Clin. exp. Immunol. (1989) **77**, 206–210

Adherence of alloreactive lymphocytes to human arterial endothelial cell monolayers

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(Accepted for publication 18 April 1989)

SUMMARY

Early events of cellular infiltration during allograft rejection involve interactions between alloreactive lymphocytes and vascular endothelium. These interactions have been studied in an *in vitro* model of lymphocyte adherence to human arterial endothelial cell (HAEC) monolayers. Alloreactive lymphocytes primed against donor HLA antigens (DPL) or unrelated HLA antigens from a third party (TPL) were studied for their adherence to HAEC derived from organ transplant donors. DPL exhibited a degree of adherence to donor HAEC which was nearly twice that of the TPL population and developed a morphologically blastoid appearance. DPL adherence was dependent on the recognition of donor HLA antigens expressed on the HAEC surface and investigation of DPL and TPL adherence at various lymphocyte concentrations showed that the binding of DPL and TPL to HAEC was saturable. Monoclonal antibody (MoAb) directed against HLA class I antigens showed inhibition of only DPL adherence to HAEC monolayers expressing donor class I HLA antigens, whereas MoAb directed against other HAEC surface antigens failed to inhibit either the DPL or TPL populations. The results of this study suggest that in a transplant situation, lymphocyte activation and adherence to the graft endothelium is increased in the context of allorecognition.

Keywords lymphocyte adherence endothelial cells HLA antigens allorecognition alloreactive T cells

INTRODUCTION

Although the presence of alloreactive lymphocytes in cellular infiltrates of human organ transplants undergoing acute rejection has been well documented, the mechanisms of lymphocyte infiltration into the allograft are not well understood. Since the vascular endothelium forms the principle barrier between the blood stream and the tissues, the development of cellular rejection requires lymphocytes to adhere and migrate through the graft endothelium. In previous studies, we have shown that T lymphocytes infiltrating human cardiac and hepatic allografts can be propagated in *in vitro* cultures supplemented with interleukin-2, a lymphokine that induces proliferation of activated T lymphocytes. Biopsies obtained during the early post-transplant period were shown to yield T cells reactive towards donor class I HLA antigens frequently, whereas later biopsies grew generally donor class II HLA-specific lymphocytes (Fung *et al.*, 1986; Zeevi *et al.*, 1986). This has led to the postulate of a sequential appearance of class I- followed by class II-specific T cells infiltrating the allograft (Duquesnoy *et al.*, 1987). Since this infiltration results from a penetration of lymphocytes through the vascular endothelium, we have developed an *in vitro* model to study the interactions between alloreactive lymphocytes and human arterial endothelial cell (HAEC) cultures derived from transplant donors. Monoclonal antibody (MoAb) binding studies have previously shown that HAEC have constitutive expression of factor VIII-related antigen and class I HLA antigens, but display appreciable levels of class II HLA antigens only after 48–72 hours of treatment with gamma interferon (γ-IFN) or lymphocyte supernatants (Colson *et al.*, 1987; Markus *et al.*, 1987).

To further study the interactions between alloreactive T cells and vascular endothelium, we have developed an *in vitro* assay on the adherence of alloactivated lymphocytes to HAEC monolayers. Here we investigate the nature of this *in vitro* adherence and the role that allorecognition of HLA antigens has in this unique lymphocyte–endothelial cell interaction.

