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BOVINE AORTA ENDOTHELIAL CELL INCUBATION WITH INTERLEUKIN 2: MORPHOLOGICAL CHANGES CORRELATE WITH ENHANCED VASCULAR PERMEABILITY

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ABSTRACT

Interleukin 2 induced alterations in the morphology of bovine aortic endothelial cells in vitro. The changes observed in confluent cultures of bovine aortic endothelial cells included retraction and elongation of cells leading to enlarged gaps between cells quantified by image analysis. Purified IL-2 (1 U/ml medium) increased the gaps between endothelial cells 3-4-fold compared with control cultures. The effect was transient, since the cells reverted to their original morphology 6-12 hours after the removal of IL-2. Correlative scanning electron microscopy (SEM) studies using fresh bovine aorta showed a dose-dependent alteration of the endothelial surface by IL-2 characterized by rounding and elongation of endothelial cells and prominent perinuclear areas. Gaps between the endothelial cells were observed when aorta samples were incubated with 2 U of IL-2/ml of medium. This was confirmed by SEM, transmission electron microscopy and Evans blue dye staining. These results suggest that IL-2 caused morphological alterations in endothelial cells that enhanced the permeability of the vascular endothelium.

KEY WORDS: Endothelial cells, bovine aorta, interleukin 2, scanning electron microscope, transmission electron microscope, interference microscopy, image analysis.

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INTRODUCTION

The vascular endothelium normally separates the blood from tissues. However, endothelial cells at the site of inflammation are known to undergo marked alterations such as retraction and loss of junctional integrity; this allows extravasation of fluids and leucocytes into the tissue. At least some of the endothelial changes at inflammatory sites may result from the local release of leucocyte products, since there is increasing evidence to show that alteration of endothelial cells in situ may be induced by lymphokines (Baldwin, 1982; Montesano et al.,1985; Sato et al., 1986). This is particularly relevant to the use of lymphokines in cancer therapy, because it has been reported that either lymphokine-activated killer cells or interleukin 2 (IL-2) increase tissue edema and vascular permeability in treated individuals (Rosenberg,1985; Lotze et al., 1985; Rosenstein et al. 1986). In vitro studies have shown that cultures of human endothelial cells exhibit dramatic changes in morphology following incubation with interleukins (Montesano et al. 1985; Groenewegen et al., 1985), tumor necrosis factor, and gamma interferon (Stolpen et al., 1986). Endothelial cells also respond to a variety of biological agents such as endotoxins and phorbol esters (Nawroth et al., 1984), arachidonic acid (Sandra et al.,1985), and alpha interferon by exhibiting a time-and dose dependent prostacyclin production that is accompanied by morphological changes in the endothelial cells. Recent studies have shown that the lymphokine IL-2 (Hall et al.,1986) also stimulates production of prostacyclin by bovine endothelial cells. The effect of IL-2 on the morphology of bovine aorta endothelial cells in vitro and ex vivo was investigated in this study. Quantification of the cell changes in vitro was performed by image analysis. Ex vivo experiments were also done to determine whether the same changes in cell morphology seen in plastic adherent cells could also be seen in endothelial cells in their normal position on the basement membrane and to determine whether these changes could be correlated with a change in vascular permeability.

Materials and Methods

Reagents

Purified human IL-2 (pIL-2) was obtained from Cellular Products, Inc., (Buffalo, NY) and Boehringer Mannheim (Indianapolis, IN). Both products elicited similar morphologic changes and prostacyclin stimulation when added to cultured bovine aortic endothelial cells (BAEC). pIL-2 from Cellular Products Inc. was used throughout the study because it was free of endotoxin as determined in our laboratory. The recombinant human IL-2 (Natural sequence) (rIL-2) was purchased from Amgen Biologicals (Thousand Oaks, CA). It was prepared from E.coli host recombinant DNA procedure and purified by chromatography. It is a non-glycosylated protein with the same 133 amino acid sequence as native human IL-2 except with the addition of a methionine residue at the amino terminus. Its endotoxin level was negligible. Both pIL-2 and rIL-2 stimulated PGI_2 synthesis in BAEC and neither compound was contaminated by IL-1, tumor necrosis factor (TNF), or interferon (IFN).

