Br. J. exp. Path. (1984) 65, 145–154

The adhesion of different cell types to cultured vascular endothelium: effects of culture density and age

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Received for publication 15 August 1983

Summary. The adhesion of lymphocytes, macrophages and resuspended smooth muscle cells to freshly subcultured bovine aortic endothelial cells is considerably greater than their adhesion to in-situ aortic endothelium when tested *in vitro*. Experiments with endothelial monolayers of different cell density and maturity suggest that this can be explained, at least in part, by two factors—firstly, an inverse relationship between macrophage, polymorph and smooth muscle cell (but not lymphocyte) adhesion and endothelial cell density, and secondly, an inverse relationship between endothelial adhesiveness and time since a culture became confluent. These observations may help to clarify the relationship between endothelial adhesiveness *in vitro* and *in vivo*, and to explain why leucocytes tend to adhere to regenerating arterial endothelium.

Keywords: cell adhesion, cell culture, vascular endothelium, cell density, cell age

The adhesion of different types of leucocyte to vascular endothelium *in vivo* has been intensively investigated because of its relevance to inflammation and to immune reactions (Schoefl 1972; Grant 1973; Anderson & Anderson 1976). The ability to grow endothelium as a cell culture has extended these studies *in vitro* with respect to lymphocytes (de Bono 1976, 1979) and granulocytes (Lackie & de Bono 1976; Beesley *et al.* 1979). Leucocyte adhesion, and leucocyte migration between endothelial cells, are easy to demonstrate *in vitro*, but there are important differences between endothelial behaviour *in vitro* and *in vivo* which have not so far been adequately explained. In-vivo leucocyte adhesion to large vessel endothelium is scanty, and adhesion to small vessel endothelium tends to be much more apparent at sites of inflammation or immunological reactivity. On the other hand, cultured bovine or porcine aortic endothelium very readily allows the adhesion of both lymphocytes and polymorphs, to the extent that substances thought to be important stimuli to leucocyte adhesion *in vivo* may have little additional effect (Lackie & Smith 1980). Perhaps cultured endothelium is more akin to its behaviour to 'inflamed' endothelium, or to the endothelium of capillaries and venules, than to the endothelium of large arteries and veins from which it is derived. If this is true, the mechanisms involved in and the environmental conditions responsible for the difference need to be critically evaluated, particularly if any extrapolation is to be made from tissue culture experiments to endothelial behaviour *in vivo*.

We have used a battery of different types of 'adhering' cell to study the adhesiveness of cultured bovine arotic endothelial monolayers under different culture conditions, with particular emphasis on the effects of endothelial cell density and on length of time

since the culture became confluent. As adhering or indicator cells we used polymorphonuclear leucocytes, blood or lymph node-derived lymphocytes, peritoneal macrophages and resuspended cultured smooth muscle cells. We deliberately chose a wide range of cell types, since our aim was to explore changes in endothelial properties in relation to its environment rather than to model a particular in-vivo process.

Materials and methods

Bovine aortic endothelium was obtained by treatment of fresh aortas with 1% w/v collagenase and cultured in Dulbecco's modified Eagle's medium (DMEM) with 20mM HEPES buffer and 15% newborn calf serum. Cells were subcultured using 0.5% w/v trypsin/1% EDTA. Cells were used in adhesion experiments after two or three passages in culture. Endothelial target morphology was controlled by light and phase contrast microscopy, and by lectin staining with fluorescein-labelled concanavalin A to detect surface overgrowth of non-endothelial cells (de Bono & Green 1983).

Lymphocytes. Lymphocytes were obtained from human peripheral venous blood by gelatin sedimentation and centrifugation over ficoll-hypaque or ficoll-urografin (SG 1.071). Bovine lymph node cells were prepared by mincing bovine para-aortic lymph nodes, straining the resulting suspension through wire mesh, and washing the resulting cells three times with DMEM.

Leucocytes. Polymorphonuclear leucocytes (PMN) were prepared from human blood by density gradient centrifugation over ficoll-hypaque. Because PMN adhesive properties may be altered by the isolation process we also studied the adhesion of polymorphs from diluted heparinized (5 units/ml) whole blood.

