THE CENTRAL AFRICAN JOURNAL OF MEDICINE

Vol. 47, No. 7 **CONTENTS** July 2001

Model for assessment of endothelial cell function and viability using the MTT dye test and [3H]

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Abstract

Objectives: To establish a reproducible *in vitro* model for evaluating endothelial cell function in clinical disease states.

Design: A prospective study of human umbilical vein endothelial cells (HUVEC) isolated and cultured.

Setting: Department of Haematology, University of Yaounde I, Cameroon and Department of Medicine University of Newcastle-upon-Tyne, England.

Results: The optimum initial cell seeding concentration, for maximal conversion of the formazan dye was $40x10^3$ cells/well, after 24 hours incubation. At concentrations above $40x103$ cells/well some inhibition of dye conversion occurred. The conversion of formazan dye was directly proportional to cell numbers for the first 48 hours only, at all cell concentrations. Thereafter, cell metabolism appeared to be inhibited.

Third passage endothelial cells (ECs) were exposed to a range of lipopolysaccharide (LPS) concentrations for one and 24 hours, prior to performing the MTT dye test. Dye conversion was observed after one hour at even the lowest concentration of LPS ($0.1\mu g/ml$), to $49,9\% \pm 5.6\%$ of unperturbed control EC, with $10x10^3$ initial seeding numbers. After 24 hours perturbation a small but statistically significant further inhibition was observed.

[3H] thymidine incorporation studies indicated that the lowest LPS concentration tested ($0.1\mu g/ml$) had a stimulatory effect on DNA synthesis at the higher cell concentration ($20x10^3$ cells/well). In the range of 1 to 100 $\mu g/ml$ of the LPS tested, there was increased DNA synthesis at all cell numbers.

Conclusion: The model may be used to monitor the effects of other agents which are known to, or could be associated with, alterations in endothelial cell function and will serve in mimicking clinical situations including hyper coagulable states.

Introduction

A highly specialised cell layer made of vascular endothelial cell (EC) lines all blood vessels and lymphatics.[1] The function of this cell layer appears to vary according to anatomical site and size of the vessel.[2] However, the basic function of supporting either anticoagulant or procoagulant activities, depending on the local conditions and circumstances, are essentially similar. Any disruption of the equilibrium that exists between the endothelial cell and the circulating blood may result in clinically significant thrombosis or haemorrhage.[3,4] Such pathophysiological insults include bacterial lipopolysaccharide (LPS) which may induce endotoxic septicaemia associated with disseminated intravascular coagulation,[5] as well as the infusion of a variety of drugs into the vascular component.[6]

To investigate the effect of such agents and toxins on the endothelial cells, it is necessary to monitor metabolic and functional aspects of the cells' behaviour. This may provide an *in vitro* model for studying clinical conditions.

The conversion of 3-(4,5-dimethylthiaxol-2-yl]-2,5 diphenyl tetrazolium bromide, or thiazol blue (MTT) to a stable formazan product, affected by mitochondrial dehydrogenases of cells may form a useful basis for such a model. The integrity of this enzyme system has been taken to represent cell viability[7,8] thus; this test was adapted for monitoring the viability of vascular endothelial cell monolayers in culture. Because proliferating cells have the ability to incorporate radionucleotides in their nuclei, this may be used as a method of monitoring ES growth and regeneration.[9] Growth and DNA synthesis of EC were thus monitored in this study, after exposure to insults using

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bacterial LPS, using [³H] tritiated thymidine incorporation studies.

Materials and Methods

Culture Technique.

