/well of substrate consisting of a 1:1 solution of TMB (3, 3', 5, 5'-tetramethyl-benzidine) and hydrogen peroxide (0.02%). OD at 630 nm was read by a ELISA reader (Dynatech Microplate Reader, Dynatech, Alexandria, VA). Appropriate controls were included in each assay. Control background OD of cells treated only with biotin-labeled $F(ab')_2$, peroxidase-labeled streptavidin and substrate was subtracted from OD readings when all reagents were present.

Detection of cytokines

TNFα and IL-1ß were measured by sandwich ELISA using cytokine specific antibodies as previously described (7). The detection limit for each cytokine was 20 pg/ml.

Statistics

Data are presented as the mean ± SEM. Significant difference was determined by the Student's t-test with Stata (Stata Corp, College Station, TX).

RESULTS

Adhesion of SEB- and TSST-activated PBMC to EC

Preliminary experiments were performed to establish the optimal concentrations of SEB and TSST for activating PBMC. Activation of PBMC by SEB or TSST did not alter the expression Of

the cell-surface markers, CD4, CD8 or OKM1. A previous report detailed the validity of the ELISA method with the conventional Cr^{51} adhesion assay (10). Figure 1 shows the adhesion of different cell types in PBMC after treatment with 100 ng/ml of SEB for various time. Three hours after treatment with SEB, the adhesion of $CD4^+$, $CD8^+$ cells and monocytes (detected with OKM1) remained unchanged as compared to untreated PBMC. A statistically significant increase in adhesion of both $CD4^+$ and $CD8^+$ T lymphocytes were observed 20 hours after SEB treatment. The level of adhesion at 44 hours was similar to that at 20 hours. Microscopic examination indicated that treatment with SEB for 20 and 44 hours increased adhesion of PBMC from 5% (untreated) to 50%. The adhesion of monocytes to EC did not change with SEB activation.

Figure 2 illustrates the adhesion pattern of $CD4^+$, $CD8^+$ cells and monocytes upon TSST activation. Results were similar to that obtained with SEB. Increased adhesion of both $CD4^+$ and $CD8^+$ cells were seen at 20 and 44 hours after TSST treatment. Visual examination showed increased adhesion from 5% to 40% after TSST activation.

Adhesion of SEB-activated PBMC to IL-1 activated EC

IL-1, a proinflammatory cytokine known to increase adhesion of leukocytes to EC, was added to EC, and the adhesion of SEB-stimulated PBMC to IL-1-activated EC was next examined. A further small increase in adhesion to IL-1-activated EC was observed with SEB-treated $CD4^+$ and $CD8^+$ cells (Figure 3). Similar results were obtained when TSST was used to activate PBMC (data not shown).

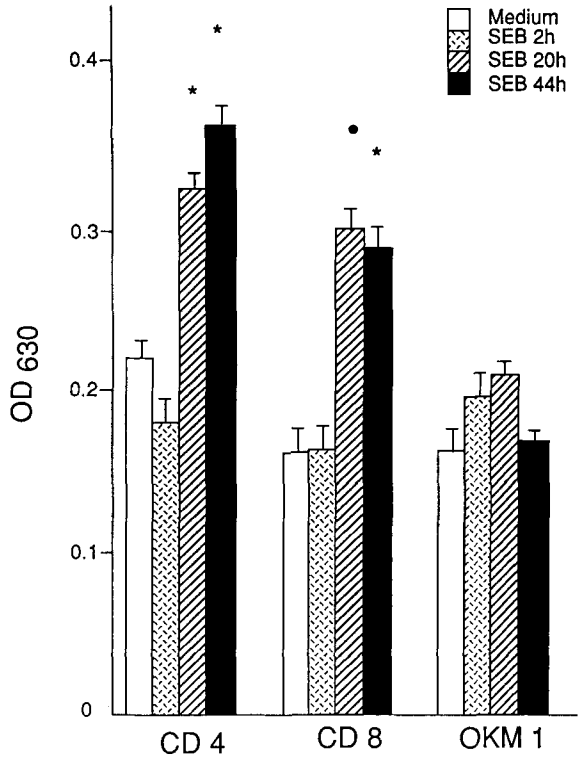

FIGURE 1. Effect of varying time of SEB treatment of human PBMC on cell adhesion to EC. PBMC were treated with 100 ng/ml of SEB for different time periods (3 h, 20 h, 44 h). Adhesion of control PBMC (treated with medium) and SEB-activated PBMC was measured by ELISA by using anti-CD4 (diluted 20-fold), anti-CD8 (diluted 20-fold) and OKM1 (diluted 1 to 400). * Denotes a statistical difference ($p < 0.05$) between control and SEB-treated groups. The data show mean ± SEM of triplicates. Results represent three experiments.