Macrophages. Human peritoneal macrophages were obtained from patients undergoing chronic ambulatory peritoneal dialysis for chronic renal failure. Clear, non-infected dialysis fluid freshly drained from the peritoneal cavity was centrifuged and the cells resuspended in DMEM. More than 70% of the resulting cells were mononuclear cells positive for α-naphthyl acetate esterase; the remainder included a few polymorphs and some desquamated peritoneal mesothelial cells.

Smooth muscle cells. Resuspended bovine aortic smooth muscle cells (SMC) were obtained from a cell line cultured in our laboratory, originally derived from bovine aortic medial cells. The cells were resuspended in trypsin-EDTA, washed in DMEM, 'clumps' of cells removed by settling, and the remaining cells resuspended in DMEM/15% calf serum for the adhesion experiments.

Adhesion to endothelial monolayers was studied in three ways.

1. A 'collecting lawn' assay with visual counting of adherent cells (Armstrong & Lackie 1975; Lackie & de Bono 1976). In this assay the 'target' cells were grown on a glass 13-mm coverslip, adhering cells (2×10^6 cells in 1 ml DMEM/15% calf serum) were added and allowed to settle under gravity for 30 min, and the coverslips then washed by perpendicular passage through an air fluid interface. Adhering cells were identified and counted by light fixation in 2% glutaraldehyde and staining with haematoxylin.

2. An assay in which the number of adhering cells was estimated by labelling them with ^{3}H-adenine. We found adenine a useful label because it is taken up by a variety of different cells and its uptake is not dependent on cell division. It probably enters a cytoplasmic nucleoside/nucleotide pool as well as, eventually, RNA and DNA. Although there is some 'leakage' from the labelled cells, re-uptake of labelled material by the target cells could be reduced by adding an excess of unlabelled adenine, and controlled by incubating additional target monolayers with the supernatant from a labelled cell suspension allowed to stand for the same time as used in the adhesion

experiment. Control experiments, in comparison with the first technique, showed that counts recovered were proportional to the number of labelled cells added, and that the excess adenine did not interfere with adhesion.

SMC were labelled by adding 3.7 kBq of ^{3}H-adenine (8.88×10^5 MBq/mmol) to approximately 6×10^6 cells in a 25-cm^2 culture flask containing 5 ml of DMEM. Labelling was continued for 16 h and the cells were then washed and incubated a further 24 h in unlabelled medium before resuspension. Unlabelled adenine was added to the final suspension to a concentration of 10^{-5}M. Lymphocytes or macrophages were incubated for 4 h at 37° with 7.4 kBq adenine, added to 2.5 ml of DMEM containing approximately 4×10^7 cells. After incubation the cells were washed and resuspended in medium containing 10^{-5}M unlabelled adenine.

Target monolayers were grown in 25 × 25 mm flat bottomed wells and approximately 2×10^6 adhering cells were added per well. After 30 min incubation cells were resuspended by agitation in a standardized way, and the wells washed with three washes of phosphate buffered saline; 0.5 ml of 1% Triton X-100 was then added to release cell-bound label, which was taken up in 10 ml of NE260 scintillant for liquid scintillation counting. Cell adhesion to native aortic endothelium was measured using squares of aorta accurately cut to fit the wells. Endothelial integrity was confirmed by silver nitrate staining and microscopic examination *en face*. Adhesion to serum-treated glass or tissue culture plastic was used as an internal control in each experiment. Experiments were run in triplicate or quadruplicate.

3. For studying adhesion to non-confluent endothelium, endothelial cells were plated at low cell density in Falcon plastic tissue culture flasks (25 cm^2 growing area) and allowed to grow until discrete 'clumps' of cells were visible. Medium (5 ml) containing 10^7 adhering cells was added and the flasks rotated on an inclined turntable (30°, 33 r/min, 10 cm from axis of rotation) for 1 h. The layer was then washed with three changes of phosphate-buffered saline, and adhering cells counted visually as in (1).

In some experiments a confluent endothelial monolayer was deliberately 'wounded' so as to study cell adhesion to the endothelial cells spreading to cover the injured area. The 'wound' was a single transverse scratch made with the end of a pasteur pipette which had been sealed and rounded in a bunsen flame. The scratch was initially about 0.5 mm wide, and endothelial continuity was restored within 4 h of wounding.