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described by Jaffe et al.,[8] with minor modifications. Umbilical cords were obtained immediately following delivery and stored in sterile phosphate buffered saline (PBS) at 18°C. The EC were released from the umbilical vein using collagenase (Worthington Biochemical Corporation), at a final concentration of 162 units/ml in PBS at 37°C for 10 minutes. Released cells were pelleted by centrifugation at 150g for five minutes at 18°C. The EC were resuspended in culture medium (TC199) containing (final concentrations) 20% foetal calf serum; penicillin and streptomycin (50 units/ml B 50 µg/ml); amphotericin B (2,5 µg/ml); L-Glutamine (2mM); and Endothelial Cell Growth Supplement (respectively from Sigma Chemical Co. Ltd, Dorset, UK). All other culture reagents were obtained from Flow Laboratories (Rickmansworth, Herts.UK). Cells were grown to confluence in 25 cm2 flasks (Sterilin, Middlesex, UK) and incubated at 37°C in an atmosphere of 5% carbon dioxide. Subsequently, adherent EC were harvested by trypsin and EDTA (0,05% to 0.02%, w/v) digestion and either sub-cultured in similar containers or (after the second passage) counted and seeded into 96-well microtitre plates (Sterilin, UK). Experiments were performed on a range of cell numbers (per well) and culture times.

MTT Assay.

After perturbation, cell monolayers were prepared by washing twice with PBS followed by the addition of fresh culture medium (200 µl/well). The MTT dye substrate in distilled buffer water (50 µl of a 2mg/ml solution), was added to each well (including an empty blank) and the microtitre plate re-incubated for four hours. Culture medium + excess MTT were aspirated from each well and discarded. This was replaced by 100 µl of di-methyl sulfoxide — DMSO (Sigma), and the plates gently agitated for two minutes to allow complete dissolution of the formazan crystals. Optical density was then determined at 540 nm (Titertek Multiskan MCC/340). All experiments were performed in triplicate (three wells for each variable) on at least two separate occasions. The means of each set of three wells retained for the final calculations.

[³H] Thymidine study.

Freshly harvested EC were sub-cultured into microtitre plates at various cell concentrations as described above. After six hours of adherence/growth, EC were exposed to various concentrations of bacterial LPS from Escherichi coli (E.coli), in triplicate wells, excluding three control wells (cells and TC199 only) and three blank wells. At timed intervals, cells were washed twice in warm PBS and the wells refilled with 200 µl of fresh culture medium containing 0.2µCi of [³H] thymidine (Amersham

International PLC, Buckinghamshire, England). Following incubation for another six hours cells were harvested using a cell harvester. This process resulted in EC lysis and washing off of the cell membranes, leaving only the nuclei (DNA and [³H] thymidine) on glass fibre paper. After drying, each sample was collected into plastic tubes and dissolved with 1ml of Scintillation fluid (LKB,UK) and the disintegration per minute counted by an LKB 1218 Rackbeta liquid Scintillation Counter.

Means ± 1 standard deviations were used to express results. Correlation analysis and Student's tests were used for statistical analyses; p values < 0.05 were considered significant.

Results

Determination of the Optimum Initial Cell Seeding Concentration.

The optimum initial cell seeding concentration, for maximal conversion of the formazan dye was 40 x 10³ cells/well, after 24 hours incubation (Figure I). At this cell concentration an optical density (OD) of 0.75 ± 0.03 was recorded. Below this cell concentration, in the range 10 to 40 x 103 cells/well, dye conversion appeared to be directly proportional to initial cell numbers (r = 0.997, Figure I). At concentrations above 40 x 103 cells/well, however, some inhibition of dye conversion occurred.

