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ORIGINAL ARTICLE

Clearance of complement by human vascular endothelial cells: effects of hypoxia/reoxygenation and IL-1β activation

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Summary

Antibody-mediated rejection is characterized by deposits of complement (C) C4 and C3 split products on endothelial cells (ECs). C3 split products are critical mechanistically and diagnostically because they are deposited in amplified quantities, bind covalently to ECs and act as ligands for leukocytes. This study was designed to determine whether cultured vascular human ECs could clear covalently bound C3 split products from their surface. An immunoglobulin M (IgM) antibody against β_2-microglobulin of major histocompatibility complex class I antigens was used to activate C in human serum. Some cells were exposed to hypoxia/reoxygenation and/or interleukin 1β (IL-1β) prior to incubation with antibody. C3b/iC3b and C3d deposition on the cell surface was measured by flow cytometry. Incubation with antibody followed by human serum caused a dose-dependent deposition of C3b/iC3b and C3d. Over half of deposited C3b/iC3b and one-third of C3d were cleared from the cell surface during a 3–7-h incubation period with human serum. Neither hypoxia/reoxygenation nor IL-1β further increased the deposition of C3b/iC3b and C3d, and only slightly modulated their rates of clearance. In summary, human ECs rapidly clear iC3b and C3d from their surface. This finding may have important diagnostic and mechanistic implications to transplantation because C3d is used as a marker of antibody-mediated rejection.

Introduction

Activation of complement (C) by antibodies initiates a highly regulated cascade of enzymatic steps that split C proteins into biologically active components that can amplify the tissue injury (reviewed in [1–3]). One important mechanism by which C activation leads to inflammation is via the covalent binding of the split products of the central C component C3 to endothelial cells (ECs), where they serve as ligands for complement receptors (CR) on neutrophils, monocytes, macrophages and B cells [4]. Moreover, once C3b, the primary split product of C3, is covalently bound to tissues, it can initiate an amplification loop through factor B. This allows C activation to continue and expand after antibodies have disappeared from the tissue by dissociation or shedding. Autologous and allogeneic tissues interrupt the inflammatory capacity of C by expressing regulatory proteins. The covalently bound C3b is rapidly altered by a succession of enzymatic steps that result in the cleavage products, iC3b and C3d. Both iC3b and C3d remain covalently bound to tissue and they are the primary ligands for the CR3 and CR2, respectively. Immunohistological stains for iC3b and C3d in tissue biopsies have provided evidence that this occurs during antibody-mediated rejection of transplants. Patients with circulating alloantibodies have been found to have extensive deposits of iC3b and C3d split products on vascular ECs [3,5,6]. The biological function of these deposits is suggested by concurrent infiltrates of neutrophils, monocytes and macrophages. Treatment with

plasmapheresis to remove antibodies can result in significant improvement of graft function with stable long-term outcome.

Certain types of cells can react to stimuli by modulating their plasma membrane. Leukocytes in suspension can shed molecules cross-linked with antibodies from their surface [7]. Similarly, leukocytes can eliminate the membrane attack complex (MAC) formed by C5b through C9 from their plasma membrane by both endocytic and exocytic processes [8–10]. The capability of adherent vascular ECs to clear covalently bound C3 split products from their surface has not been explored. In this study, we hypothesized that vascular ECs may also clear C3 split products from their surface without intervention of responding leukocytes as a mechanism to control inflammation. We further examined the effects of hypoxia and reoxygenation as well as the proinflammatory cytokine IL-1β on the retention of iC3b and C3d deposited by IgM antibody against major histocompatibility complex class I antigens on cultured human umbilical vein ECs (HUVECs). Furthermore, the effects of C activation and the subsequent response of ECs on the expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 were studied, because activation of ECs by sublytic C levels was shown to result in an upregulation of cell adhesion molecules for leukocytes [11,12].

