

**Short communication**

**TEST OF ENDOTHELIAL MONOLAYER INTEGRITY IN THE PRESENCE OR ABSENCE OF ENDOTOXIN AND ANTI-TNF- $\alpha$  MONOCLONAL ANTIBODIES**

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**ABSTRACT:** Monolayer cultures of human umbilical vein endothelial cells were established and tested for the effects of endotoxin treatment and anti-TNF- $\alpha$  monoclonal antibodies following endotoxin treatment on albumin permeability and electrical conductance. Application of 40  $\mu\text{g}(\text{ml})^{-1}$  of endotoxin on human umbilical cord endothelial cell culture monolayer increased transendothelial albumin permeability significantly ( $P < 0.001$ ) and decreased transendothelial electrical resistances significantly ( $P < 0.001$ ). When 10  $\mu\text{g}(\text{ml})^{-1}$  of anti-TNF- $\alpha$  monoclonal antibodies was added after endotoxin, the effects of the latter on albumin permeability and electrical resistance were significantly decreased ( $P < 0.001$ ). This suggested that the monolayer sheet can be disturbed by endotoxin-induced TNF- $\alpha$  from endothelial cells. TNF- $\alpha$  can disintegrate the endothelial cell monolayer during septicemia. This can be abrogated using anti-TNF- $\alpha$  monoclonal antibodies.

**Key words/phrases:** Anti-TNF- $\alpha$  monoclonal antibodies, electrical resistance, endothelial monolayer, endotoxin, permeability

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## INTRODUCTION

Endothelial cells are found to be physiologically and metabolically dynamic, capable of performing a number of important functions in the body such as mediation of the regulation of blood pressure, and control of blood coagulation. In order to study them in detail, it is possible to isolate the cells from blood vessels using digestive enzymes such as collagen and trypsin, and grow them in culture flasks using appropriate media containing antibiotics. Endothelial cells grown on culture as a continuous sheet-like monolayer can be used as model for capillaries.

Endothelium is a single layer of epithelial cells that lines the inner wall of blood vessels. In the past, endothelial cells were perceived as passive, metabolically inert and permeable barriers. However, they are now known to be metabolically and physiologically dynamic having multiple functions (Curzen *et al.*, 1994).

In order to maintain the potency of blood vessels and the fluidity of the circulating blood, endothelial cells synthesize and release a number of anticoagulants including thrombomodulin, heparin sulphate and prostacyclin (Danner *et al.*, 1991). Endothelial cells can produce pre-coagulants such as thrombin, factor-II, factor-III, factor-V, platelet activating factor, and von Willebrand factor (Figer *et al.*, 1966). Endothelial cells produce substances that can regulate vascular tone such as endothelium derived relaxing factor, endothelin, and angiotensin converting enzyme (Cruzen *et al.*, 1994).

Endothelium plays an integral role on the acute inflammatory response by producing certain inflammatory mediators such as histamine, serotonin, bradykinin, and prostaglandins (Parrillo, 1993). Endothelium can also express cell surface adhesive substances called selectins (glycoproteins) which can selectively adhere with integrin of neutrophils during transendothelial migration (Hirokawa *et al.*, 1994).

Abnormalities of endothelial cell structure and function contribute to the development of such diseases as thrombo-embolism, atherosclerosis, and vasculitides (Castillo and Sanchez, 1993; Bone, 1994).

In order to investigate all these functions of endothelial cells and pathological conditions associated with them, researchers have developed techniques of isolation, purification and culturing of endothelial cells. Endothelial cells can be isolated from pulmonary arteries, aorta, coronary arteries, renal vessels and umbilical vein during delivery (Figer *et al.*, 1966; Booyse *et al.*, 1975). Once isolated, endothelial cells can be cultured on a culture media plated on a membrane support to form a confluent monolayer that represents an *in vitro* capillary model (Booyse *et al.*, 1975; Chuang *et al.*, 1990). This enables one to measure an *in vitro* transendothelial migration of neutrophils, and permeability for macromolecules such as plasma proteins. All measurements of transendothelial electrical resistance are means of testing endothelium monolayer integrity (Baetscher and Brune, 1983; Chuang *et al.*, 1990).

Endotoxin, a lipo-polysaccharide (LPS) in its chemical nature, is a cell wall component of Gram-negative bacteria. It can stimulate macrophages, neutrophils and endothelial cells to produce such cytokines as TNF- $\alpha$  and II-1. These cytokines are chemo-attractants for neutrophils during inflammation and they are also known to be central mediators of septic shock (Tracey and Fong, 1987). Endothelial cells, in response to endotoxin, TNF- $\alpha$  and II-1, are well known to produce large amount of endothelium derived relaxing factors (EDRF). EDRF is now known to be nitric oxide (Hirokawa *et al.*, 1994). Nitric Oxide is a potent vasodilator biological molecule and it is suspected to be the ultimate mediator of septic shock (Moncada *et al.*, 1991; Castillo and Sanchez, 1993; Bone, 1994). During septic shock, the capillary endothelial permeability is pronounced resulting in hypotension secondary to fluid extravasation (Baetscher and Brune, 1983). Researchers suggested that all those cascades of events for the genesis of sepsis can be abrogated if anti-TNF- $\alpha$  monoclonal antibodies are applied on *in vitro* model (Tracey and Fong, 1987; Lowery, 1993).