Figure I : Effect of initial cell seeding numbers on MTT conversion after 24 hours culture.
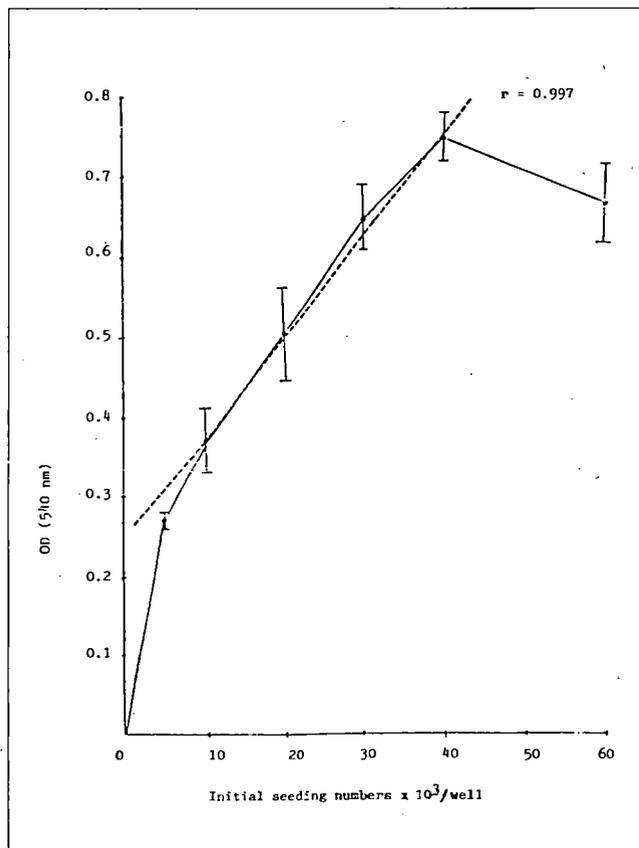
Determination of the Optimum Duration of Culture.

The MTT assay was performed on EC after culture for variable periods (one to four days) and with a range of initial cell seeding numbers per well (5 to 40 x 10^3 cells/well). The conversion of the formazan dye was directly proportional to cell numbers for the first 48 hours only, at all cell concentrations tested. Thereafter, cell metabolism appeared to be inhibited (Figure II). In the range 10 to 40 x10^3 cells, initial seeding concentration over the first 48 hours and metabolic activity was linearly related (r=0.997).

*Figure II: Effect of culture duration on MTT conversion (cell numbers/well x10^3).*
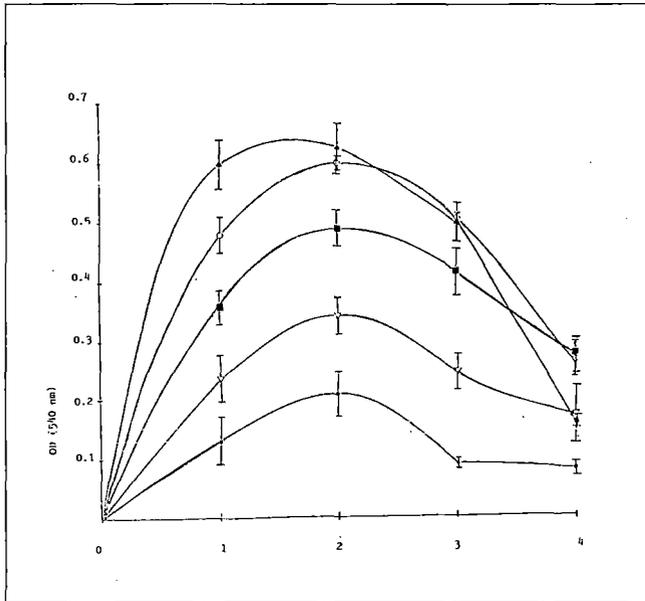
[●] = 5; [▽] 10; [■] = 20; [○] = 30; [▲] = 40.

Parameters were defined for subsequent experiments based on the above data: cell seeding concentrations of 10 and 20 x 10^3 EC/well, and optimum culture times of one and 24 hours. Under these conditions the assay system could be used to detect both inhibition and augmentation of metabolic activity.

