Immunology 1994 **81** 538–545

Adhesion and penetration properties of human lymphocytes acting on allogeneic vascular endothelial cells

R. A. BLAHETA, M. SCHOLZ, N. P. HAILER, J. BEREITER-HAHN,* A. ENCKE & B. H. MARKUS
*Department of General Surgery, Hospital of J. W. Goethe University and *Institute of Cellular Kinematics, Department of Biology, Frankfurt/Main, Germany*

SUMMARY

Lymphocyte infiltration through vascular endothelium is one important step in the course of graft rejection. To investigate this process more exactly we established a monolayer invasion assay which enabled us to discriminate between adherent and penetrated cells. Detailed studies of adhesion and penetration kinetics of peripheral blood lymphocytes (PBL) acting on allogeneic human umbilical vein endothelial cells (HUVEC) were carried out by combined phase contrast and reflection interference contrast microscopy. Between 30 and 35% of all PBL attached to HUVEC after 4 hr. Out of these less than 10% penetrated. When HUVEC were prestimulated for 2 hr by interferon (IFN)-α, -β, -γ or interleukin (IL)-1, PBL adhesion in the early phase of cellular attachment to endothelial cells was accelerated. Overall adhesion however did not increase. Long-term pretreatment of HUVEC for 72 hr with IFN-γ or IL-1 also modified PBL–HUVEC interactions. However, a 72-hr pretreatment with IFN-α or -β did not influence lymphocyte binding behaviour. PBL penetration was not only accelerated but also enhanced by IFN-α, -β, -γ, irrespective of whether HUVEC were prestimulated for 2 hr or PBL and cytokines were added simultaneously to HUVEC. On the other hand IL-1 was not able to enhance the amount of penetrated cells but only accelerated the infiltration process. Up-regulation or *de novo* expression of the adhesion molecules ICAM-1 (intercellular adhesion molecule), ELAM-1 (endothelial leucocyte adhesion molecule) and VCAM-1 (vascular cell adhesion molecule) did not parallel PBL binding kinetics. Therefore an ICAM-, ELAM- and VCAM-independent modulation in the early phase of lymphocyte attachment to endothelium seems likely. The lymphocyte cytoskeleton may have a role in this process.

INTRODUCTION

Lymphocytes play a central role in the outcome and course of an immune response. They can emigrate from blood into lymphoid tissue by adhering to and penetrating through the specialized endothelial walls of post-capillary high-endothelial venules.[1] When inflammation has occurred lymphocytes can move directly from blood to the site of inflammation.[2] A prime example of inflammation is the process of graft rejection characterized by a massive infiltration of responder lymphocytes into the donor graft.[3] This process is accompanied by an enhanced release of cytokines [interferons (IFN), interleukins (IL)] and an up-regulation or new expression of several adhesion molecules on cellular membranes.[4]

Although studies on lymphocyte traffic have been very extensive the correlation to cytokine release and adhesion molecule expression has never been fully clarified. Moreover, *in vitro* assays established measure lympocyte binding to endothelial cell monolayers but fail to distinguish between adhesion and penetration.[5-8]

The present study was designed to investigate the cellular adhesion and penetration processes separately to obtain a better understanding of inflammatory events occurring after organ transplantation. The results clearly demonstrate that a distinct proportion of human lymphocytes do attach to allogeneic endothelial cells but only a small amount actually penetrate the monolayer. Soluble mediators act differently on both processes. Moreover, the adhesion molecules ICAM-1 (intercellular adhesion molecule),[9] VCAM-1 (vascular cell adhesion molecule)[10] and ELAM-1 (endothelial leucocyte adhesion molecule)[11] (also known as E-selectin) assumed to participate in lymphocyte–endothelial cell interactions are obviously not responsible for the onset of the cellular infiltration process.

Received 20 September 1993; revised 17 November 1993; accepted 23 December 1993.

Abbreviations: ELAM, endothelial leucocyte adhesion molecule; HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.

Correspondence: Dr R. Blaheta, J. W. Goethe-University-Hospital, Dept. of General Surgery, Transplant-Immunology Laboratory, Bldg 23 A, EG 7, Theodor-Stern-Kai 7, D-60590 Frankfurt/Main, Germany.

MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVEC)

Human umbilical veins were washed with phosphate-buffered saline (PBS) and endothelial cells detached by chymotrypsin (α-chymotrase; Hasenclever, Bonn, Germany; 10 min). The iso-

lated endothelial cells were pooled and grown in Medium 199 (M199; Biozol, München, Germany) containing heparin (Liquemin N 5000; Roche, Basel, Switzerland), gentamycin (50 mg/ml; Gibco, Karlsruhe, Germany), HEPES buffer (HEPES 1 M; Seromed, Berlin, Germany), 10% fetal calf serum (FCS) (Gibco), 10% pooled human AB serum (kindly provided from the Blood Bank of The Red Cross, Frankfurt, Germany) and endothelial cell growth factor (ECGF, 75 mg; Boehringer, Mannheim, Germany). The cell cultures were serially passaged (1:3 split ratios) in tissue culture flasks (Falcon Primaria, 75 cm^2; Becton Dickinson, Heidelberg, Germany). Subcultures from passages 3–7 were selected for experimental use.

Peripheral blood lymphocytes (PBL)

Human PBL were isolated from heparinized blood of healthy volunteer donors by Ficoll–Hypaque centrifugation. PBL were suspended at 1×10^6 cells/ml in HUVEC medium. The cell suspension was transferred to culture flasks (Falcon Primaria, Becton Dickinson) and incubated overnight in a humidified atmosphere at 37° and 5% CO_2. Monocytes were depleted by adhesion to the plastic of the culture flask. Viability of the PBL was >95% as assessed by trypan blue exclusion.