Results

Lymphocytes, macrophages, resuspended smooth muscle cells and polymorphonuclear leucocytes all adhered less well to native arotic endothelium than to freshly subcultured, low density endothelial monolayers (Fig. 1). Because native large vessel endothelium grows at a high cell density compared with endothelial cultures, we compared cell adhesion to endothelial cultures of different cell densities but similar 'age' since subculture. This was done in three ways: by plating endothelial cell suspensions with different cell concentrations; by plating out 'clumps' of endothelium at low density so as to allow different densities to develop in the same culture; and by 'wounding' an already confluent layer so as to compare adhesion to the cells which had spread out to cover the injured area with adhesion to their more compact neighbours. Results will be considered for each type of adhering cell in turn.

Lymphocytes

These showed either a *positive* relationship between lymphocyte adhesion and endothelial density (Figs 2 & 3) or at higher endothelial densities, independence between the two. There was no excess adhesion to the edges of the wounded endothelium. We cannot exclude the possiblity that lymphocyte adhesion might decline at very high endothelial densities, as we were unable to culture

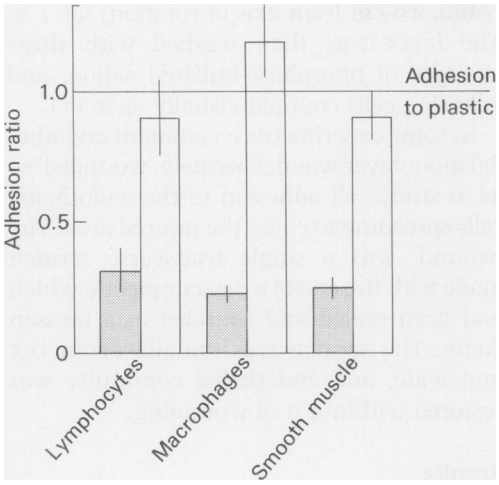

Fig. 1. Comparison of the adhesion of lymphocytes, macrophages and resuspended smooth muscle cells to fresh in-situ aortic endothelium (▦) and to cultured bovine aortic endothelial cells (□). Density of the in-situ endothelium was approximately 2500 cells/mm^2 (higher than *in vivo* because the vessel wall was not under tension). Density of the cultured cells was approximately 400 cells/mm^2, and they were used 48 h after subculturing. Results are means (bars of SD) of quadruplicate experiments 'standardized' with respect to adhesion to tissue culture plastic.

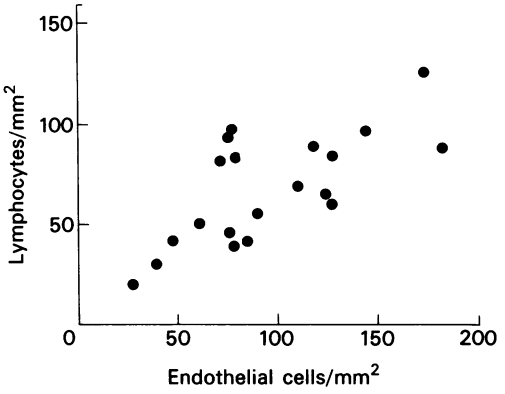

Fig. 2. Plot of lymphocyte adhesion against endothelial cell density in a single culture flask containing endothelial cells in 'clumps' at different densities.

endothelium satisfactorily at densities of greater than 1500 cells/mm^2.

Macrophages

These showed a *negative* relationship between adhesion and endothelial density, i.e. they adhere more readily to low-density monolayers. They also tend to accumulate at the edges of wounded endothelial monolayers or of growing clumps of endothelium (Figs 4 & 5).

Resuspended smooth muscle or endothelial cells

These showed a similar negative relationship between adhesion and endothelial cell density (Fig. 6). We were initially concerned that this might simply reflect greater 'access' to

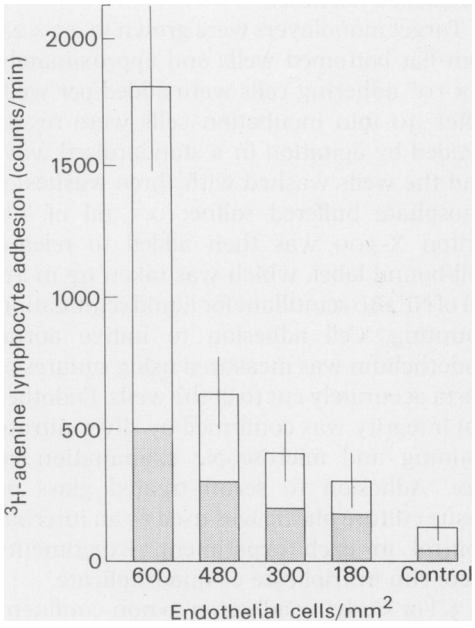

Fig. 3. ^{3}H-adenine labelled lymphocyte adhesion to 48-h cultures of bovine aortic endothelium plated at different endothelial cell densities. Approximate endothelial densities were determined by optical counts in parallel cultures fixed and stained with haematoxylin. Shaded areas show adenine uptake from lymphocyte supernatants.