Production of cytokines by SEB- or TSST-activated PBMC

The production of proinflammatory cytokines by PBMC upon stimulation with SEB or TSST was next examined. Abundant levels of TNFα (1600 pg/ml with SEB, 1200 pg/ml with TSST) and IL-1ß (700 pg/ml with SEB, 400 pg/ml with TSST) were produced by PBMC upon stimulation with SEB or TSST.

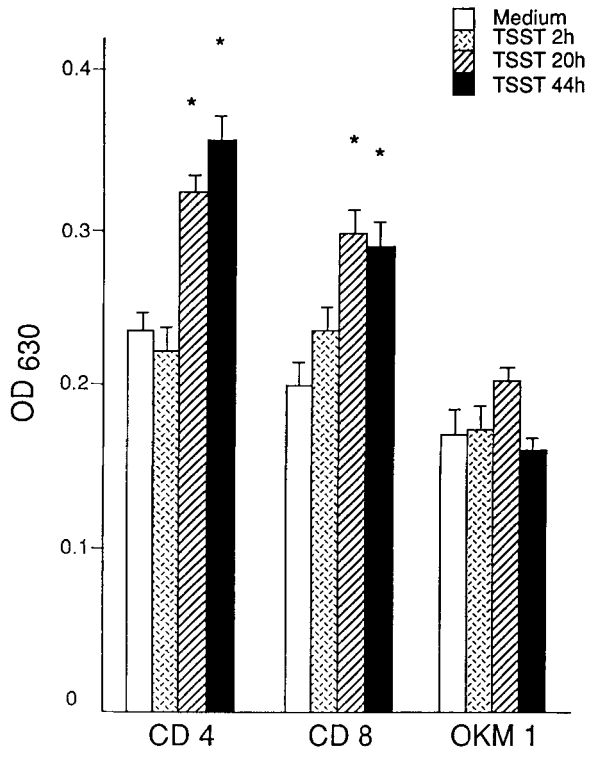

FIGURE 2. Effect of varying time of TSST treatment of human PBMC on cell adhesion o EC. PBMC were treated with 200 ng/ml of TSST for different time periods (2 h, 20h, 44 h). Adhesion of control PBMC (treated with medium) and TSST-treated PBMC was measured as in figure 1. * Denotes $p < 0.05$ between control and TSST-treated samples. The data show mean ± SEM of triplicates and represent three experiments.

DISCUSSION

A simple, sensitive method to measure leukocyte adhesion to EC is described here. Monoclonal antibodies to a specific cell-surface marker on the adhered cell were used to tag the cell. Biotin-conjugated $F(ab')_2$ anti-mouse Ig and peroxidase-labeled streptavidin were used to enhance the sensitivity and specificity