Materials and methods

Antibodies

Monoclonal mouse anti-human β_2-microglobulin [immunoglobulin M (IgM) κ], r-phycoerythrin (PE)-conjugated mouse anti-human ICAM-1 (IgG_1,κ), PE-conjugated mouse anti-human VCAM-1 (IgG_1,κ), and isotype control (PE-conjugated mouse IgG_1,κ) were purchased from Pharmingen (San Diego, CA, USA). Polyclonal fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C3d was purchased from DAKO (Glostrup, Denmark). Polyclonal FITC-conjugated goat anti-human C3 was purchased from ICN Cappel (Aurora, OH, USA). It has been shown that this polyclonal antibody to C3 antibody recognizes membrane-bound C3b and iC3b [13]. FITC-conjugated goat anti-mouse IgM [$F(ab')_2$ fragment, μ chain specific] was obtained from Jackson Immunoresearch (Westgrove, PA, USA). The monoclonal mouse IgM isotype antibody from clone TU99 can bind either free or complexed β_2-microglobulin (Pharmingen).

Preparation of human serum

Whole blood was collected from healthy volunteers by venipuncture of the antecubital vein into 10 ml Vacutainer collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) without any anticoagulants. The venipuncture procedures were examined and approved by the Joint Committee on Clinical Investigation at the Johns Hopkins School of Medicine. Blood was allowed to clot for 45 min at room temperature, before it was transferred to 15 ml centrifuge tubes (Sarstedt, Newton, NC, USA) and spun for 10 min at 1 500 × *g*. The top layer was removed from each tube, pooled, and spun for an additional 5 min to remove residual red blood cells. Normal human serum (NHS) was immediately placed on ice, aliquoted and frozen at −80 °C. NHS obtained from the same three volunteers was used for all experiments. Prior to each experiment, some amount of serum was heat-inactivated at 56 °C for 30 min [heat-inactivated human serum (HIHS)] to render C inactive.

HUVEC tissue culture

Primary noncryopreserved HUVECs were purchased from GlycoTech (Rockville, MD, USA). Cells were grown in T75 flasks (Becton Dickinson) in EGM-2 complete growth medium with 2% fetal bovine serum (FBS), growth supplements and antibiotics (Clonetics, San Diego, CA, USA) in a 37 °C incubator (5% CO_2/95% air). Medium was replaced every other day. Upon confluence, ECs were rinsed with warmed HEPES-buffered saline solution (Clonetics) and detached by incubating with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Clonetics) for 2 min, before addition of trypsin neutralizing solution (Clonetics). Cells were dislodged by gentle tapping and pipetting, centrifuged for 5 min at 200 × *g*, and seeded into either T75 flasks or 60 mm tissue culture dishes (Corning, Corning, NY, USA). Cells were used up to the fifth passage.

HUVEC exposure to hypoxia/reoxygenation

Endothelial cells in 60 mm tissue culture dishes were prepared for hypoxic treatment by washing with Hanks-balanced salt solution (HBSS; Biofluids, Rockville, MD, USA) and adding 2 ml of basal media (EBM-2; Clonetics) supplemented with 5% heat-inactivated FBS (Sigma, St Louis, MO, USA), that had been previously warmed and equilibrated with a hypoxic gas mixture (5% CO_2/95% N_2; Puritan Bennett, Linthicum Heights, MD, USA). Samples were then placed in a humidified air-tight modular incubator chamber (Billups-Rothenburg, Del Mar, CA, USA) that was flushed with 100% N_2 at 2 psi for 10 min prior to switching to the hypoxic gas mixture, as previously described [14]. Briefly, to ensure an O_2-free environment, the hypoxic gas mixture was passed through a catalytic deoxygenator (Gas Purification Technology, Manalapan, NJ, USA) before entry into the chamber. Partial O_2 pressure was monitored using a Clark-style O_2 analyzer (Hudson