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MATERIALS AND METHODS

Preparation and culture of endothelial cells

Human aorta specimens were obtained from cardiac and hepatic organ donors. Samples were transported in sterile HBSS (Hanks' balanced salt solution without Ca and Mg, GIBCO,

Table 1. Effect of initial lymphocyte concentration on the numbers and percentage of adherent lymphocytes

Total cells added/ml	No. of adherent lymphocytes ($\times 10^3$) DPL	TPL	Adherent cells (%) DPL	TPL	DPL:TPL	P*
5×10^3	0·24±0·07	0·15±0·06	47·7±13·9	30·0±13·0	—	NS
10^4	0·45±0·1	0·18±0·07	44·7±13·2	17·7±8·9	—	NS
5×10^4	2·3±0·17	1·1±0·1	46·1±3·5	22·5±2·8	2·05	<0·001
10^5	3·6±0·5	1·8±0·2	36·5±5·0	18·4±2·2	1·98	<0·005
5×10^5	6·1±0·5	4·3±0·3	12·3±1·0	8·7±0·7	1·42	<0·02

Varying concentrations of DPL or TPL were added to the HAEC monolayers for 18 h at 37°C. Nonadherent lymphocytes were removed and data from triplicate assays was pooled and presented as means±s.e.

* Mann–Whitney rank sum test comparing DPL and TPL populations.

Grand Island, NY) containing 56 μg/ml gentamicin (Gibco) and 10 U/ml heparin (O'Neal, Jones & Feldman, St Louis, MO). In addition, donor splenocytes were obtained, separated on a Ficoll-Hypaque gradient, and stored in liquid nitrogen prior to use.

HAEC were harvested and cultured using methods previously described (Jarrell *et al.*, 1984). Briefly, specimens were clamped, washed with sterile HBSS, infused with a 0·1% collagenase (Sigma, St Louis, Mo) solution and incubated for 30 min at 37°C. After flushing with HBSS, HAEC were sedimented at 1200 rev/min for 10 min and resuspended in 2·5 ml in endothelial cell media with sera consisting of medium M199 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), 10% pooled human serum, 4 mM L-glutamine (B & B/Scot Lab, Fisheville, RI), 24 mM HEPES-buffer (B & B/Scott Lab), 56 μg/ml gentamicin, 250 μg/ml fungizone (Gibco), 10 U/ml heparin and 100 μg/ml endothelial cell growth factor (ECGF) (Collaborative Research, Lexington, MA). Primary cultures were established in 25-cm² tissue culture flasks (Costar, Cambridge, MA) and incubated at 37°C in a 5% CO_2 humidified atmosphere.

Endothelial cell origin was verified using three separate criteria: (1) all cultured HAEC exhibited a typical monolayer with cobblestone morphology under light microsopy; (2) flow cytometric analysis with antibody against human factor VIII-related antigen (Atlantic Antibodies, Scarborough, ME) demonstrated >95% positive staining in all HAEC cultures; and (3) Weibel–Palade bodies, cytoplasmic organelles unique to endothelial cells (Weibel & Palade, 1964), were detected by electron microscopy.

Preparation of human lymphocytes

Human lymphocytes were isolated from peripheral venous blood by Ficoll-Hypaque gradient separation, washed two times in RPMI 1640 (B & B/Scott Lab), and suspended in tissue culture media (RPMI 1640 supplemented with 10% pooled human serum, 4 mM L-glutamine, 24 mM HEPES-buffer, and 56 μg/ml gentamicin). Mixed lymphocyte cultures (MLC) were set up in upright 25-cm² flasks using equal quantities (10^7 cells in 7 ml) of responder cells and irradiated (2000 rad) stimulator cells of either donor or HLA unrelated third party origin. MLC cultures were supplemented with media on days 6, 8 and 10 and were used for adherence assays on days 12–14, following documentation of the MLC specificity.

Table 2. Effect of HLA antigen presentation on the specificity of the adherent lymphocyte population

Lymphocyte population	Cells adherent to designated HAEC monolayer (%)* Donor HAEC	Third party HAEC	Unrelated HAEC
DPL	22·0±2·3	11·9±1·6	14·7±1·7
TPL	15·5±1·8	24·7±2·4	13·4±0·9
P†	<0·01	<0·02	NS

* DPL and TPL adherence was compared on six HAEC monolayers from each of the respective donors, third party or HLA class I unrelated individuals.

† DPL and TPL adherence on the individual HAEC monolayers was analysed by the Mann–Whitney rank sum test.