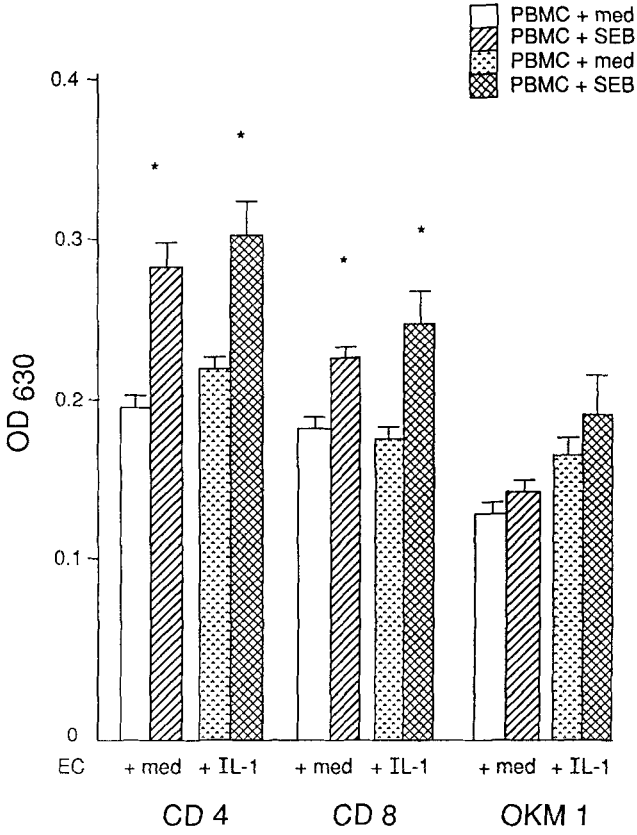

FIGURE 3. Adhesion of PBMC to untreated and IL-1-activated EC. EC was treated with 1 ng/ml IL-1 for 5 h. PBMC was treated with medium (control) or SEB for 20 h. Adhesion assay was performed as in figure 1. * Denotes a statistical difference ($p < 0.05$) between treated and corresponding untreated control groups. The data show mean ± SEM. Results represent three experiments.

of this assay. This method was applied to the study of superantigen-activated PBMC.

This report shows for the first time, that SEB- or TSST-activated T cells bind EC better than normal, unstimulated T cells. Adhesion of both $CD4^+$ and $CD8^+$ T lypmhocytes to EC were increased by superantigen activation of T cells, whereas monocyte

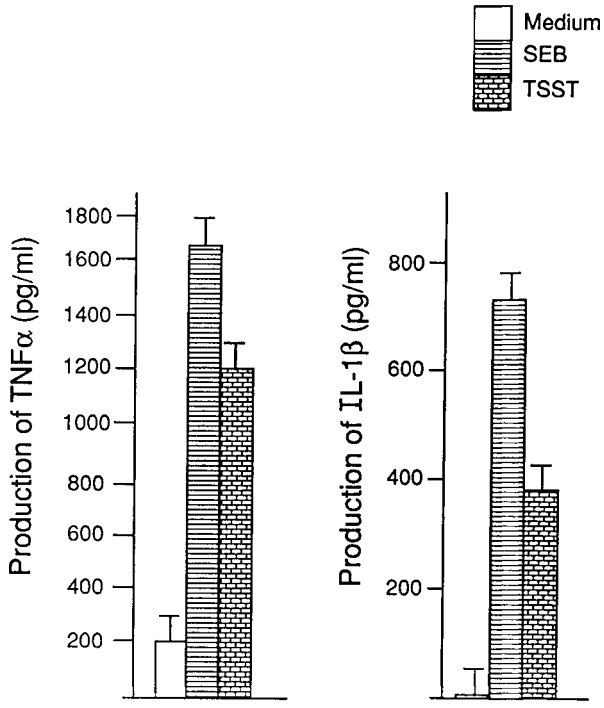

FIGURE 4. Production of TNFα and IL-1ß by SEB and TSST stimulated PBMC. PBMC were treated with SEB or TSST for 20 h. Supernatants were then assayed for TNFα and IL-1ß by ELISA. Data show mean ± SEM of duplicates in ELISA and represent levels of cytokine from three donors.

adhesion was not affected. Adding the proinflammatory cytokine, IL-1, to EC increased the adhesion further. It is well documented that IL-1 induces the expression of adhesion molecules, ICAM-1, ELAM, and VCAM-1 on EC (1-3). Both ICAM-1 and VCAM-1 contribute to the adhesion of monocytes and lymphocytes through their interaction with leukocyte cell surface molecules, CD11/CD18 complex, and VLA4. The mechanism of increased T cell adhesivity by SEB is presently unknown. Because integrin activation was achieved through engagement of TCR (1,3), it is

likely that superantigens generated intracellular signals that led to activation of cell-surface molecules to a higher affinity state as well. An increase in the number of adhesion receptors on T lymphocytes could also account for the enchancement of attachment of these cells to EC. Preliminary investigations showed increased expression of VLA4 on SEB- and TSST-activated PBMC.

The data presented here demonstrate clearly that superantigens treatment increased the adhesivity of T lymphocyte to EC. Proinflammatory cytokine production by PBMC after superantigen activation could increase the adhesivity of EC for lymphocytes further. This might partly be responsible for toxic shock induced by these bacterial proteins.

ACKNOWLEDGEMENTS

I thank Dr. John Ortaldo for OKM1 mAb, Marilyn Buckley for technical assistance, Katheryn Kenyon for editorial assistance, and David Elia for preparation of graphs.

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