RCI, Temecula, CA, USA) placed in the media contained in the tissue culture container. Starting partial pressure was 150 mmHg and dropped to 10 mmHg (1% O_2) inside the chamber within the first 10 min of hypoxia. Temperature was maintained inside the chamber at 37 °C during the gas purge using a heating pad, and afterwards by placing the chamber into the 37 °C tissue culture incubator. After 3 h of hypoxia, reoxygenation was initiated by returning the cells to their starting O_2 tension. ECs that were to remain under normoxic conditions (N) were given similar treatment, with the exception that they received media that had been equilibrated with a 5% CO_2/95% air gas mixture, and were then placed into the 37 °C tissue culture incubator.

HUVEC exposure to IL-1β, IgM and human serum

At the beginning of reoxygenation, both hypoxic and normoxic EC monolayers were supplemented with either 0.5 ml of recombinant IL-1β (0.2 ng/ml final concentration; Sigma) diluted in EBM-2 or EBM-2 with no other additives. The cells were then placed in the 37 °C tissue culture incubator for 30 min before removal to be supplemented with either 0.5 ml of anti-β_2-microglobulin antibody (1 μg/ml final concentration) diluted in EBM-2 or EBM-2. The total volume after this step was 3 ml in each dish. The antibody was allowed to bind for an additional 30 min. ECs were then washed with HBSS and incubated with 2 ml of EBM-2 containing either 20% NHS or 20% HIHS. This incubation step lasted either 3 or 7 h, for a total of 4 or 8 h of reoxygenation (Fig. 1).

Quantification of C deposition and adhesion molecule expression by flow cytometry

Endothelial cells were washed with PBS without Ca^{2+} and Mg^{2+} (Sigma) and incubated with 4 ml PBS containing 25 mM HEPES and 10 mM EDTA (Sigma) for 20 min at 37 °C. Cells were dislodged by pipetting and rinsing twice with PBS. After centrifugation for 5 min at 200 × *g*, cells were resuspended in cold PBS containing 0.5% bovine serum albumin (Sigma) and 0.02% sodium azide (FACs buffer). After adjusting the cell concentration (*c.* 2.3×10^6 cells/ml), the cells were stained using the following antibodies: FITC-anti-C3 (1 μl per 200 μl of sample; 1:200 dilution), FITC-C3d (1:50), FITC-IgM (1:100),

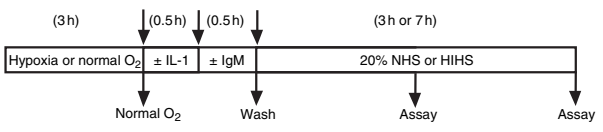

Figure 1 Schematic diagram of experimental design. For details, see Materials and methods.

PE-ICAM-1 (1:50), PE-VCAM-1 (1:50), and PE-isotype control (1:50). Antibody binding was carried out for 30 min at 4 °C in radioimmunoassay tubes (Becton Dickinson). Samples were then washed twice using cold FACs buffer and centrifugation, before being resuspended in 100 μl of FACs buffer for analysis on a FACscan flow cytometer (Becton Dickinson). Fluorescence was measured on a log scale and expressed as geometric mean fluorescence intensity (GMFI). The cells that were gated stained almost entirely negative for propidium iodide (5 μg/ml in PBS; data not shown).

Statistics

Flow cytometry data were analyzed based on the normalized GMFI for three independent experiments. Normalized GMFI refers to normalization by the normoxic, HIHS control after subtraction of autofluorescence. Error bars indicated standard errors (SE). Data for deposited C3b/iC3b and C3d, as well as for surface expression levels of ICAM-1 and VCAM-1, were analyzed by four-way ANOVA (N or H/R; ±IL-1β; ±IgM; NHS or HIHS), and data for bound IgM were analyzed by two-way ANOVA (±IgM; NHS or HIHS). Multiple comparisons were conducted using *t*-tests with a Bonferroni correction. Analyses were performed using Intercooled Stata software (Stata Corp., College Station, TX, USA). $P \leq 0.05$ were considered significant.