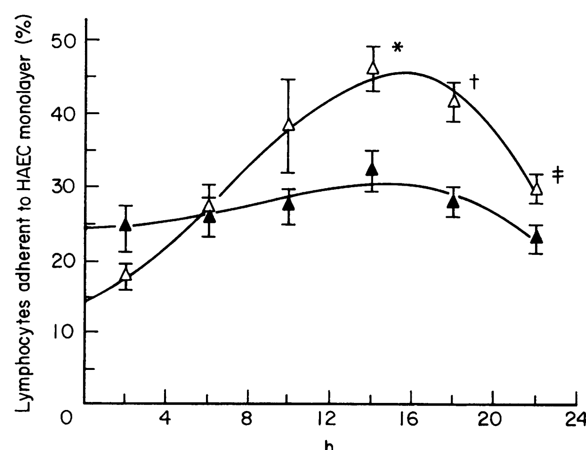

Fig. 1. Effect of lymphocyte-endothelial co-culture time on the percentage of adherent lymphocytes. DPL (△) or TPL (▲) were each added to three separate HAEC monolayers of donor origin at a concentration of 10^5/ml for various time periods. * $P<0·01$; † $P<0·005$; and ‡ $P<0·05$.

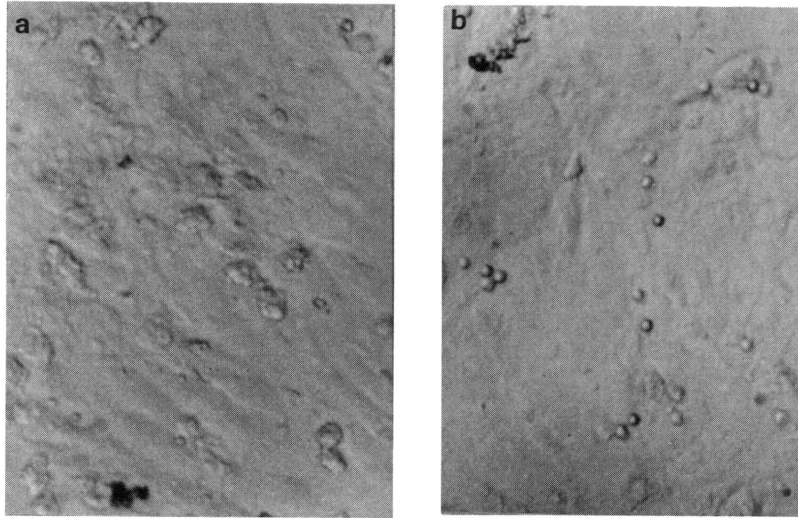

Fig. 2. Photograph of direct microscopic assessment of the DPL (a) and TPL (b) populations after 18 h co-culture on identical HAEC monolayers. Note blastoid morphology of DPL as compared with TPL. Both populations appeared nonblastoid at 2, 6 and 10 h (not shown).

The specificity of the MLC responder cells was determined by primed lymphocyte testing (PLT) on day 10, as previously described (Sheehy *et al.*, 1975; Zeevi *et al.*, 1986). Briefly, 2×10^4 MLC responder cells were incubated for 72 h with 10^5 irradiated lymphocytes from the original stimulator or appropriate controls in a U-bottomed, 96-well plate. On day 12, wells were pulsed with 1 μCi ^{3}H-labelled thymidine, and harvested 18 h later with a cell harvester (Skatron, Sterling, VA). Responder cells were defined as reactive towards a given stimulator cell when the total incorporated ^{3}H-thymidine count ± 2 s.d. was greater than background.