Monolayer invasion assay

Round cover glasses (22 mm diameter) were treated with 3-aminopropyl-triethoxysilan (Sigma, München, Germany; 2%, in acetone) at 20°. After 1 hr the cover glasses were washed in distilled H_2O, dried and each placed into one well of six-well multiplates (Falcon Primaria, Becton Dickinson). Cultured HUVEC were trypsinized and transferred to the prepared multiplates. When confluency was reached 1×10^6 PBL was added carefully to each well. After incubation for various time periods (if not otherwise indicated: 0, 1, 2, 4, 8, 12, 24, 48 and 72 hr at 37°) the multiplates were washed four times with warmed (37°) M199 to remove all non-adherent lymphocytes. The remaining cells were fixed with glutardialdehyde (Merck, Darmstadt, Germany; 1%). Bound PBL (adherent and penetrated cells) were counted in five different fields ($5 \times 0{\cdot}25$ mm^2) using a phase contrast microscope (20 × objective). Penetrated PBL were recognized and counted using reflection interference contrast microscopy. Combined phase contrast and reflection interference contrast microscopy provided direct evaluation of (1) percentage of all PBL adhering to HUVEC and (2) percentage of adherent PBL penetrating through the HUVEC monolayers.

Cytokines

IFN-α (1000 U/ml), IFN-β (1000 U/ml), IFN-γ (Sigma; 500 U/ml) or IL-1 (Seromed; 10 U/ml) were added to the PBL–HUVEC co-cultures in four different variations (I–IV).

Variations I and II. HUVEC were pretreated with cytokines for a short (2 hr—variation I) or long (72 hr—variation II) period. After 2 or 72 hr, respectively, cytokine-containing medium was removed and PBL were added to HUVEC in cytokine-free medium.

Variation III. PBL were added to HUVEC monolayers together with cytokines. Following PBL addition both adherent and penetrated cells were counted after 0, 1, 2, 4, 8, 12, 24, 48 and 72 hr if not otherwise indicated.

Variation IV. To investigate the adhesion and penetration process depending on cytokine incubation time PBL were added to HUVEC after variable time-points (0, 1, 2, 4, 8, 12, 24, 48 and 72 hr following IFN-γ incubation of HUVEC). After 2 hr non-adherent cells were washed off in each case and adherent and penetrated cells counted.

Adhesion molecule staining

Cultured HUVEC were trypsinized and transferred to 96-well multiplates (Falcon Primaria, Becton Dickinson). HUVEC were stimulated with cytokines IFN-α, -β, -γ or IL-1 for 2 or 72 hr, respectively. After 0, 1, 2, 4, 8, 12, 24, 48 and 72 hr following cytokine incubation 96-well multiplates were washed and cells fixed (Primafix, Camon, Wiesbaden, Germany). The adhesion molecules ICAM-1, ELAM-1 and VCAM-1 were marked by monoclonal antibodies (clone BBIg-I1, BBIg-E6 or BBIg-V1, respectively; all Biermann, Bad Nauheim, Germany). For conventional immunohistochemical staining HUVEC were then incubated with blocking serum, biotinylated anti-mouse IgG, avidin and biotinylated horseradish peroxidase (HRP; Vectastain, ABC-kit, Camon). Staining intensity was investigated by phase contrast microscopy and subsequently related to steadily increasing intensity values ranging from '0' (no staining intensity) to '7' (very high staining intensity). In addition, quantitative analysis was performed in triplicate by fluorometric measurements. HUVEC stained with primary antibody were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zymed, Ade Laborbedarf, München, Germany) for 1 hr at 4° and fluorescence intensity evaluated using a Cytofluor (Cytofluor 2300 system, Millipore, Eschborn, Germany; $\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm).

Staining of lymphocyte subsets

To investigate the role of different PBL subpopulations in the cellular binding process HUVEC were trypsinized and transferred to 24-well multiplates (Falcon Primaria, Becton Dickinson). When confluency was reached isolated PBL were added to the monolayer. After 8 and 72 hr culture plates were washed four times with warmed (37°) M199 to remove all non-adherent lymphocytes. The remaining cells were dried overnight and then fixed with ice-cold (−20°) methanol/acetone. PBL were marked by the monoclonal antibodies anti-CD5 (Leu-1), anti-CD8 (Leu-2a), anti-CD4 (Leu-3a), anti-CD16 (Leu-11b) and anti-CD22 (Leu-14; all Becton Dickinson). Cells were then incubated with blocking serum, biotinylated anti-mouse IgG, avidin and biotinylated HRP (Vectastain, ABC-kit, Camon). Percentage of positively stained PBL were evaluated by phase contrast microscopy.

Statistics

Each experiment was repeated five times if not otherwise indicated. All figures show data of one representative experiment. Data represent the mean values of five counts. Mean SD were *c.* 10%.

RESULTS

Effect of cytokines (2 hr prestimulus or continuous stimulus) on PBL adhesion and penetration

As shown in Fig. 1 significant adhesion of PBL to unstimulated HUVEC was detectable after 1 hr (6·1%). A plateau was reached after 4 hr (32·1%) which lasted up to 72 hr. Simulta-