Results

IgM antibody causes a dose-dependent deposition of C3b/iC3b and C3d

An IgM monoclonal antibody to β_2-microglobulin was used to activate C. This ensured that the antibody and C deposition would be achieved independent of the HLA type of the HUVECs. Deposition of C3b/iC3b and C3d on HUVECs was dependent on the concentration of IgM antibody to β_2-microglobulin. Binding of IgM antibodies to HUVECs increased linearly with the concentrations of the IgM monoclonal antibody to β_2-microglobulin from 0.1 to 5 μg/ml (Fig. 2a). These concentrations of IgM antibody activated C in NHS as evidenced by deposition of C3 split products on the ECs. Deposition of C3b/iC3b and C3d increased exponentially after HUVECs were treated with concentrations of the IgM antibody from 0.1 μg/ml to 1 μg/ml (Fig. 2b,c). An IgM concentration of 1 μg/ml was chosen for subsequent experiments because it caused nearly maximal deposition of both C3b/iC3b and C3d on the HUVECs and allowed ready detection of clearance of these ligands from the plasma membranes. Control HUVECs treated with IgM monoclonal antibodies and incubated in HIHS demonstrated no significant C3b/iC3b or C3d deposition (not shown).

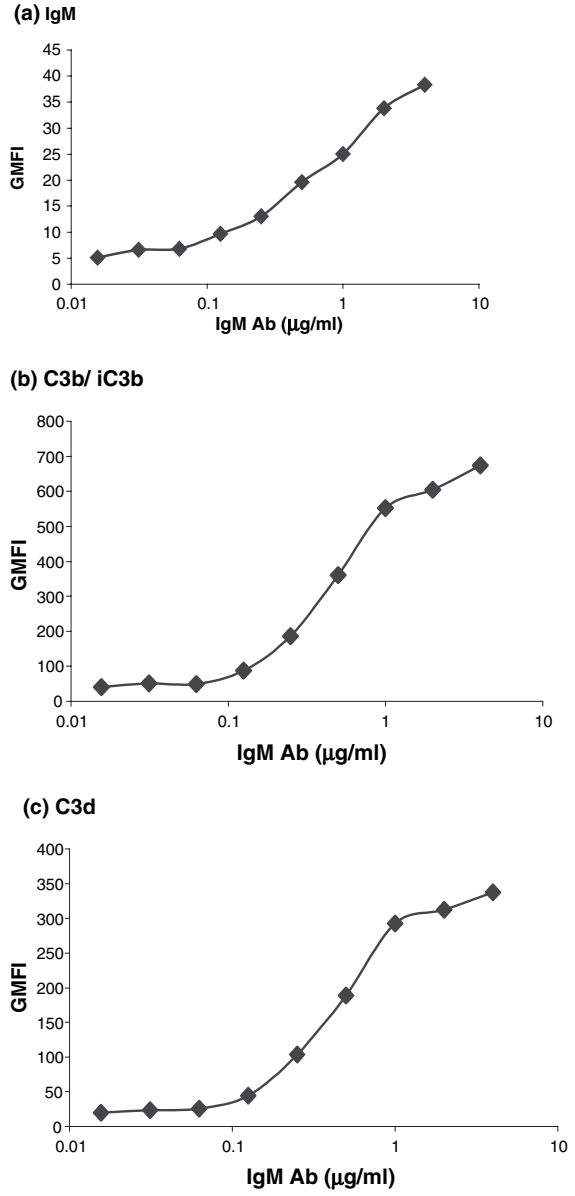

Figure 2 Deposition of IgM, C3b/iC3b and C3d on HUVECs as a function of the concentration of IgM antibody specific for β_2-microglobulin. (a) Geometric mean fluorescence intensity (GMFI), after correction for autofluorescence, for IgM bound to ECs 3 h after incubation with different dilutions of IgM antibodies to β_2-microglobulin. (b) and (c) GMFI for C3b/iC3b and C3d bound to ECs 3 h after incubation with different dilutions of IgM antibodies to β_2-microglobulin.