Cultured endothelial cells

Bovine aortic endothelial cells (BAEC) were obtained from freshly excised bovine thoracic aortas as previously described (Booyse et al.,1975). All experiments were performed with cloned confluent endothelial cells at passage 5, which had been maintained in RPMI 1640 containing 100 U/ml penicillin, 50 µg/ml streptomycin, 200 U/ml neomycin, and 20% heat inactivated fetal bovine serum. Cultures of BAEC were grown to confluence in T-25 culture flasks and were prescreened for degree of confluence before treatment with either pIL-2 or rIL-2. Subconfluent and overgrown cultures were excluded from the experiment. Fresh medium was applied to the endothelial cells 24 h before each experiment. The cells were incubated for various times at 37°C in the presence or absence of IL-2. After the incubation, the medium was decanted and the cells were fixed for image analysis.

Bovine Aorta

Tubular aortic segments were obtained from freshly slaughtered cattle on the day of the experiment. The aortas were immersed in phosphate buffered saline and were transported to the laboratory on ice. The segments were cut longitudinally to expose the lumen and were placed on a template device (Eldor et al., 1981), avoiding unwanted distortion of the tissue. The template allowed incubation of the endothelial surface of the aorta with several agents simultaneously. The template was placed in a CO_2 incubator for 2 h at 37°C in the presence or absence of test agents. At the end of the incubation time, the tissues were fixed directly by adding the appropriate fixative into each well of the template. The tissue from each well was punched out carefully using a sharp cork borer. The samples were transferred to fresh fixative in individual vials and processed for electron microscopy.

Scanning electron microscopy

The aorta samples were rinsed with serum-free medium and fixed with 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight at 4°C. The samples were washed and processed for SEM using the O-T-O technique (Bucana et al., 1976). The samples were dehydrated in increasing concentrations of ethanol, substituted with Freon 113, critical point-dried in dry CO_2 in a Polaron critical point dryer (Polaron Instruments Inc., Hatfield, PA), and sputter-coated with gold-palladium alloy in a Balzer MED 010 vacuum evaporator (Balzer Union Inc., Hudson, NH). The samples were then examined in an AMRAY 1000A scanning electron microscope (Amray, Inc., Bedford, MA). Parallel samples were processed for TEM.

Transmission electron microscopy

The glutaraldehyde-fixed samples were treated with 1% tannic acid for 20 min , washed 3 times, and fixed with 1% OsO_4 for 1 h. The samples were washed with distilled water 4-5 times and were fixed with aqueous 1% uranyl acetate for 1 h, washed 3 times with distilled water, and dehydrated in graded concentrations of ethanol. The samples were then rinsed with absolute ethanol 3 times, infiltrated with graded concentrations of Spurr's low-viscosity medium in absolute ethanol, and embedded in pure Spurr's resin. The blocks were polymerized in a 60°C oven for at least 12 h. Thin sections were cut with glass knives, stained with lead citrate for 5 min in an LKB Ultrostainer (LKB Instruments, Rockville, MD), and examined in a JEOL 1200-EX electron microscope (JEOL, Inc., Peabody, MA) at an accelerating voltage of 60 kilovolts.

Image analysis

Samples for image analysis were plated in triplicate. Cultures of BAEC were fixed with 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer for 1 h at room temperature. The samples were rinsed with buffer and fixed according to the O-T-O method (Bucana et al., 1976). This procedure enhanced the contrast of cells and facilitated the delineation of cell boundaries from the background. The samples were examined with a 40X objective in a Nikon Diaphot TMD inverted microscope (Nikon Inc., Garden City, NY) equipped with a Hoffman modulation contrast system (Modulation Optics Inc., Greenvale, NY). Semi-automatic image analysis was performed in a Zeiss IBAS Image Analyzer (Carl Zeiss, Inc., Thornwood, NY) using commercially available software. Direct electronic measurement of gap formation in confluent endothelial cell cultures is based on the difference in optical density of the area occupied by cells and the exposed plastic . It is a modification of the grid assay developed by Laposata et al. (1983) to measure gap formation in endothelial cells grown in vitro. The image is captured and enhanced electronically. Delineation of the cell boundaries was achieved by the superimposition of a grey scale (0 to 256, where 0 is black and 256 is white) to the enhanced image and optical densities of the cells and the exposed plastic was determined interactively. Delineation of the cell boundaries was done interactively because when cells retract, a residue and remnants of cytoplasmic processes are left behind thus increasing the optical density of the exposed plastic. The area of the exposed plastic was designated the intercellular gap area. The ratio of the gap area to the total area measured and multiplied by 100 was expressed as Area Percent. When cells in confluent cultures retract and elongate, overlapping of cell processes occur and