Adherence of human lymphocytes to HAEC monolayers

Confluent monolayers of HAEC were established in serum-free EC media in a 96-well, flat bottomed tissue culture plate (No. 25860, Corning Glass Works, Corning, NY). To verify their identity during HAEC coculture, lymphocytes were labelled prior to the adherence studies with a vital stain which did not affect lymphocyte adherence (data not shown). 10 μl of the staining solution (7 mg of hydroethidine in 1 ml of *N,N*-dimethylacetamide, Polysciences, Warrington, PA) were added to 10^6 lymphocytes suspended in 1 ml of phosphate-buffered saline (PBS) and incubated in darkness for 30 min. Lymphocytes were washed in PBS, and viability was shown to be $>95\%$ before being resuspended to the appropriate concentrations and added to the HAEC monolayer.

Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere for 2–22 h. The 96-well plate was then inverted and centrifuged at 15 ***g*** for 5 min at room temperature. The number of adherent lymphocytes was assessed at $\times 200$ using a Leitz Diavert inverted microscope, and results were expressed as the mean number or the mean percentage $\pm$ s.e.m. of lymphocytes added initially that remained adherent to the HAEC monolayers. Analysis of triplicate assays from two different HAEC monolayers revealed s.d. $< 10\%$. Statistical significance between adherent populations tested was analysed by the Mann–Whitney *U*-test.

MoAb blocking of lymphocyte adherence

MoAb directed against class I HLA antigens [w6/32 (Pel-Freeze, Rogers, AR)], class II HLA antigens [TU35 (A. Ziegler, Tübingen, FRG) and CA2.206 (D. Charron, Paris, France)], lymphocyte function-associated (LFA) antigens (a gift from T.A. Springer, Boston, MA) or mouse IgG (Cooperbiomedical, Malvern, PA) specificities were used at a final dilution of 1:40 in serum-free endothelial cell medium. HAEC monolayers, or lymphocyte populations for the LFA-1 experiments, were pre-incubated with antibody for 30 min before lympocytes were added for the adherence assay described above.

RESULTS

Effect of alloreactive lymphocyte concentration on HAEC monolayer adherence

Two populations of alloactivated lymphocytes were generated: donor-primed lymphocytes (DPL) and third-party primed lymphocytes (TPL), exposed to HLA antigens different from those of the donor.

Various concentrations (5×10^3 to 10^6 lymphocytes/ml) of DPL and TPL were co-cultured with HAEC monolayers for 18 h at 37°C before being inverted and subjected to a dislodgement force of 15 ***g*** for 5 min. In all assays where the lymphocyte concentration exceeded 5×10^5/ml, extensive damage to the HAEC monolayers was seen, and they were excluded from data analysis. The adherent DPL and TPL populations exhibited a dose-dependent response to the initial lymphocyte concentration added to the HAEC monolayer. However, the percentage of DPL and TPL adherent suggested that a saturation of the available lymphocyte binding sites occurred around 5×10^5/ml (Table 1).

Effect of lymphocyte–endothelial co-culture duration on HAEC monolayer adherence

DPL or TPL (10^5/ml) were co-cultured on HAEC monolayers for various time periods (2, 6, 10, 14, 18 or 22 h) at 37°C before being inverted and subjected to a dislodgement force. Although