## Determination of Standard LPS Perturbation.

Using the above parameters, third passage EC's were exposed to a range of LPS concentrations for one and 24 hours, prior to performing the MTT dye test. Dye conversion was observed after one hour at even the lowest concentration of LPS (0.1µg/ml), to 49,9% ± 5.6% of unperturbed control EC, with 10 x10^3 initial seeding numbers. Interestingly, a similar degree of inhibition was observed with the same dose of LPS, but twice the initial seeding numbers (20 x 10^3): to 47.9% ± 5.0% (of unperturbed controls), and only a modest further inhibition occurred at higher concentrations of LPS. However, after 24 hours perturbation a small but statistically significant further inhibition was observed. This was to 36.7% ± 7.3% with 0.1µg LPS/ml and 10 x10^3 initial cell seeding number; and to 26.2% ± 6.9% with the same concentration of LPS and 20 x10^3 cells (p<0.02 at all LPS concentrations) — Figure III.

Figure III(a): Effects of LPS on EC after one hour and 24 hours exposure.

Concentration of LPS µg/ml)
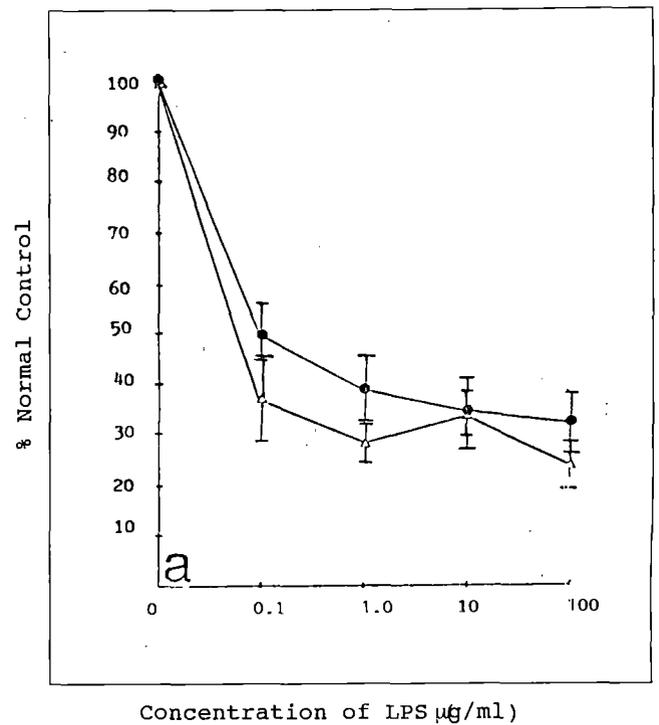
a) = 10 x10^3 cells

Figure III(b): Effects of LPS on EC after one hour and 24 hours exposure.

Concentration of LPS µg/ml)
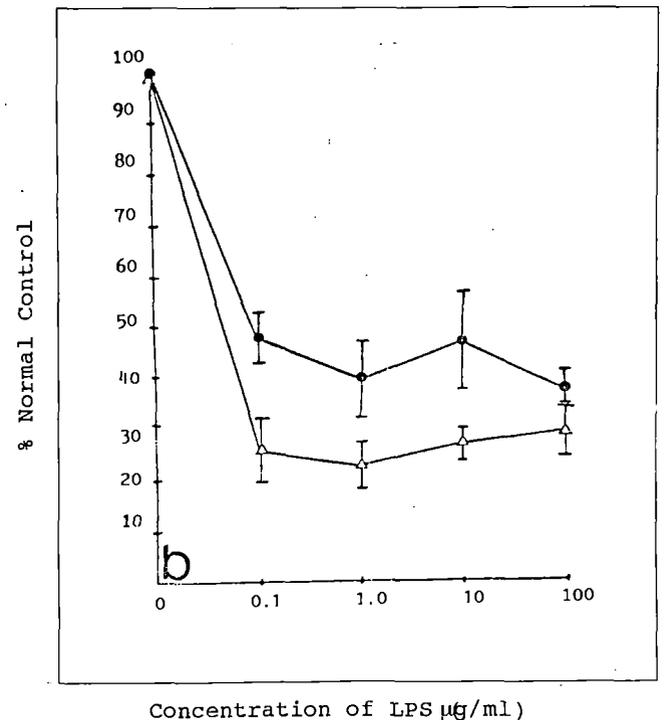
*b) = 20 x 103 cells/well.*

[³H] thymidine incorporation studies indicated that the lowest LPS concentration tested (0.1 µg/ml had a stimulatory effect on DNA synthesis at the higher cell concentration (20 x 10^3 cells/well). This was not observed

using 10×10^3 cells/well. However, thereafter, in the range of 1 to 100 µg/ml of LPS tested, there was increased DNA synthesis at all cell numbers (Figure IV).