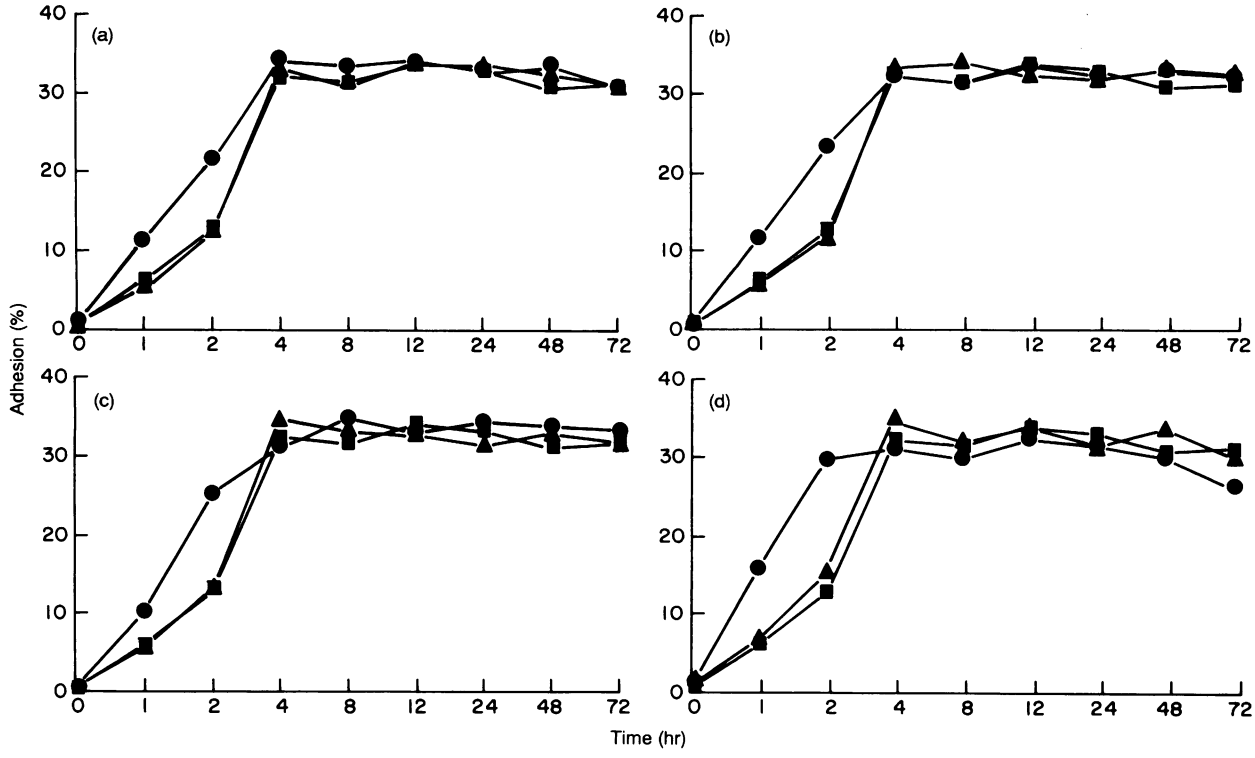

Figure 1. Adhesion kinetics of PBL. Lymphocytes were added to allogeneic HUVEC. Non-attached cells were washed off after different time periods (hr) and bound cells counted by phase contrast microscopy (%). Pretreatment of HUVEC with either IFN-α (a), IFN-β (b), IFN-γ (c) or IL-1 (d) for 2 hr led to a significant increase in the binding rate during the early phase of the adhesion process. Simultaneous stimulation did not change the adhesion behaviour compared to the control. Results of one out of five distinct experiments that gave similar data. Each point represents the mean of five counts. Control, no stimulation (■); 2 hr prestimulation (●); simultaneous stimulation (▲).

neous addition of cytokines and PBL to HUVEC did not change cellular adhesion behaviour. However, when HUVEC were pretreated with cytokines for 2 hr marked differences in the early phase of PBL attachment could be observed. Compared to the control, adhesion rate after 2 hr differed by +240% (IL-1 stimulation), +170%, +180% or +200% (IFN-α, -β, -γ stimulation), respectively. After 4 hr the adhesion rate of unstimulated PBL approximated the adhesion rate of stimulated cells. Therefore, the cytokines used did not enhance overall PBL adhesion but accelerated the early phase of attachment.

Effect of cytokines (2 hr prestimulus or continuous stimulus) on PBL penetration

Two per cent of adherent cells penetrated unstimulated HUVEC after 2 hr. The penetration curve was steepest between 2 and 4 hr but maximum values were obtained after 8–24 hr. Between 8 and 9% of adherent or less than 3% of the total number of PBL had penetrated at this time (Fig. 2). Addition of IFN-α, -β or -γ to the HUVEC cultures resulted in a significant enhancement of the penetration rate compared to the control, irrespective of whether a short or a continuous stimulus was used (Fig. 2). The penetration rate after 4 hr increased by +300% (IFN-α or -γ stimulus) or +270% (IFN-β stimulus). Continuous stimulus was effective over the whole period measured whereas a 2-hr prestimulus became ineffective after 24–48 hr (Fig. 2a–c). Interestingly, IL-1 treatment did not cause enhancement but only acceleration of the penetration process in the early phase of lymphocyte emigration (Fig. 2d).

Effect of long-term pretreatment with different cytokines on PBL adhesion and penetration

PBL were added to HUVEC monolayers and adherent and penetrated cells counted after 0·5, 1, 2, 4, 8 and 12 hr. A 72-hr prestimulus with IFN-α, -β did not change the adhesion and penetration rate. A 72-hr prestimulus with IFN-γ or IL-1, however, was able to influence both processes (Fig. 3). With regard to lymphocyte adhesion IFN-γ-and IL-1-mediated effects were most distinct after 1 and 2 hr, and were more pronounced in the case of pretreatment with IL-1. Penetration experiments again demonstrated enhancing properties of IFN-γ and accelerating properties of IL-1.

Effect of cytokine incubation time on PBL adhesion and penetration (variation IV)

To investigate possible changes in the velocity of the adhesion and penetration processes PBL were added for only 2 hr to HUVEC at different time-points ranging from 0 to 72 hr after stimulation with IFN-γ (Fig. 4). The rate of adherent cells per 2 hr did not change between 0 and 1 hr; the rate of penetrated cells per 2 hr did not change between 0 and 4 hr following IFN-γ incubation. A marked increase could be observed when lymphocytes were added to HUVEC after 2 or 8 hr, respectively. Interestingly, the penetration kinetics paralleled the kinetics of IFN-γ-evoked ICAM-1 up-regulation (Fig. 5c).