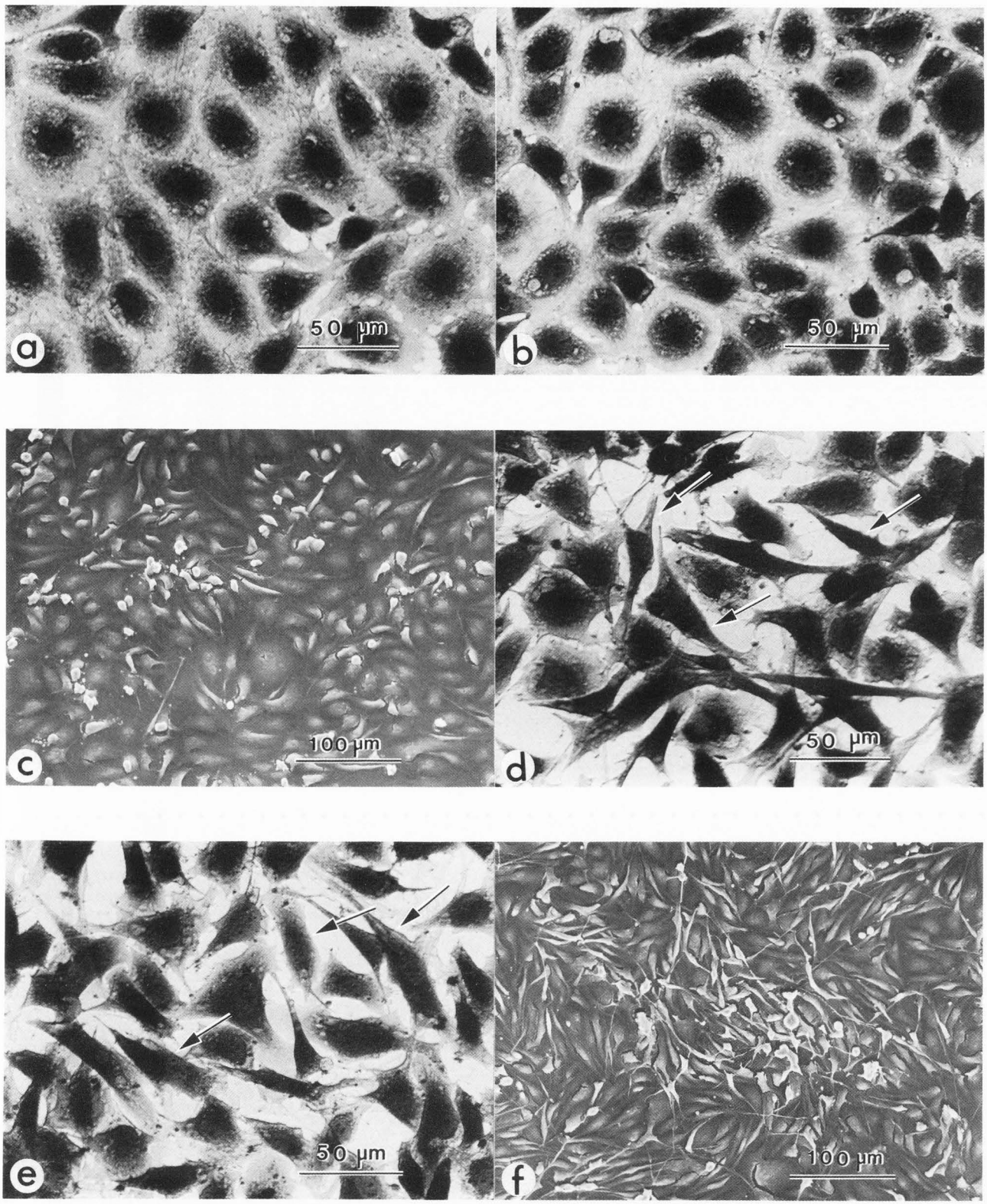

Fig. 1. Hoffman modulation contrast micrograph of confluent culture of control BAEC at 6 and 12 h (a and b) and of cultures treated with 1 U IL-2 for 6 and 12 h (d and e). Cultures treated with IL-2 exhibit numerous retracted cells (arrows) resulting in wide gaps between cells. SEM of dense cultures show polygonal cells in the control (c) and elongated cells in the IL-2 treated cultures (f).

gap areas increase accordingly. All samples were coded and scored in a blind fashion to remove bias. Samples were plated in triplicate, and 5 areas (0.04 mm 2 each) from each flask were analyzed. Only areas containing about the same number of cells per field were measured. The following parameters were measured: (a) the sum of gap areas (b) the area of the field, and (c) the ratio of (a) to (b) multiplied by 100 (Area Percent). Mean and standard deviations of each sample were obtained.