Figure IV: The effect of LPS on the incorporation of [³H] thymidine by HUVEC, expressed as % control counts per minute (cpm).
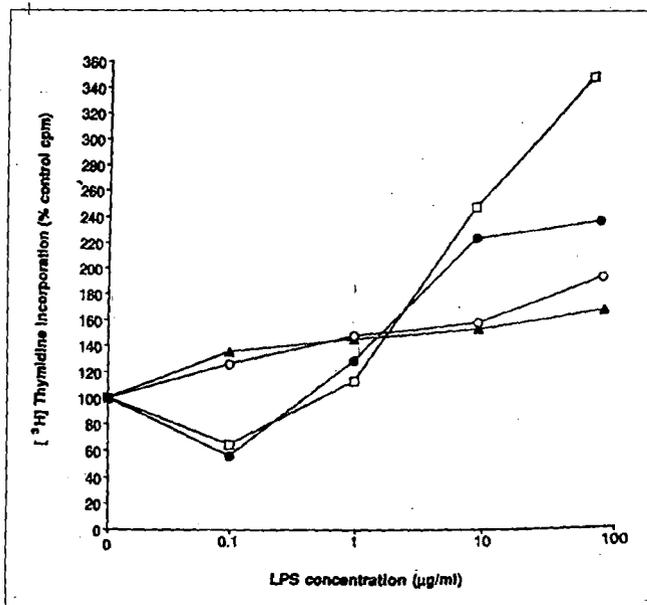
Each point represents the mean of counts from three different wells.
● *=10 x 10³ cells exposed to LPS for one hour; ☐ = 10 x 10³ cells for 24 hours; ○ = 20 x 10³ cells for one hour; and ▲ = 20 x10³ cells for 24 hours.*

Discussion

Endothelial cells possess a heterogeneous group of functions with which they maintain the integrity of the vascular tree. A number of agents, of which the most commonly used in experimental conditions is LPS, derived from the endotoxin of *E. Coli,* are capable of perturbing EC.[9,10] Such perturbation leads to an enhancement of the procoagulant activity of endothelial cells, possibly by modulating normal metabolic pathways.[11]

The MTT test has been shown to be an important method for monitoring variations in cell metabolism.[7] Such methods are required for rapid and accurate screening of clinically used agents/drugs which may influence the metabolic state of a cell. The MTT test is capable of offering a general view of the cells' viability in the presence of such agents, which may have a variety of activities and side effects.

Thus, these preliminary studies to adapt this test for use with EC have shown that MTT dye conversion was directly proportional to initial cell seeding numbers over the range one to 40×10^3 cells/well. Similarly, in time sequence studies, dye conversion was efficient over the 48 hours only. At cell concentrations greater than 40×10^3 cells/well, or with incubation times greater than 48 hours, further MTT dye conversion was inhibited. It is possible that this reflects a culture-overcrowding phenomenon, as

previously described.[12] These data suggest that, within these limits, this assay is both sensitive and reproducible, and may be used to detect both inhibition and augmentation of EC metabolic activity either the normal or perturbed states. Furthermore, the results shown in Figure III demonstrate the profound and reproducible effect of LPS on the cells' metabolism, as reflected by a reduction in MTT conversion after an exposure to as little as 0.1µg/ml of LPS, at cell concentrations of 10 and 20 x 10³ cells/well. Thus, the model may be used to monitor the effects of other agents which are known to, or could be associated with, alterations in the endothelial cell function and will serve in mimicking clinical situations including hypercoagulable states.

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