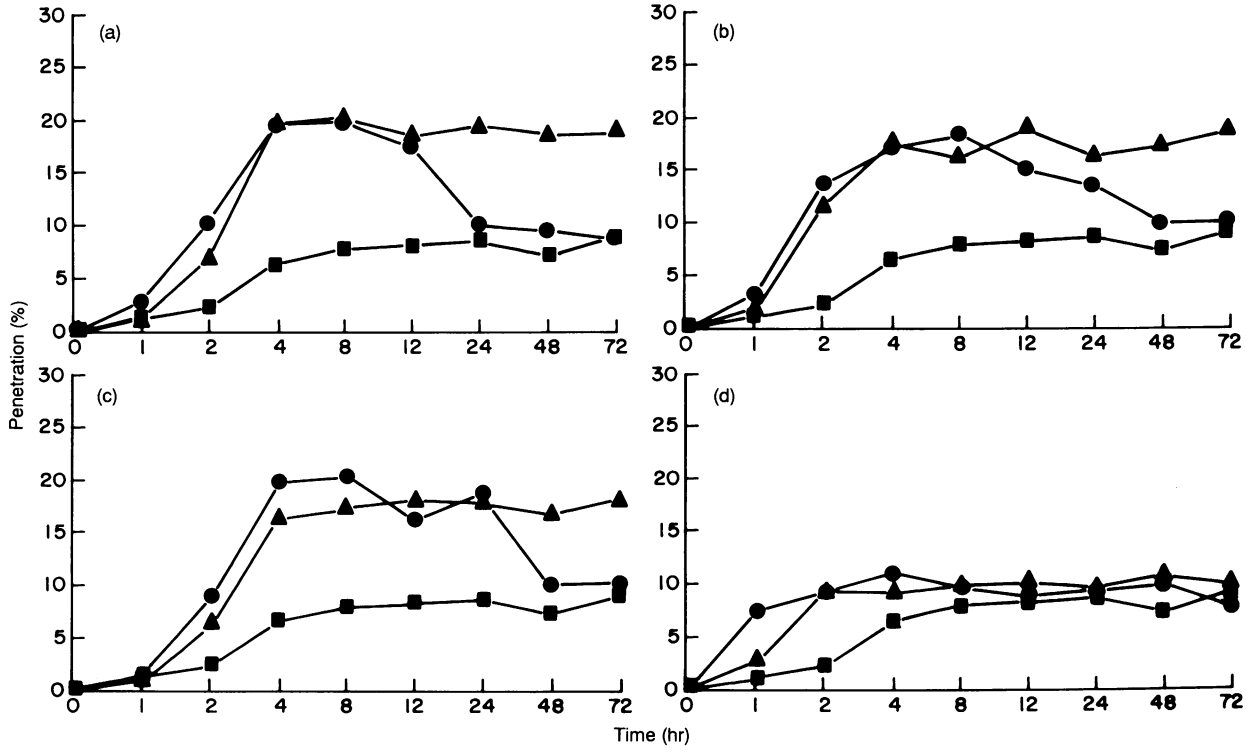

Figure 2. Penetration kinetics of PBL. Lymphocytes were added to allogeneic HUVEC. Non-attached cells were washed off after different time periods (hr) and penetrated cells evaluated by combined phase contrast–reflection interference contrast microscopy (%). With regard to IFN-α (a), IFN-β (b) and IFN-γ (c) PBL penetration rate could be enhanced whether a short or continuous stimulus was used. However continuous stimulus led to long-lasting changes in penetration behaviour, whereas a 2-hr prestimulus influenced lymphocyte penetration only at 24 hr. In strong contrast IL-1 treatment altered the penetration rate only in the early phase of the emigration process. In addition maximum values never exceeded maximum control values (d). Results of one representative experiment of five. Each point is the mean of five counts. Control, no stimulation (■); 2 hr prestimulation (●); simultaneous stimulation (▲).

Kinetics of adhesion molecule expression

Continuous stimulation of HUVEC by the different cytokines resulted in quantitatively higher ICAM-1, ELAM-1 or VCAM-1 expressions than after 2 hr stimulation. However the time-course of molecule expression was similar regardless of whether a continuous or short-term stimulus was used. Cytokines up-regulated ICAM-1 expression on endothelial cell membranes after 4 hr when IL-1 was used (Fig. 5d) or after 8 hr when IFN-α, -β or -γ were added (Fig. 5a–c). ELAM-1 expression was initiated between 4 and 12 hr by IL-1 (Fig. 6a). VCAM-1 did not respond to IFN but a weak expression on HUVEC occurred with IL-1 (Fig. 6b). As opposed to ICAM-1, VCAM-1 and ELAM-1 expressions were not distributed homogeneously on the endothelial cell monolayer.

Quantification of lymphocyte subset profiles

To examine which population of lymphocytes preferably adhered to HUVEC monolayers bound lymphocytes were stained in an early (8 hr) and late (72 hr) phase of adhesion with monoclonal antibodies anti-CD5, anti-CD8, anti-CD4, anti-CD16 and anti-CD22. Table 1 shows the mean results of five experiments. The pattern of bound PBL did not change between the early and late phases of the cellular emigration process. However, when compared to distribution in peripheral blood, a shifting of the ratio T/B lymphocytes in favour of helper T cells was obvious.

DISCUSSION

Lymphocyte emigration from blood into the donor organ is an important step in rejection processes. *In vivo* and *in vitro* assays were established to gain better insight into the mechanisms of lymphocyte–endothelial cell interactions. Stamper and Woodruff[5] and Woodruff *et al.*[6] demonstrated specific lymphocyte binding to endothelium on frozen sections of rat lymph nodes. Zweiman *et al.*[8] measured the percentage of ^{51}Cr-labelled lymphocytes binding to confluent endothelial cell monolayers in flat-bottomed microwells. However, our method of combined phase contrast and reflection interference contrast microscopy allowed us to distinguish PBL that only adhered to HUVEC from PBL that had actually penetrated through the monolayer.

Under cytokine-free conditions 30–35% of all PBL, mostly CD4$^+$ cells (Table 1), adhered to HUVEC monolayers. Cavender[12] and Oppenheimer-Marks and Ziff[13] reported similar binding rates. Masuyama *et al.*[14] estimated the proportion of adherent T cells to be only 2–3%. They, however, removed not only non-adhering but also loosely bound lymphocytes by washing the culture plates on a magnetic stirrer.

We calculated the total PBL penetration rate to be $< 3\%$. It seems that out of all attached cells only a minor proportion will bind firmly to endothelial cells. Of these a small percentage will penetrate. Because the monoclonal antibodies used in our assay did not penetrate well through the HUVEC monolayer subset profiles of penetrated PBL could not be investigated.