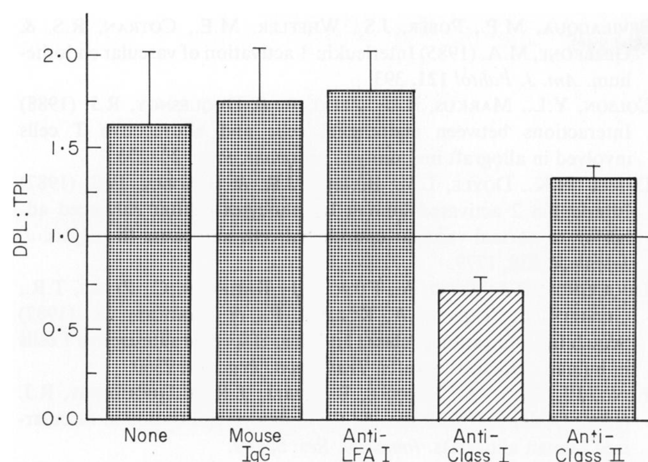

Fig. 3. Effect on DPL adherence to class I positive HAEC monolayers by the presence of various MoAb. HAEC monolayers (or lymphocyte populations for anti LFA-1 experiments) were preincubated with the designated MoAb for 30 min before lymphocyte-HAEC co-culture. Lymphocyte co-culture was done in the continued presence of the MoAb at a final dilution of 1:40. Nonadherent lymphocytes were removed after 18 h and data from triplicate assays in 2–6 experiments was pooled and presented as the ratio of adhering DPL/adhering TPL $\pm$ SE. Only anti-class I MoAb was able to decrease DPL adherence to the levels seen in the TPL population. This inhibition was statistically significant to $P<0{\cdot}02$ when compared with IgG controls.

no statistically significant differences between DPL and TPL adherence was noted during the first 10 h of co-culture, DPL adherence exceeded TPL adherence by over 50% following 14–18 h, decreasing by 22 h as monolayer damage became evident (Fig. 1). In contrast, TPL adherence remained relatively constant with the decrease seen at 22 h failing to reach statistical significance. Although < 10% of the DPL and TPL exhibited a blastoid appearance when initially added to the HAEC monolayers, by 18 h more than 90% of DPL exhibited a blastoid appearance and many appeared to have migrated through the endothelial monolayer (Fig. 2). The adherence of unprimed lymphocytes to HAEC was similar to TPL adherence, and neither population exhibited morphological changes during the 18-h co-culture (data not shown).

Allospecificity of alloreactive lymphocyte adherence to HAEC monolayers

The possibility that increased DPL adherence could be due to innate differences in adherence among different alloreactive lymphocyte populations was investigated by comparing lymphocyte adherence with HAEC monolayers displaying various HLA antigens. DPL and TPL populations were co-cultured on HAEC monolayers presenting HLA antigens in common with the donor, with a third party or with an unrelated individual. Increased lymphocyte adherence was exhibited by the DPL population only when exposed to the HLA antigens of the donor HAEC monolayer (Table 2). Similarly, the TPL population demonstrated a blastoid morphology and increased adherence only when co-cultured on an HAEC monolayer presenting the third party alloantigens. This enhanced lymphocyte adherence was specific to the HAEC monolayer of the appropriate HLA type as there was no statistically significant difference between DPL and TPL adherence to an HLA monolayer.

DPL adherence is presumably related to the allorecognition of HLA antigens on donor endothelium. Therefore, MoAb that prevent allorecognition were used to inhibit HLA-specific interactions required for this increased adherence. The adherence of DPL to class I positive donor HAEC was inhibited only by class I antigen blockade, whereas TPL adherence remained unaffected by anti-class I, anti-class II, anti-LFA-1 or mouse IgG MoAb (Fig. 3).

DISCUSSION

The early events of cellular rejection involve interactions between lymphocytes and vascular endothelium. These interactions consist of various phases including recognition, adherence, activation, increased vascular permeability and eventually penetration of lymphocytes through the small vessel endothelium. However, immunophenotypic diversity of endothelium from various human tissues has been conclusively demonstrated (Turner *et al.*, 1987). This heterogeneity required lymphocyte–endothelial interactions to be studied with a human endothelial source obtained from the transplanted organ with the same immunologic phenotype as the microsvascular endothelium. *In vitro* cultures of HAEC derived from aortic specimens of transplant donors have been previously shown to be immunologically similar to non-lymphoid small vessel endothelium in that both strongly express factor VIII-related antigen and class I HLA antigens whereas, class II HLA antigen expression occurs when induced by γ-IFN or lymphocyte supernatants (Colson *et al.*, 1987; Markus *et al.*, 1987). The adherence assay described here focuses on the physical binding of alloactivated lymphocytes to cultured HAEC. Experimental conditions were determined whereby DPL underwent morphological change and showed significantly greater adherence to HAEC than did TPL.