Results

BAEC in culture formed a fairly homogeneous monolayer of epithelial-like polygonal cells with few intervening spaces (Figs. 1 a-c). Treatment of BAEC with 1 U/ml of IL-2 elicited marked retraction and elongation of cells (Figs. 1 d,e, f). These changes were observed as early as 6 h after the addition of IL-2 (Fig. 1 d). Retraction and elongation of BAEC were accompanied by an increase in intercellular spaces or gaps between the cells. Gap formation was dependent on the cell density of the confluent monolayer. The maximum gap response varied inversely with the cell density of the monolayer; therefore it was imperative that cultures were prescreened to ensure that confluence had not been reached before the experiment was started. The area occupied by the gaps in relation to the total area of the field examined, expressed as Area Percent, was quantified by image analysis. A significant increase in Area Percent was observed in cultures treated with a minimum dose of 1 U/ml of IL-2. This phenomenon was observed to be a time- and dose-dependent phenomenon that occurred as early as 6 h after the addition of 1 U/ml of IL-2, peaked at 12 h, and returned to control values at 24 h. However, these morphological changes persisted for a longer time in the presence of higher concentrations of IL-2 (Fig. 2). The morphological changes of BAEC returned to normal when IL-2 was removed from the incubation medium. The changes could easily have been missed if the experiments had been terminated at later times. Studies using rIL-2 showed that higher concentrations were required to produce transient morphological changes in BAEC that did not persist as long as the changes observed after incubation with pIL-2 (Fig. 3).

Results from the ex vivo experiments correlated very well with the in vitro experiments. Control bovine aortas showed a fairly uniform tightly apposed monolayer of polygonal endothelial cells that were smooth and almost devoid of cytoplasmic processes on the exposed surface (Fig. 4a). Incubation of the endothelial surface of the bovine aorta with increasing concentrations of IL-2 showed partial rounding of endothelial cells that gave the impression that the endothelial cells were raised from the basement membrane (Figs. 4 b-d). The nuclear region was more prominent, and the treated endothelial cells became more elongated than the control cells (Fig. 4c). Distinct gaps between cells were seen in the endothelial surface incubated with 2U/ml of IL-2 (Fig. 4d, arrows) but no desquamation was observed. The

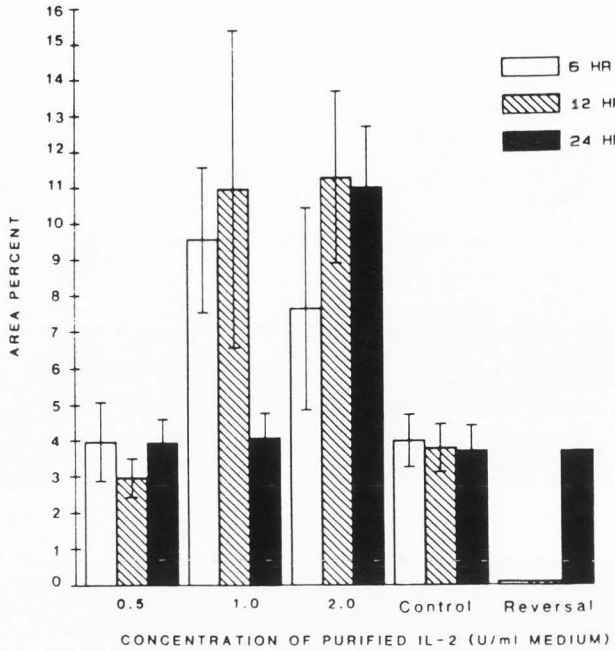

Fig. 2. Effect of various concentrations of IL-2 on gap sizes (Area Percent) of in vitro cultures of BAEC with time measured by image analysis. Samples were examined at 6 h (□), 12 h (▨), and at 24 h (■). Removal of IL-2 from the incubation medium restored the normal morphology of BAEC (Area Percent =3.66, S.D. = 0.86) 18 h later.