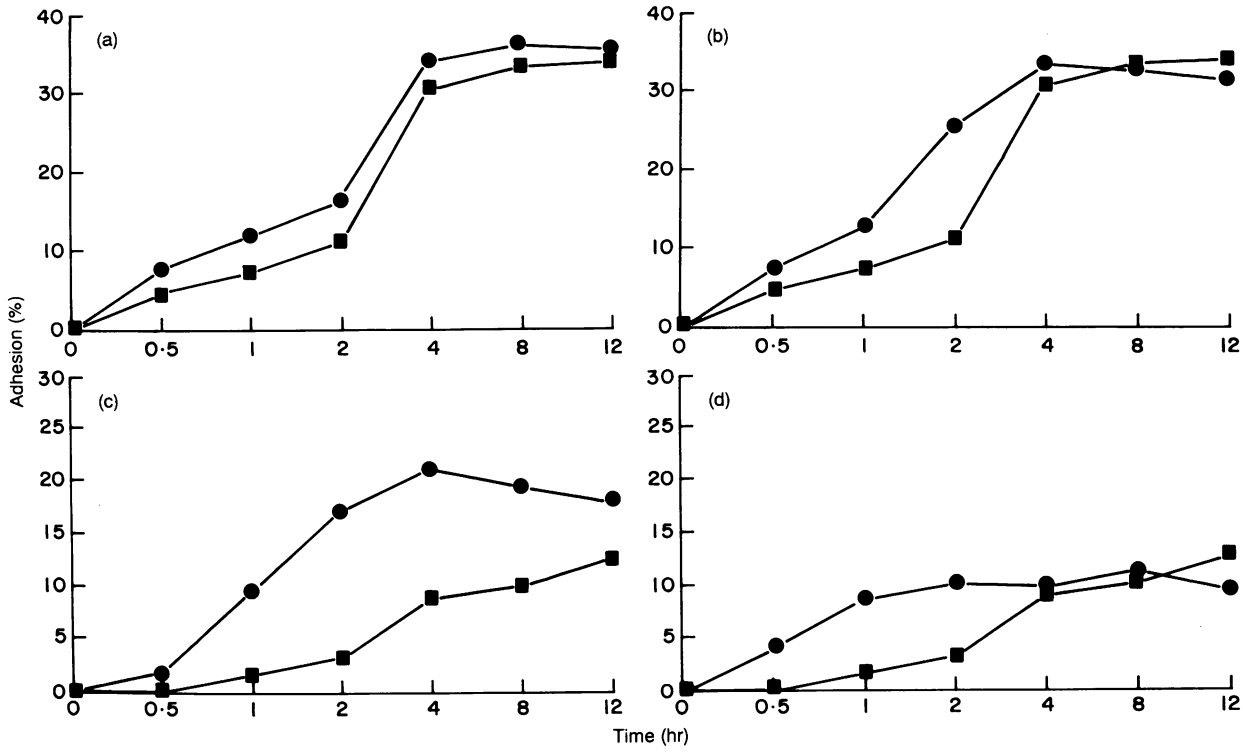

Figure 3. Modulation of adhesion and penetration by long-term stimulation. HUVEC were prestimulated for 72 hr with either IFN-α, -β, -γ or IL-1 and both adherent and penetrated cells evaluated as described in the Materials and Methods. Changes in PBL infiltration rates could be observed when endothelial cells were pretreated with IFN-γ (a,c) or IL-1 (c,d) (but not with IFN-α, -β). Results of one out of five experiments that gave similar data. Each point is the mean of five counts. Control, no stimulation (■); 72 hr prestimulation (●).

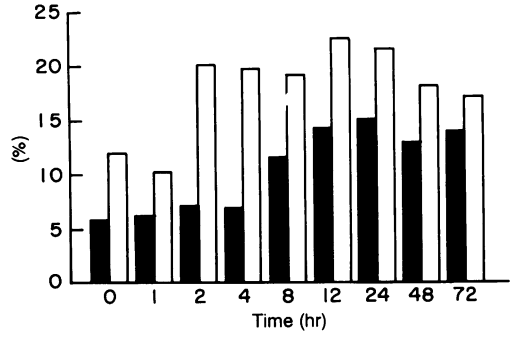

Figure 4. Adhesion and penetration velocity of human PBL. Lymphocytes were added to IFN-γ-stimulated endothelial cells after different time periods (hr) and washed off after 2 hr. Both adhesion (□) and penetration (■) rates per 2 hr were augmented when PBL were added to the culture plates between 2 and 72 or 8 and 72 hr, respectively. Results of one out of five similar results. Each column represents the mean of five counts.

Lymphocyte adhesion to endothelial cells could be modified when endothelial cells were pretreated with cytokines for 2 hr but not when cytokines and PBL were added to HUVEC simultaneously. This coincides with other reports revealing pretreatment of human endothelial monolayers with IFN-γ or IL-1 to be necessary for enhanced leucocyte binding.[13,15-17] Analysis of our kinetic studies might now explain this difference. As shown in Fig. 1 significant lymphocyte adhesion to HUVEC occurred within 60 min of the addition of PBL and reached a plateau after 4 hr. In a preliminary investigation the nonbound cells, harvested between 4 and 72 hr, did not adhere to a second endothelial monolayer indicating that adhesion was limited not by endothelial cells but by lymphocyte subpopulations (data not shown). Thus cytokines are only able to interfere in the early process of PBL adhesion when maximum binding has not yet occurred (although their basic activity is sustained for a longer period of time). We conclude that their mechanisms of action consist more in accelerating than in enhancing the lymphocyte binding process. The cytokine effect was most pronounced when PBL were counted after 2 hr, that is 4 hr after adding the cytokine impulse. If cytokines and PBL had been added simultaneously to HUVEC maximum alterations of the adhesion rate should, therefore, have been expected after 4 hr. However at that time the maximum amount of (the suited) PBL was already adherent and therefore could not be further enhanced by IFN-α, -β, -γ or IL-1, respectively.