The objective of the present work is to test the hypotheses that:

1. endotoxin increases albumin passage through the endothelial monolayer;
2. endotoxin decreases transendothelial electrical resistance; and
3. anti TNF- $\alpha$  monoclonal antibodies decrease or revers the above two effects of endotoxin.

## MATERIALS AND METHODS

Human umbilical vein endothelial cells were isolated with the help of trypsin enzymatic digestion of the intima. The endothelial cells were grown in a culture flask following the standard procedures adapted from Maryama (1963). The culture media was consisting of a 1:1 mixture of M-199 & RPM 1640 (containing L-glutamine, GIBCO) supplemented with 20% heat inactivated fetal calf serum (GIBCO). After the initial culturing of the endothelial cells in a monolayer, the culture was detached using trypsin (0.25%) in order to transfer them to another culture plate (Fig. 1a) in serial passages. The trypsinized endothelial cells in suspension were seeded over polycarbonate filter membrane support and kept in the incubator. After six days, the formation of endothelial monolayer was examined under high power objective microscope to check if it was ready for experimentation.

### *Test of endothelial monolayer permeability for albumin*

To evaluate the functional integrity of human umbilical vein endothelial cell monolayer, 30  $\mu\text{l}$  of  $^{125}\text{I}$  labelled human serum albumin in 10% fetal calf serum was added to 100  $\mu\text{l}$  medium in the upper compartment (Fig. 1a). Thirty minutes later, a 30  $\mu\text{l}$  sample was taken from the lower chamber beneath the filter for  $^{125}\text{I}$  labelled albumin determination using LKB-1240 Gamma counter. The ratio of the total count added to the upper chamber to the total count in the lower chamber is indicative of the percent albumin diffusion. This was calculated using the following formula:

$$\% \text{Diffusion} = \frac{\text{Total count in the lower chamber}}{\text{Total count added on the upper chamber}} \times 100$$

Measurements were made four times at the interval of 30 minutes. Transendothelial albumin permeability was challenged by adding 40  $\mu\text{g}(\text{ml})^{-1}$  endotoxin alone and endotoxin with 10  $\mu\text{l}(\text{ml})^{-1}$  anti-TNF- $\alpha$  monoclonal antibodies after endotoxin.

### *Transendothelial electrical resistance measurement*

Transendothelial monolayer electrical resistance was measured with a volt metre using two miniature AgCl electrodes. One electrode was placed in the upper chamber (Fig. 1b). Readings in ohms were taken directly from the ohmmeter.

The known resistance of the filter membrane support was subtracted from the total resistance in order to determine the resistance of the endothelial cell monolayer. Resistance measurements were done before and after the addition of endotoxin alone as well as after the addition of endotoxin plus anti-TNF- $\alpha$  monoclonal antibodies.

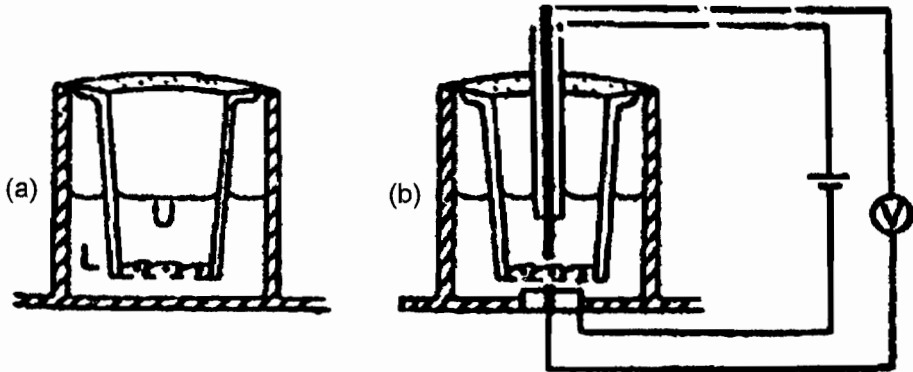


Fig. 1. Schematic view of the assay system for measuring the permeability (a) and transendothelial electrical resistance (b) of monolayers of human umbilical vein endothelial cell culture (L, lower chamber; U, upper chamber).

## RESULTS AND DISCUSSION

Results of transendothelial permeability for albumin and transendothelial electrical resistance were recorded from ten repeated measurements and presented in graph forms as shown in Figs 2 and 3, respectively.

Following controlled measurement, it was found that endotoxin (LPS) increased significantly ( $P < 0.001$ ) albumin passage through the monolayer. Endotoxin stimulates endothelial cells and macrophages to present TNF- $\alpha$ , which in turn disrupts monolayer integrity, increasing its leakiness, leading to hypotension. The above finding is supported by Furie *et al.* (1987) suggesting that during bacterial infection of the human body, both trans-capillary neutrophil migration and leakiness to plasma proteins will be increased secondary to this fluid extravasation, as a consequence of which circulatory shock will develop.