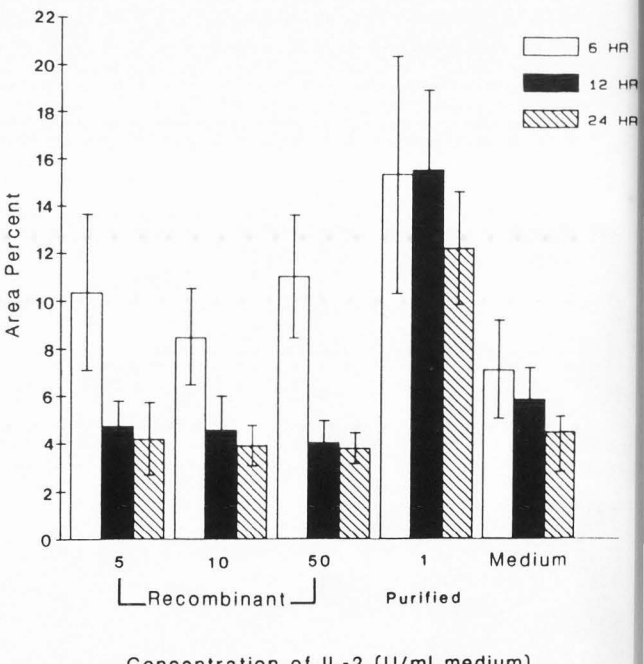

Fig. 3. Effect of various concentrations of rIL-2 on gap sizes of in vitro cultures of BAEC at 6 h (□), 12 h (■) and 24 h (▨) after the addition of the test agents. Control cultures incubated with 1 U of pIL-2 or with fresh medium were also included in the assay.