As a consequence of cytokine-evoked acceleration more lymphocytes might be able to penetrate through the endothelium. Surprisingly, the emigration process was enhanced by IFN-α, -β or -γ, but not by IL-1. This finding is of importance because IL-1, but not IFN-α, -β or -γ, could be shown to express adhesion molecules ELAM-1 and VCAM-1 on HUVEC membranes (see below). Oppenheimer-Marks and Ziff[13] measured lymphocyte migration through HUVEC monolayers into nitrocellulose filters located below the monolayer. They observed an IFN-α or -γ evoked increase in PBL migration and provided evidence that IFN-γ-enhanced migration was not due to

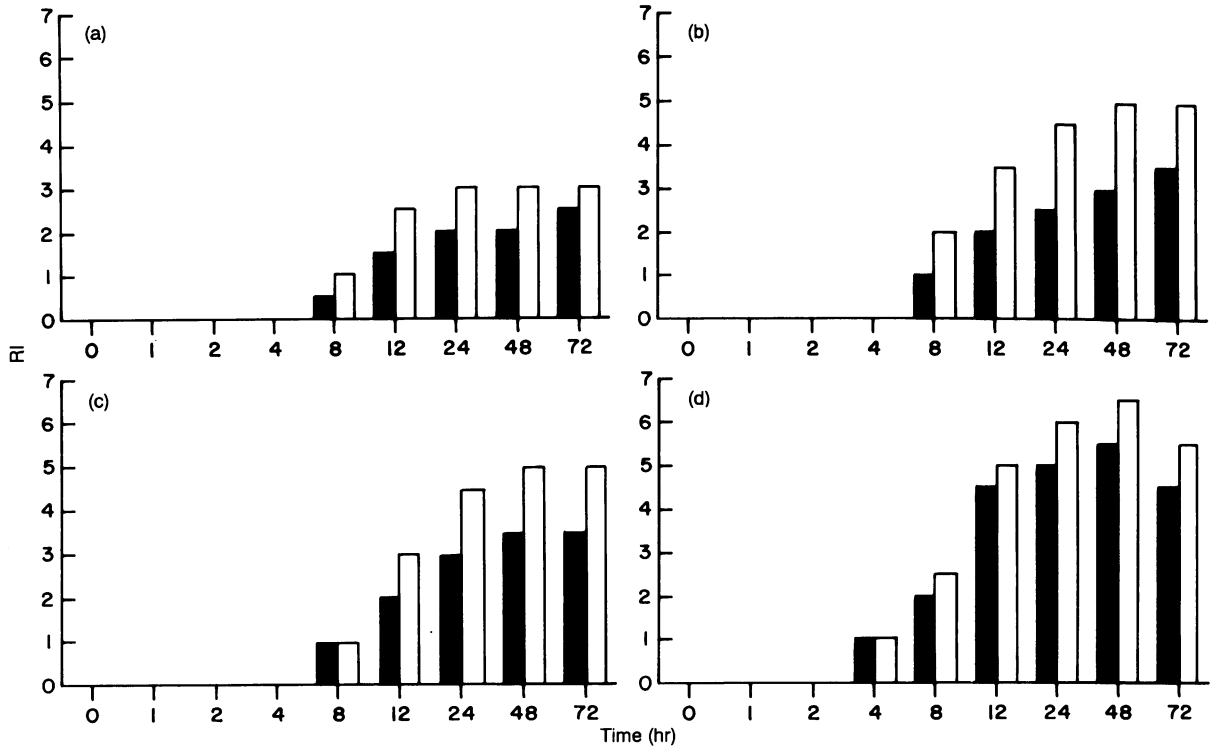

Figure 5. ICAM-1 up-regulation. Endothelial cells were treated for 2 or 72 hr with IFN-α (a), IFN-β (b), IFN-γ (c) or IL-1 (d), respectively. Cells were fixed and stained after different time periods (hr) as described in the Materials and Methods and the staining intensity evaluated by phase contrast microscopy (RI = relative intensity). ICAM-1 up-regulation did not depend on the cytokine incubation time [2 hr incubation (□); 72 hr stimulation (■)]. When IFN were used in the assays staining became positive after 8 hr, when IL-1 was added after 4 hr. Mean of five experiments. Mean SD was < 10%.

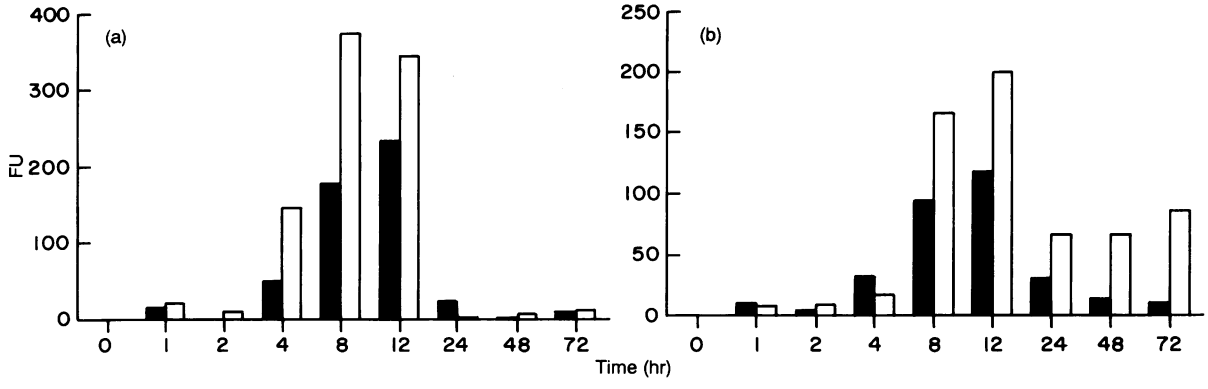

Figure 6. Fluorometric detection of ELAM-1 and VCAM-1 expression. Endothelial cells were incubated with IL-1 for 2 (■) or 72 hr (□) as explained in the Materials and Methods. ELAM and VCAM expression were quantified by Cytofluor analysis detecting the amount of fluorescence/well (FU = fluorescence unit). Each column represents the mean value of six (ELAM-1) or eight (VCAM-1) tests. SD of each assay was < 10%.

increased binding of T cells to HUVEC, but rather to an action on the endothelial cells themselves.