As shown in Fig. 2, the effect of endotoxin (LPS) was antagonized by anti-TNF- $\alpha$  monoclonal antibodies. A ten  $\mu\text{l}(\text{ml})^{-1}$  anti-TNF-monoclonal antibodies decreased albumin flux significantly ( $P < 0.001$ ).

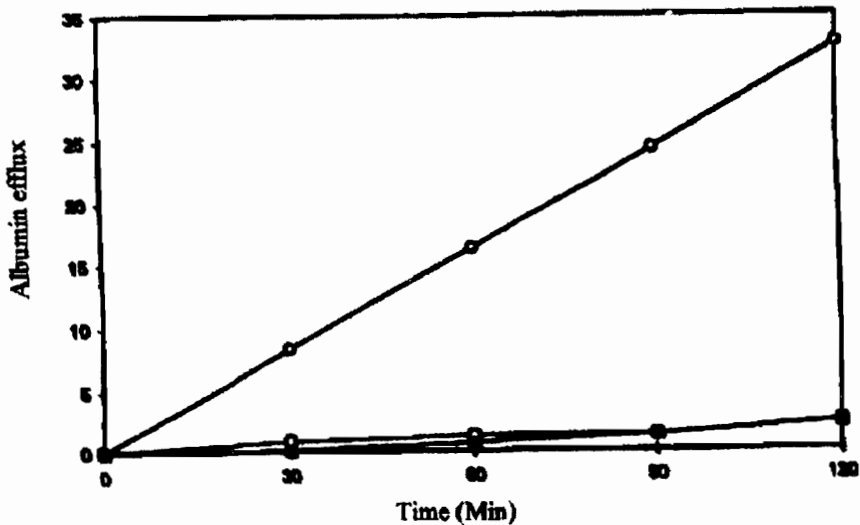


Fig 2. **Albumin permeability of endothelial cell culture.** The time source change in albumin permeability of monolayer culture of endothelial cells following the application of endotoxin (LPS, 40  $\mu\text{l}$ ) alone (—○—), endotoxin plus anti-TNF- $\alpha$  monoclonal antibodies (—□—) and control values recorded in the absence of test chemicals (—△—).

Transendothelial electrical resistance measured before and after the endothelial cell monolayer was treated with LPS [40  $\mu\text{l}(\text{ml})^{-1}$ ] decreased apparently because LPS disintegrated the confluent endothelial monolayer of the culture (Fig. 3). This effect is significantly reversed by treatment with anti-TNF- $\alpha$  monoclonal antibodies.

In conclusion, endotoxin increases albumin permeability of cultured endothelial cell monolayer. This is because LPS and cytokines such as TNF- $\alpha$  and interleukin alter endothelial cell permeability. Anti-TNF- $\alpha$  monoclonal antibodies reverse the endotoxin-mediated increase in endothelial permeability and decrease in electrical resistance, suggesting that endothelial cell monolayer functional integrity alteration is mediated via TNF- $\alpha$ .

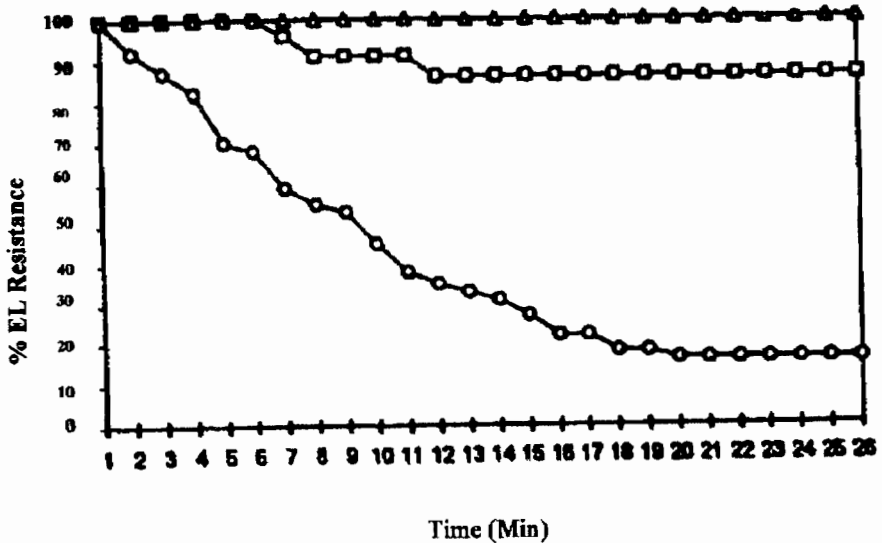


Fig. 3. Electrical resistance of endothelial cell culture. The time course change in electrical resistance of monolayer culture of endothelial cells following application of endotoxin (LPS, 40 μl) alone (—○—), endotoxin plus anti-TNF-α monoclonal antibodies (—□—) and control values recorded with isotonic saline (—△—).

ACKNOWLEDGEMENTS

This experiment was done in Sweden, Karolinska Institute, Department of Physiology. We thank all people working in the cell culture lab who helped us carry out this experiment.

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