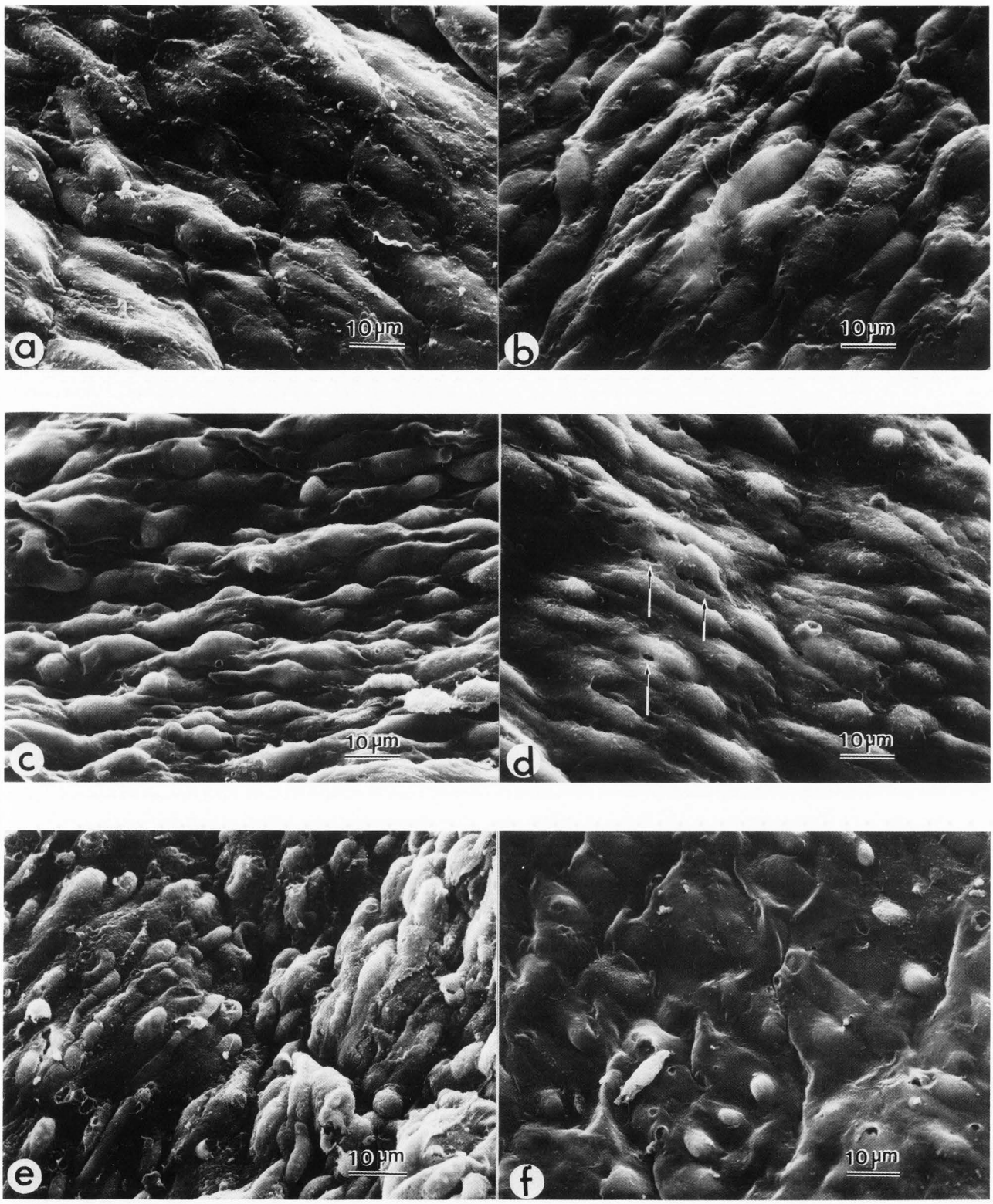

Fig. 4.
SEM micrographs of bovine aorta control (a), bovine aorta treated with 0.5 U IL-2 (b), 1 U IL-2 (c), and 2 U IL-2 (d). Gaps between cells were prominent in Fig. 4d. The effect of IL-2 on the morphology of BAEC was similar to aorta treated with the calcium ionophore, A23187, (e), but not with cytochalasin D (f). All samples were incubated for 2 h.

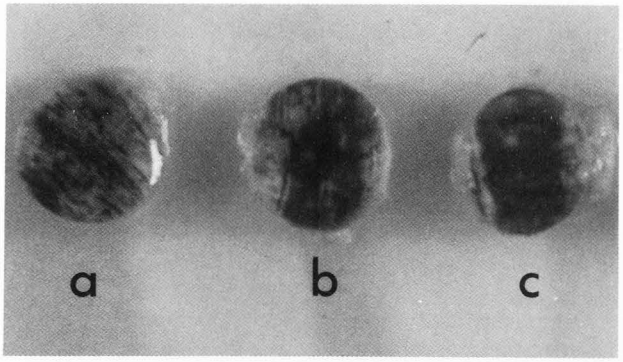

Fig. 5. Evans blue dye staining of control (a), cytochalasin D treated aorta (b) and IL-2 treated (c) bovine aorta. Note markedly increased dye in IL-2 treated aorta.

cell surface remained smooth and no increase in cytoplasmic processes was observed. These morphologic changes were similar to those induced by ionophore A23187 (Fig. 4e) but were different from cytochalasin induced changes (Fig. 4f). Functionally, the endothelial surface exposed to IL-2 was observed to be more permeable to Evans blue dye than was the control surface (Fig. 5).

Correlative TEM studies on parallel samples showed that control BAEC were extended and formed a thin monolayer with simple intercellular junctions (Figs. 6 a-b) By contrast, BAEC from IL-2 treated samples showed retracted cells with convoluted intercellular junctions (Figs. 6 c-d). Although no systematic serial sectioning was done, convoluted cellular interdigitations were observed from five different areas of the embedded block. The significance of the convoluted cellular interdigitations is not known at the present time and would require further study. The tangential sections shown here