Although prestimulation of endothelial cells for 2 hr was necessary to change the adhesion and penetration behaviour of human lymphocytes, a long-term preincubation of HUVEC for 72 hr by IFN-α or -β for 72 hr showed no effect. It seems that the influence of IFN-α and -β on cellular emigration is only of limited duration. In contrast, IFN-γ and IL-1 show long-term activity. Hence we can distinguish between three classes of cytokines. Class I (short efficacy): IFN-α and -β; class II (long efficacy) IFN-γ, which accelerates PBL adhesion and enhances PBL penetration; class III (long efficacy) IL-1, which accelerates both PBL adhesion and penetration but does not enhance penetration.

The cellular emigration process seems to be correlated with up-regulation or *de novo* synthesis of several adhesion molecules. ICAM-1 was shown to have an effect at the level of lymphocyte adhesion mediated by endothelial cells.[18-20] Rice *et al.*[21,22] suggested that VCAM-1 may be a central mediator of lymphocyte recruitment into inflammatory sites. In addition T-

Table 1. Distribution pattern of PBL subtypes attached to endothelial cell monolayers†

Subtype	CD5	CD8	CD4	CD16	CD22
Distribution after 8 hr (%)	90	< 5	60–70	< 1	10
Distribution after 72 hr (%)	> 90	2–5	70	< 1	5–10
Distribution in peripheral blood (control, in %)	60–80	15–25	30–40	5–15	15–25

† PBL ($0{\cdot}5$–1×10^6/ml) were added to HUVEC monolayer. Non-adherent cells were washed off after 8 or 72 hr, respectively. Lymphocyte subpopulations were stained using monclonal antibodies (see the Materials and Methods) and the percentage with respect to the total cell number evaluated. The distribution pattern of attached cells was compared to the distribution pattern in peripheral blood. The results are mean values of five different tests. SD was below 10%.

cell infiltration was accompanied by the expression of ELAM-1, as described recently.[23,24]

Our analysis revealed an increased expression of ICAM-1, VCAM-1 and ELAM-1 independent of duration of the cytokine stimulus. Observation of positively stained cells by phase contrast microscopy showed homogeneous ICAM-1 but heterogeneous ELAM-1 and VCAM-1 staining. This may indicate that there are several endothelial cell subpopulations of which some are better qualified to express ELAM or VCAM molecules than others.

Because lymphocytes adhered to HUVEC before up-regulation or new expression of ICAM-1, ELAM-1 or VCAM-1 was detected on endothelial cell membranes, these molecules cannot be responsible for beginning the adhesion process. This assumption is in strong contrast to other investigations which assume adhesion molecules to be central mediators for lymphocyte adhesion.[14,18,21,22,25] However, extensive kinetic studies have not been carried out. The possibility that subtle (undetectable) increases in one or more of those molecules can nevertheless stimulate adhesion should be excluded, because blocking antibodies to ICAM-1, ELAM-1 or VCAM-1 had no effect on lymphocytes in this early adhesion period (data not shown). We hypothesize that cytokines generate an adhesion molecule-independent process before or immediately after attachment of PBL to endothelial cell membranes. This process might include, at least in part, the cytoskeletal organization of lymphocytes. Changes in cytoskeletal structures may trigger the increase in PBL adhesiveness and allow spreading and movement over the endothelial cell substratum; videoscope analysis confirmed that the early phase of PBL attachment to endothelial cells was in fact associated with changes in cell shape and locomotion (data not shown). An interesting explanation that supports our hypothesis is presented by Dustin and Springer.[26] They showed that activation of lymphocytes by the T-cell receptor converted LFA-1 (lymphocyte function-associated molecule 1) to a high avidity state in the early phase of lymphocyte adhesion. Based on these findings they proposed an interaction between lymphocyte LFA-1 in the high avidity state and endothelial ICAM-1, driving the adherence–non-adherence equilibrium towards stable adherence. Because LFA-1 integrates the extracellular environment with the cytoskeleton,[27–29] either a parallel or time shifted regulation of cytoskeletal organization and LFA-1 by intracellular second messengers should be taken into consideration.

On the other hand, ICAM-1, ELAM-1 and VCAM-1 may be responsible for stabilizing the late binding phase and/or regulating the penetration process. This hypothesis is based on the observation that at the same time as ICAM-1 up-regulation was evoked by IFN-γ, PBL penetration velocity was augmented.

It should also be mentioned that the interplay between HUVEC and PBL may involve not only physical interactions by the activation of the cytoskeleton and (later) receptor expression, but also the production of soluble chemoattractants. In this regard HUVEC are known to produce IL-8, which in turn is induced by IL-1.[30] IL-8 was recently described to modulate T-lymphocyte trafficking.[31]

In conclusion these results present new insights into the course of cellular infiltration and its correlation to cytokine release and adhesion molecule expression. Ongoing studies should investigate possible mechanisms responsible for the modulation of the early adhesion phase. Knowledge of such triggering events might be helpful in understanding the rejection mechanisms and allow for better immunosuppressive regimens.

ACKNOWLEDGMENTS

We wish to thank R. Schroeder and E. Harbich for excellent technical assistance. This work was supported in part by Dr Paul und Cilli Weill-Stiftung.

REFERENCES

1. Duijvestijn A. & Hamann A. (1989) Mechanisms and regulation of lymphocyte migration. *Immunol. Today*, **10**, 23.
2. Jutila M.A., Berg E.L., Kishimoto T.K., Picker L.J., Bargatze R.F., Bishop D.K., Orosz C.G., Wu N.W. & Butcher E.C. (1989) Inflammation-induced endothelial cell adhesion to lymphocytes, neutrophils, and monocytes. *Transplantation*, **48**, 727.
3. Kupiec-Weglinski J.W. & Tilney N.L. (1989) Lymphocyte migration patterns in organ allograft recipients. *Immunol. Rev.* **108**, 63.
4. Springer T.A. (1990) Adhesion receptors of the immune system. *Nature*, **346**, 425.
5. Stamper H.B. & Woodruff J.J. (1976) Lymphocyte homing into lymph nodes: *in vitro* demonstration of the selective affinity of recirculating lymphocytes for high endothelial venules. *J. exp. Med.* **144**, 828.
6. Woodruff J.J., Katz I.M., Lucas L.E. & Stamper H.B. (1977) An *in vitro* model of lymphocyte homing: membrane and cytoplasmic events involved in lymphocyte adherence to specialized high endothelial venules of lymph nodes. *J. Immunol.* **119**, 1603.
7. Yu C.-L., Haskard D.O., Cavender D., Johnson A.R. & Ziff M. (1985) Human gamma interferon increases the binding of T lymphocytes to endothelial cells. *Clin. exp. Immunol.* **62**, 554.
8. Zweiman B., Moskovitz A.R. & Lisak R.P. (1982) Quantitative assessment of the adherence of normal human blood mononuclear cells to vascular endothelial cell monolayers. *Cell. Immunol.* **68**, 165.