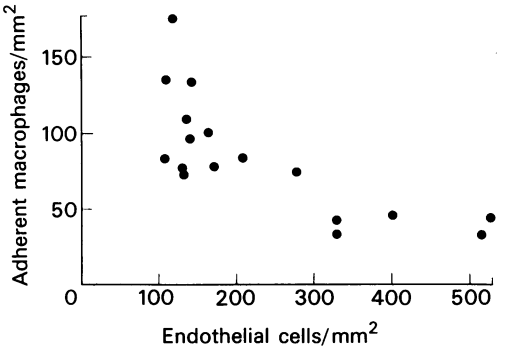

Fig. 4. Plot of macrophage adhesion against endothelial cell density in a single culture flask containing bovine aortic endothelium at different densities.

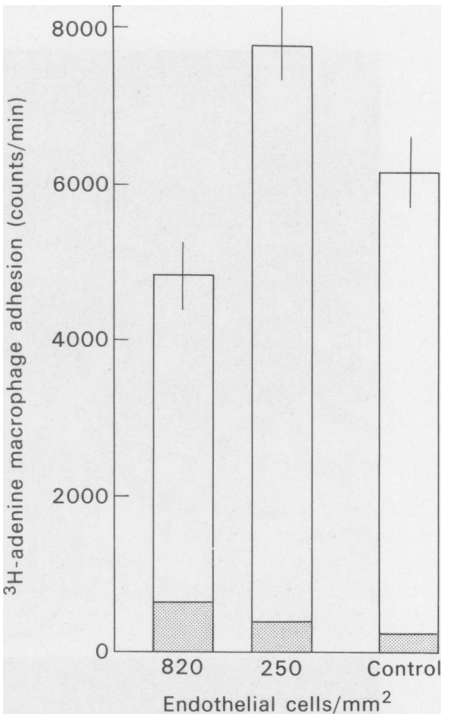

Fig. 5. Macrophage adhesion to 48-h cultures of bovine aortic endothelial cells at different cell densities. Shaded areas show adenine uptake from macrophage supernatants.

the substratum in the less dense endothelial cultures, but adhesion to low-density endothelium was actually greater than to glass or plastic (Fig. 7). Polymorphonuclear leucocytes showed an inverse relationship between adhesion and endothelial density, and also showed increased adhesion at cut endothelial edges (Fig. 8).

We also examined cell adhesion to endothelial monolayers at different times after they had reached confluence. Smooth muscle cells and macrophages showed a progressive decline in adhesion with age in culture (Figs 9 & 10). Lymphocyte adhesion also declined with culture age, although there was sometimes a transient increase possibly related to an increase in endothelial density. Polymorphs were not tested in this system.

We were not able to demonstrate an effect of culture supernatants from cultures of different age or density on cell adhesion to glass or plastic—it is possible that this lack of effect might be due to the instability of a potential mediator, but this would not explain the local differences in adhesion in cultures of varying endothelial density. Indomethacin (10^{-5}M), heparin sulphate (2–5 units/ml) or trypsin inhibitor were also without effect.

Discussion

We have found that the surface adhesive properties of cultured bovine aortic endothelium alter with both the cell density of the culture and with the length of time since subculturing. This probably represents a true change in surface properties rather than a humoral effect mediated by a soluble cell product. Vlodavsky *et al.* (1979*a*) have shown that confluent endothelial monolayers exhibit a specific protein on the cell surface which is not present in subconfluent cultures, but the relationship of this to our observations on cell adhesion is at present speculative. Endothelial surface area is known to control a number of intracellular functions, including cell division (Folkman &