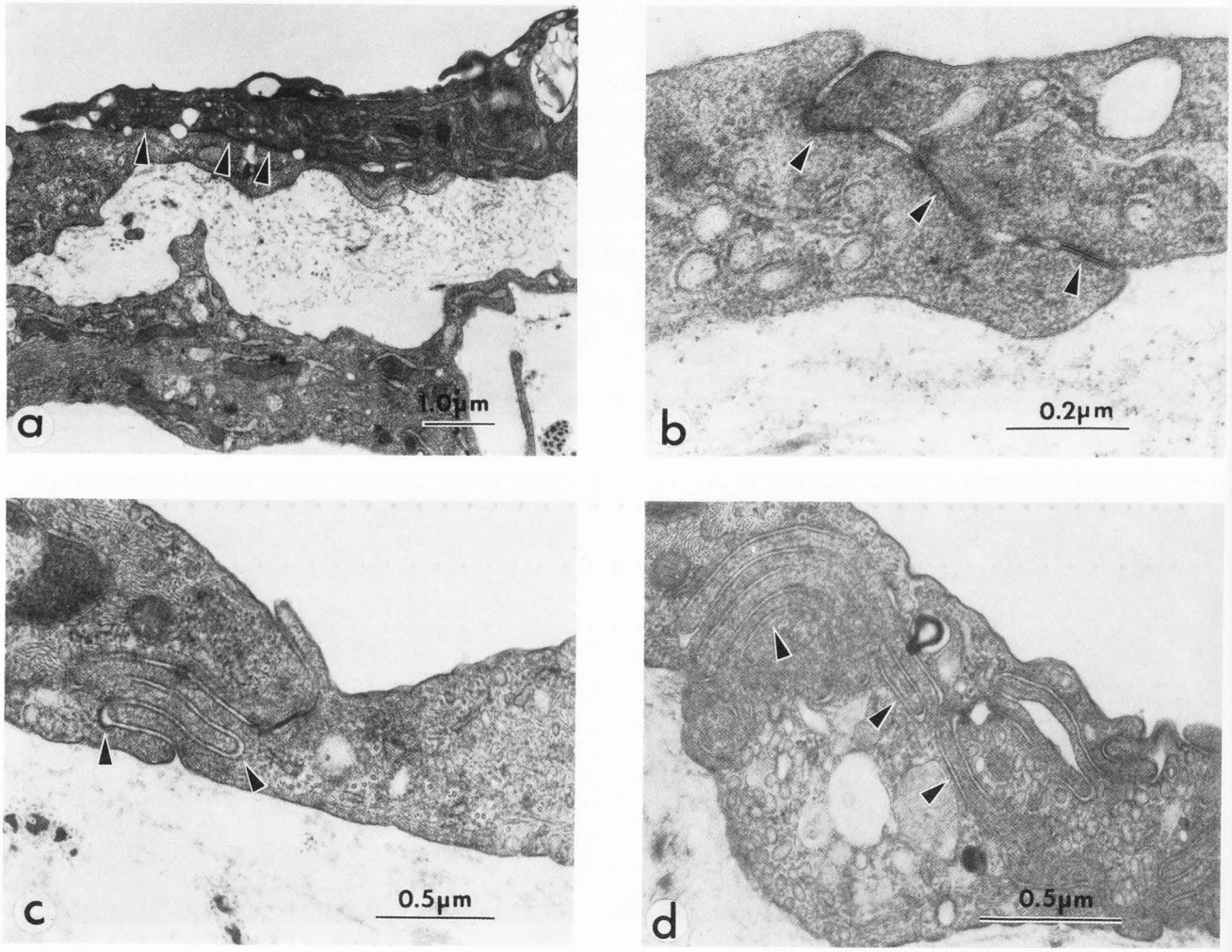

Fig. 6. TEM of cell junctions (arrows) of bovine aorta incubated with various concentrations of IL-2 for 2 h. In control aorta the intercellular junction is shown as a simple overlapping of endothelial cells (a,b), whereas cell junctions of aorta incubated with >1 U of IL-2/ml medium usually show convoluted cellular interdigitations (c,d).

corroborated the SEM observation that cells treated with IL-2 were rounded and appeared to retract from adjoining cells and the basement membrane.

Discussion

The present study shows that IL-2 elicits intercellular gap formation in cultured bovine endothelial cells. This gap response is time- and dose-dependent and cell density-dependent, and exhibits reversibility upon removal of IL-2 from the medium. The effect of rIL-2 appears to be of shorter duration than that of purified IL-2. The difference in potency between pIL-2 and rIL-2 could be due to the fact that pIL-2 is a glycosylated protein while the rIL-2 is either non-glycosylated or has different glycosylation sites.

The morphological changes observed in the cultured BAEC are corroborated by the ultrastructural results obtained from the ex vivo bovine aorta experiments. Intercellular gap formation is seen to be due to retraction of the endothelial cells from each other whereby large junctional gaps are created. Formation of intercellular gaps leads to increased vascular permeability as shown by the increased permeability of IL-2 treated aorta to Evans blue dye. Because IL-2 induces a dramatic morphological change in endothelial cells, it is inferred that IL-2 can elicit pathophysiologic events modulating vascular permeability in vivo. Extrapolation of findings from in vitro to in vivo studies is difficult to make and we are aware that other cells such as smooth muscle cells may also be affected by lymphokines.

Laposata et al. (1983) demonstrated the formation of gaps in primary cultures of human endothelial cells following incubation with the vasoactive substances histamine and thrombin. Our results with IL-2 are similar to the gap formation induced by thrombin but not that induced by histamine. It is conceivable that most agents that alter the permeability of vascular endothelium do so because they act directly on the vascular endothelial cells (Majno et al., 1969, Shasby et al., 1984). Majno et al. (1969) have shown that inflammatory mediators produced marked but transient endothelial cell separation in the postcapillary venules subsequent to active endothelial cell contraction that created large junctional gaps. However, it is known that some agents known to increase vascular permeability such as bradykinin and serotonin failed to induce gap formation in vitro. We have recently shown that IL-2 receptors are expressed in bovine endothelial cells (K. Frasier-Scott, personal communication). Therefore, endothelial cell retraction induced by IL-2 is likely to be a receptor-mediated event.