9. ROTHLEIN R., DUSTIN M.L., MARLIN S.D. & SPRINGER T.A. (1986) A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J. Immunol.* **137,** 1270.
10. RICE G.E. & BEVILACQUA M.P. (1989) An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. *Science,* **246,** 1303.
11. BEVILACQUA M.P., STENGELIN S., GIMBRONE M.A. & SEED B. (1989) Endothelial leucocyte adhesion molecule 1: An inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science,* **243,** 1160.
12. CAVENDER D.E. (1989) Lymphocyte adhesion to endothelial cells *in vitro*: Models for the study of normal lymphocyte recirculation and lymphocyte emigration into chronic inflammatory lesions. *J. invest. Dermatol.* **93,** 88S.
13. OPPENHEIMER-MARKS N. & ZIFF M. (1988) Migration of lymphocytes through endothelial cell monolayers: augmentation by interferon-γ. *Cell. Immunol.* **114,** 307.
14. MASUYAMA J., MINATO N. & KANO S. (1986) Mechanisms of lymphocyte adhesion to human vascular endothelial cells in culture. *J. clin. Invest.* **77,** 1596.
15. BEVILACQUA M.P., POBER J.S., WHEELER M.E., COTRAN R.S. & GIMBRONE M.A. (1985) Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leucocytes, monocytes, and related leucocyte cell lines. *J. clin. Invest.* **76,** 2003.
16. CAVENDER D.E., HASKARD D.O., JOSEPH B. & ZIFF M. (1986) Interleukin 1 increases the binding of human B and T lymphocytes to endothelial cell monolayers. *J. Immunol.* **136,** 203.
17. MORZYCKI W., SADOWSKA J. & ISSEKUTZ A.C. (1990) Interleukin-1 and tumor necrosis factor α induced polymorphonuclear leucocyte–endothelial cell adhesion and transendothelial migration *in vitro*: the effect of apical versus basal monolayer stimulation. *Immunol. Lett.* **25,** 331.
18. BOYD A.W., NOWOTNY J.R., WICKS I.P., SALVARIS E., WELCH K. & WAWRYK S.O. (1989) The role of accessory molecules in lymphocyte activation. *Transpl. Proc.* **21,** 38.
19. BOYD A.W., WAWRYK S.O., BURNS G.F. & FECONDO J.V. (1988) Intercellular adhesion molecule 1 (ICAM-1) has a central role in cell–cell contact-mediated immune mechanisms. *Proc. natl. Acad. Sci. U.S.A.* **85,** 3095.
20. DUSTIN M.L., GARCIA-AGUILAR J., HIBBS M.L., LARSON R.S., STACKER S.A., STAUNTON D.E., WARDLAW A.J. & SPRINGER T.A. (1989) Structure and regulation of the leucocyte adhesion receptor LFA-1 and its counterreceptors, ICAM-1 and ICAM-2. *Cold Spring Harb. Symp. Quant. Biol.* **54,** 753.
21. OSBORN L., HESSION C., TIZARD R., VASSALLO C., LUHOWSKYJ S., CHI-ROSSO G. & LOBB R. (1989) Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell,* **59,** 1203.
22. RICE G.E., MUNRO J.M. & BEVILACQUA M.P. (1990) Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. *J. exp. Med.* **171,** 1369.
23. DAML N. K., EBERHARD C. & VAN DER VIEREN M. (1991) Direct interaction with primed $CD4^+$ $CD45R0^+$ memory T lymphocytes induces expression of endothelial leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1 on the surface of vascular endothelial cells. *Eur. J. Immunol.* **21,** 2915.
24. SHIMIZU Y., NEWMAN W., GOPAL T.V., HORGAN K.J., GRABER N., BEALL L.D., VAN SEVENTER G.A. & SHAW S. (1991) Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *J. Cell Biol.* **113,** 1203.
25. FUGGLE S.V., SANDERSON J.B., GRAY D.W.R., RICHARDSON A. & MORRIS P.J. (1993) Variation in expression of endothelial adhesion molecules in pretransplant and transplanted kidneys—correlation with intragraft events. *Transplantation,* **55,** 117.
26. DUSTIN M.L. & SPRINGER T.A. (1989) T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature,* **341,** 619.
27. KISHIMOTO T.K., O'CONNOR K., LEE A., ROBERTS T.M. & SPRINGER T.A. (1987) Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell,* **48,** 681.
28. HYNES R.O. (1987) Integrins: a family of cell surface receptors. *Cell,* **48,** 549.
29. KISHIMOTO T.K., LARSON R.S., CORBY A.L., DUSTIN M.C., STAUNTON D.E. & SPRINGER T.A. (1989) The leucocyte integrins. *Adv. Immunol.* **46,** 149.
30. SICA A., MATSUSHIMA K., VAN DAMME J., WANG J.M., POLENTARUTTI N., DEJANA E., COLOTTA F. & MANTOVANI A. (1990) IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. *Immunology,* **69,** 548.
31. BARKER J., JONES M.L., MITRA R.S., CROCKETT-TORABE E., FANTONE J.C., KUNKEL S.L., WARREN J.S., DIXIT V.M & NICKOLOFF B.J. (1991) Modulation of keratinocyte-derived interleukin-8 which is chemotactic for neutrophils and T lymphocytes. *Am. J. Pathol.* **139,** 869.