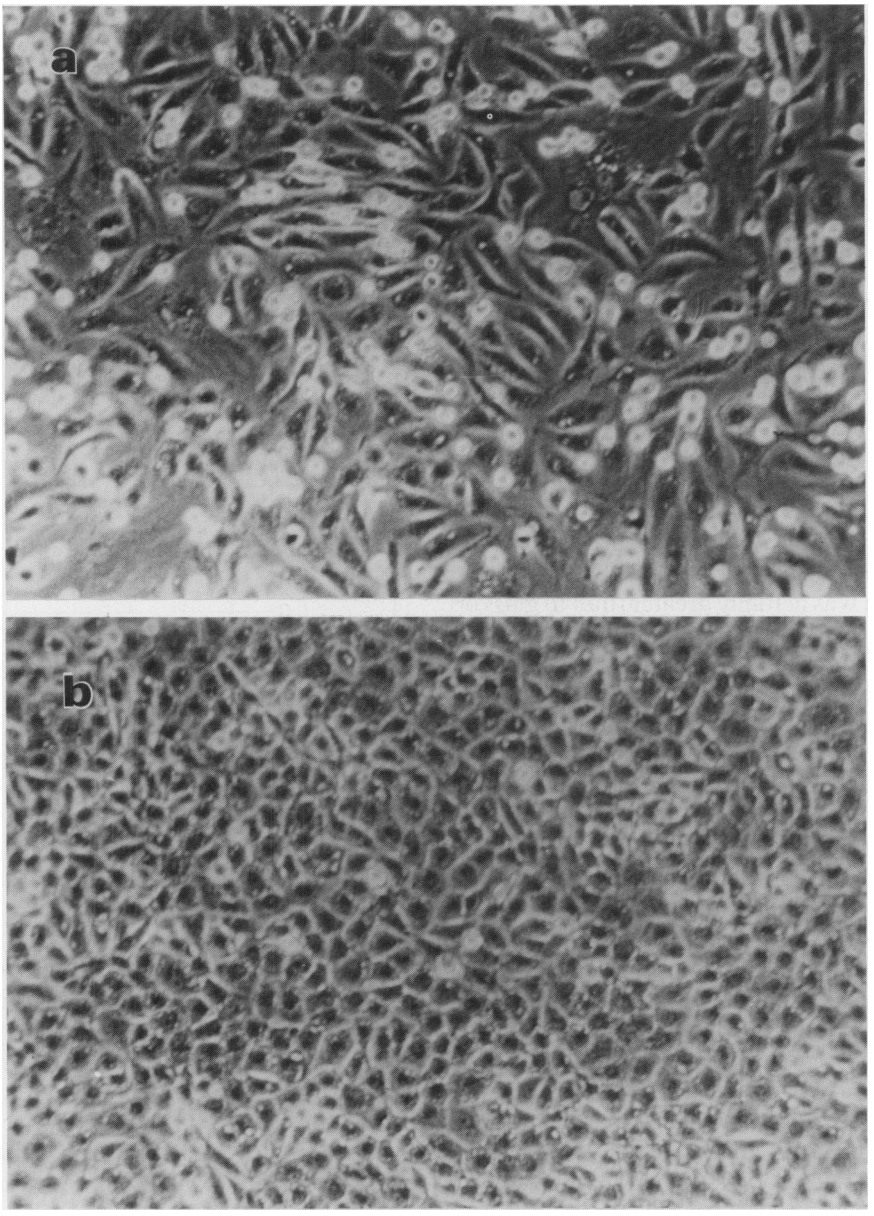

Fig. 6. Photomicrographs of SMC adhering to (**a**) low density endothelial culture (approx. 210 cells/mm^2) and (**b**) high density endothelial culture (approx. 800 cells/mm^2). The SMC are the light-coloured dots in these phase-contrast photomicrographs taken after 30 min incubation of the monolayers with resuspended smooth muscle cells ($\times$ 150).

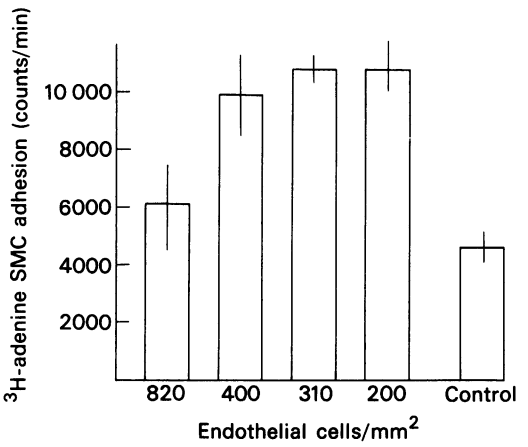

Fig. 7. Smooth muscle cell adhesion to endothelial monolayers of different cell density 48 h after subculturing.