Morphological changes in endothelial cells have been shown by Stolpen et al. (1986) to be accompanied by alterations in the cytoskeletal organization that may be influenced by extracellular calcium concentration (Shasby and Shasby, 1984). We provide indirect evidence for the involvement of calcium in initiating the morphological changes by the use of a calcium ionophore, A23187, that produces changes similar to those observed with IL-2. We suggest that IL-2 may trigger calcium influx into the endothelial cells, which in turn induces cytoskeletal rearrangement and morphological change. Studies on the effect of Il-2 on human endothelial cells are in progress; therefore, no correlations can be made at this time.

The use of either lymphokine activated killer cells or IL-2 in clinical cancer studies has been shown to induce fluid retention in cancer patients (Rosenberg, 1985; Rosenstein et al., 1986). The short half-life of IL-2 in the circulation was compensated for by administering IL-2 either as a continuous infusion or 2-3 times daily (Lotze et al.,1985). Furthermore, massive doses of IL-2 (5,000 to 100,000 U) were used in these human patients and in animal experiments (Rosenberg, 1985). Although we did not use high doses of IL-2 in our studies, we speculate that continuous infusion of IL-2 would induce marked changes in the endothelial surface of blood vessels. These vascular changes may account for the adverse reactions, such as capillary leakage problems and pulmonary edema, that are often observed in cancer patients receiving this modality of therapy.

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Discussion with Reviewers

R. Laschi: Fig. 4c shows an endothelial area (bovine treated with 1 U of IL-2) which appears to be more damaged than that seen in Fig. 4d (incubation with 2 U of IL-2). This apparently does not agree with data reported in Fig. 2. Have you some comment on the matter?

Authors: Although the cells in Fig. 4c appear to be raised and rounded, the gaps between cells are most prominent in Fig. 4d (arrows in micrograph). The values shown in Fig. 2 refer to values obtained in vitro cultures of BAEC.

R. Laschi: You state that gaps between the endothelial cells were observed when aorta samples were incubated with >1 U of IL-2/ml medium. However, your Figs. 6c-6d at TEM show a lot of convoluted cell interdigitations as well as well-developed and complete tight and gap junctions (not described in the text). In view of these findings, your general statement seems to be a contradiction.

F. W. Orr: What structural features in the transmission electron micrographs (Fig. 6) are likely to correlate with the gaps observed in the scanning electron micrographs (Fig. 4d)?

Authors: By transmission electron microscopy, gaps between endothelial cells in the aorta segments were seen as exposed basement membrane and connective tissues. A section across several cells with gaps in between exhibited an alternating pattern of cell-connective tissue-cell. The exposed connective tissue varied according to the size of the gap between cells. Examination of consecutive sections showed cells that are far apart from each other followed by sections showing cells touching each other and exhibiting complex junctions shown in Figs. 6c-6d.

F. W. Orr: The authors should describe the methods by which they determined that the IL-2 was free of endotoxin, IFN, and TNF.

Authors: The reagents were found to be free of endotoxin based on the standard Limulus amebocyte assay (M.A. Bioproducts, Walkersville, MD). The reagents were also analyzed by HPLC and lymphocyte assay to check for TNF and IFN and were found to be free of the above compounds.

F. W. Orr: Have the authors excluded the possibility that some of the observed effects reflect toxicity?

Authors: Parallel in vitro samples have been tested for viability by the trypan blue dye exclusion test and were found to be >95% viable. In addition, the reversal experiments showed that removal of IL-2 from the medium allowed the cells to revert back to normal morphology, thus indicating that the cells are still viable after 24 h incubation with IL-2.

P. W. Bankston: Increased permeability in inflammation is an exclusively venular event unless endothelial cells are heavily damaged in other vessels. Do you intend to imply by your results that the site of increased permeability after intravenous IL-2 administration includes large vessel walls? Are your results applicable at all to the in vivo situation where small vessels are the likely sites of increased extravasation which leads to edema?

Authors: The authors are aware that endothelial cells vary according to species and location in the vascular system. Therefore, we cannot make a direct correlation between our results and in vivo effects of IL-2. We can only speculate that a similar effect may occur in vivo but, clearly, more studies are needed to verify the effect of IL-2 and other biological agents on endothelial cells. A recent paper by Cotran et al. (Cotran et al., 1987, J. Immunol. 139:1883-1888) showed that intravenous administration of IL-2 in humans led to activation of certain antigens in the capillary endothelial cells, whereas IL-2 administered in vitro to cultured human umbilical vein endothelial cells failed to induce the same antigens. It is clear that a logical comparison can only be made when both in vivo and in vitro systems are derived from the same source and species.