Although our studies have shown a two-fold higher adherence of DPL as compared with TPL, there is still a considerable degree of TPL adherence to HAEC which ranged from 8·0 to 32·2%. The TPL values were similar to those observed with unprimed peripheral lymphocytes in our studies, as well as those adhering to human unbilical vein endothelial cells, as reported previously (Yu *et al.*, 1985; Haskard, Cavender & Ziff, 1986; Damle *et al.*, 1987). Optimal experimental conditions for DPL and TPL adherence were dependent on the numbers of lymphocytes added to the HAEC cultures. The best results were obtained with 5×10^3 to 10^4 lymphocytes (5×10^4/ml to 10^5/ml) added to a monolayer of approximately 10^4 HAEC. At lower lymphocyte numbers, standard errors were increased as too few cells adhered to HAEC for reliable quantitation. At higher lymphocyte numbers the percent adherence began to fall, suggesting a saturation of potential binding sites. This finding supports the saturation ratio of 5:1 reported by Bender *et al.* (1987) in a non-allogenic assay using endothelium from newborn preputial skin. Although much of the DPL adherence can be attributed to non-allospecific binding, increased DPL binding supports the existence of additional binding sites on allogeneic endothelium and/or increased affinity for allospecific lymphocytes.

Kinetic studies showed a progressive increase in DPL adherence exceeding TPL adherence as early as 10 h but not reaching statistical significance until 14 h. Our experience has shown an optimal incubation time of 14–18 h to differentiate between DPL and TPL adherence. The decrease in DPL

adherence between 18 and 22 h coincides with a decrease in the viability of HAEC, probably as a consequence of the damage caused by cytotoxic cells in DPL. Although a decrease in TPL adherence was also noted, HAEC monolayer toxicity was not detected and the decrease failed to reach statistical significance. After adherence, DPL but not TPL underwent morphologic changes and developed a blastoid appearance. Although blastogenic transformation suggests a further activation of DPL after exposure to HAEC presenting donor alloantigens, it is unlikely that all these cells are alloreactive. Rather, it is our hypothesis that the blastoid morphology exhibited by 90% of the DPL population is the *in vitro* correlate lymphocytes, which has been shown to depend on the presence of an allogeneic cellular interaction (Hanto *et al.*, 1982).

The dependence of DPL adherence on donor HLA antigen expression on HAEC was confirmed by demonstrating increased adherence only when co-cultured on HAEC monolayers displaying alloantigens from the donor. Furthermore, only class I-specific MoAb showed significant inhibition of DPL adherence, whereas no effect was seen on TPL adherence. Although anti-LFA-1 MoAb have been shown to inhibit general lymphocyte endothelial cell adherence by 20–40% in this assay at 1 h (unpublished observation) and in other short-term assays, it has been ineffective in activated endothelial systems where interleukin 1 is present (Haskard *et al.*, 1986). Therefore, it is possible that the failure of anti-LFA-1 MoAb to block lymphocyte adherence in an 18-h co-culture is due to the fact that cultured endothelium endogenously produce interleukin 1 (Moissec, Cavender & Ziff 1986) and become activated in 4–6 h (Bevilacqua *et al.*, 1985). Studies are currently in progress to characterize the specificity and nature of the DPL and TPL adhering to HAEC with and without induced class II antigen expression.

ACKNOWLEDGMENTS

The authors wish to thank the Pittsburgh Transplant Foundation for their invaluable aid in donor sample procurement. We would also like to thank Drs Bartley Griffith, Thomas Starzl, Robert Hardesty, Carlos Esquivel, Shunzaburo Iwatsuki, Alfredo Trento, Robert Gordon and Andrei Steiber for their cooperation.

This work was supported by the American Association of University Women Education Foundation American Fellowship Program (to Y.C.), the Deutsche Forschungsgemeinschaft Research Fellowship Program (to B.M.) and by grants HL36416 and A123467 from the National Institutes of Health and American Heart Association, Western Pennsylvania Chapter.

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