Moscona 1978) and it is possible that a metabolic alteration rather than a change in surface might control adhesiveness. The apparent dichotomy between lymphocyte adhesion, which increases with increasing endothelial density and that of other cell types which is inversely related to density may reflect a fundamental difference in adhesion mechanisms, or simply a quantitative difference in that a greater endothelial density than we were able to obtain would be needed to inhibit lymphocyte adhesion. Recently subcultured endothelium was more adhesive to all types of cells than endothelium which had reached maximum cell density several days previously.

The conditions in our experiments were different from those used by Vlodavsky *et al.* (1979*b*) in the production of 'phenotypically altered' endothelium using serum-poor medium. Our cultures retained an 'epithelial' morphology, showed little platelet adhesion, did not demonstrate excessive surface accumulation of concanavalin A, and did not significantly alter their adhesive properties on supplementation with bovine brain fibroblast growth factor or endothelial growth factor.

Large vessel endothelium *in vivo* has a high endothelial cell density and a relatively slow endothelial turnover rate. Bovine arterial endothelial cell densities are in the range 1500–3000/mm^2 in material fixed at physiological intraluminal pressure (Repin *et al.* 1982). Lower endothelial cell densities, or more extended endothelial cells, may be found in proliferating capillaries or regenerating aortic endothelium (Poole *et al.* 1958). 'Giant' endothelial cells are also a feature of ageing endothelium, and are sometimes seen near atheromatous plaques. It is interesting that macrophage, and to a lesser extent polymorph, adhesion to regenerating large vessel endothelium *in vivo* has been observed in a number of studies (Poole & Florey 1958; Ramsay *et al.* 1982) in circumstances where the haemodynamic situation would make it difficult for local humoral factors to have an effect. An alteration in the adhesive properties of 'nascent' endothelium may therefore have some physiological role, particularly since macrophages are capable of secreting growth factors for vascular wall cells (Glenn & Ross 1981) and may play an important role in the biology of atheromatous lesions (Gerrity 1981).

The principal implications of these results are firstly, that they provide a partial explanation for previous observations of disproportionate cell adhesion to cultured endothelium compared with ex-vivo large vessel endothelum, and secondly that they emphasize the need for careful specification of endothelial density and culture conditions if cultures are to be compared with endothelium *in situ*. It is also possible that identification of the molecular components responsible for the changes in adhesion may eventually provide a clinically useful way of distinguishing between 'nascent' and 'mature' endothelium *in vivo*.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Great Britain. We thank Miss Joyce Giblett for typing the manuscript.

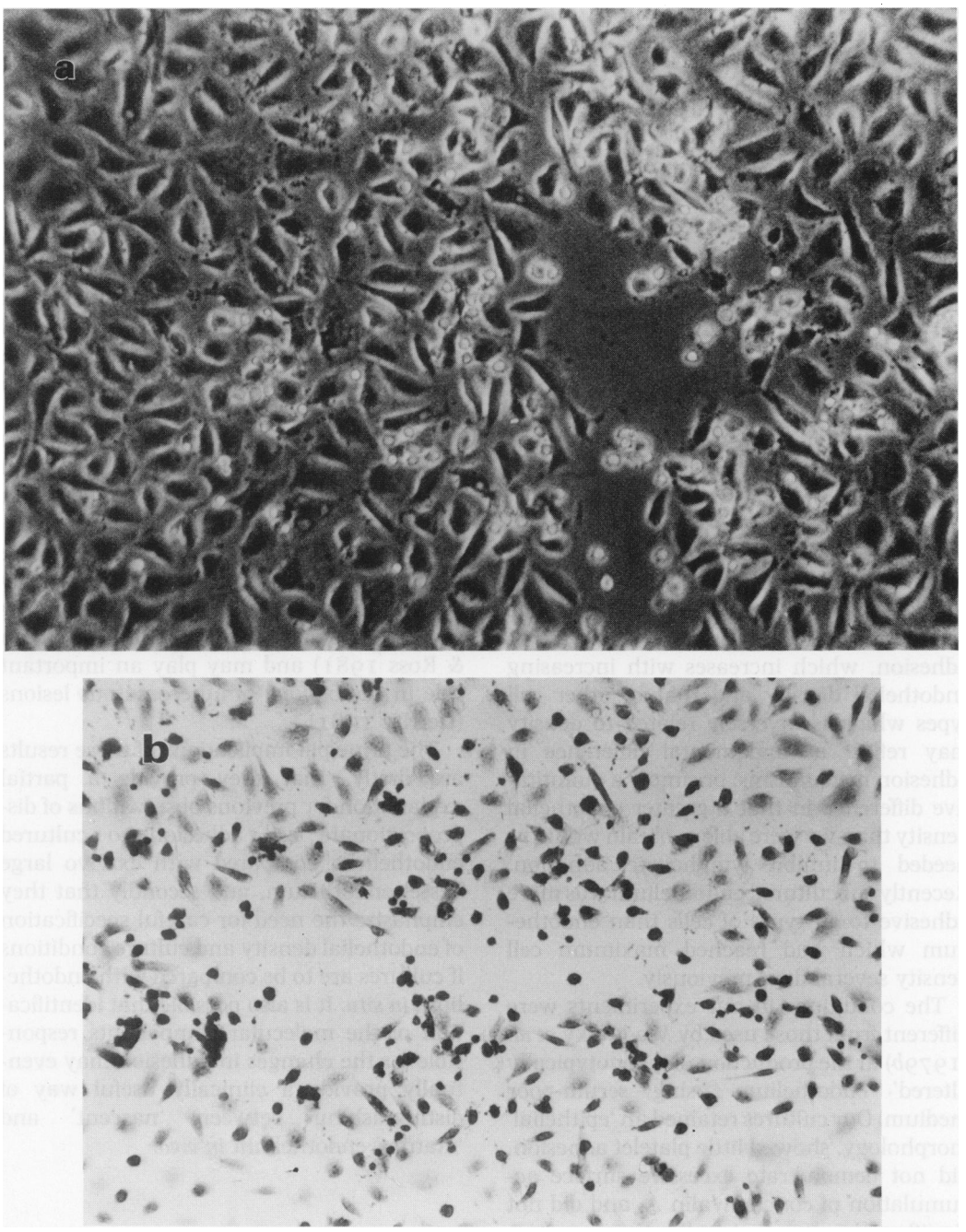

Fig. 8. PMN adhesion at wounded endothelial edges. A confluent endothelial monolayer was scratched with a glass rod, and endothelium allowed to spread into the gap for 2 h. Fresh human blood with a PMN count of 8×10^9/l was anticoagulated with heparin, diluted 1:1 with DMEM and incubated on a turntable with the scratched monolayer for 1 h at 37°C. Phase-contrast appearances are shown (**a**) where the SMC appear light (× 250) and a haematoxylin-stained preparation (**b**) where the SMC appear dark (× 150).

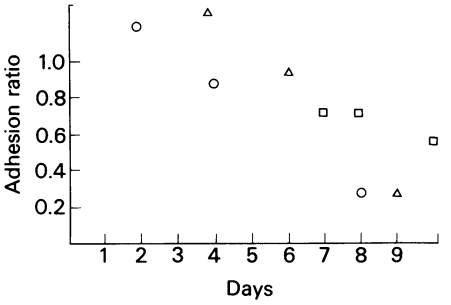

Fig. 9. Plots of SMC adhesion against age of endothelial culture for three separate experiments. Each point represents mean of 10 counts of SMC adhering to endothelium divided by mean of 10 counts of the same SMC adhering to glass.

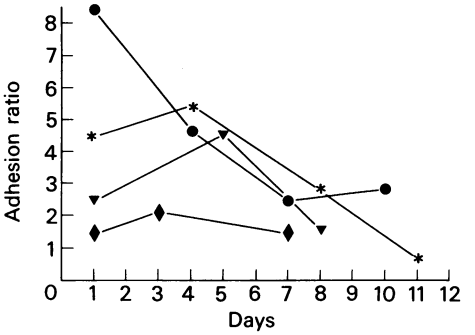

Fig. 10. Adhesion ratios for lymphocyte adhesion to endothelium divided by lymphocyte adhesion to tissue culture plotted against time since subculturing.

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