Clearance of IgM, C3b/iC3b, and C3d from EC membranes

As expected the IgM antibodies released or were shed from the surface of the ECs with time. The staining intensity (normalized by dividing by the fluorescence corresponding to N, no IgM, no IL-1β, HIHS for 3 h) decreased by at least half from 3–7 h after the ECs were treated with IgM monoclonal antibodies in the presence of either NHS or HIHS (Fig. 3). Therefore, there was no evidence that activation of C altered the clearance or release of IgM from the membrane of the ECs. During this same time period, the C3b/iC3b normalized fluorescence also decreased by over half and the C3d staining decreased by over one-third in spite of the continuous availability of normal serum as a source of C (Fig. 4a,c). However, changes in normalized deposition of C3b/iC3b and C3d for NHS samples without prior incubation with IgM antibodies, and for all HIHS samples with or without prior incubation with IgM antibodies were negligible (Fig. 4a,c).

Effects of hypoxia/reoxygenation on clearance of IgM, C3b/iC3b, and C3d from EC membranes

Although hypoxia/reoxygenation has been reported to increase the deposition of C3b/iC3b [13,15], it did not result in significant deposition of C3b/iC3b or C3d in the absence of IgM antibodies. Neither did it increase C3b/iC3b or C3d deposition caused by IgM antibodies. In fact, it significantly decreased the deposition of C3b/iC3b measured at 3 h after treatment with IgM antibodies, and slightly decreased the deposition of both C3b/iC3b at 7 h and C3d at 3 and 7 h after treatment with IgM antibodies (Fig. 4a,c). Therefore, hypoxia/reoxygenation marginally decreased the rate of clearance (normalized fluorescence/h) of C3b/iC3b and C3d from ECs after treatment with

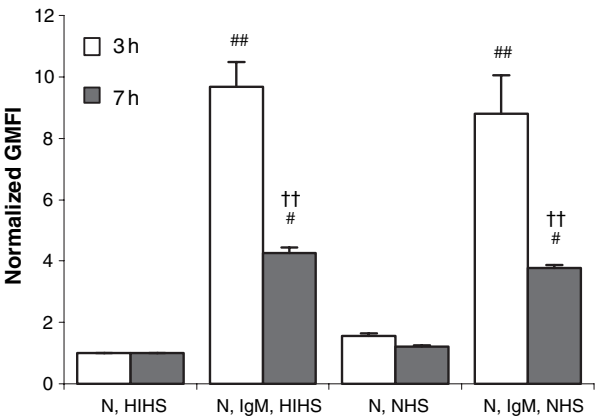

Figure 3 Staining of normoxic, non-IL-1β-stimulated HUVECs for IgM. Normalized GMFI measurements refer to the GMFI after normalization by the N, HIHS control. Error bars represent mean ± SE of $n = 3$ independent experiments. Symbols denote the following comparisons: # to the same condition without IgM; † to the same condition at 3 h. Single symbols correspond to $P < 0.05$, and double symbols to $P < 0.001$.

IgM in the absence of IL-1β (Fig. 4a,c). Hypoxia/reoxygenation also did not affect the binding or clearance of IgM from the surface of ECs (not shown).

Effects of IL-1β on deposited IgM, C3b/iC3b, C3d, and adhesion molecule expression

The proinflammatory cytokine IL-1β activated HUVECs as evidence by a dose-dependent increase in both ICAM-1 and VCAM-1 expression (Fig. 5). At a concentration of 0.2 ng/ml, IL-1β increased the expression of ICAM-1 and VCAM-1 significantly, but did not alter the binding of IgM antibodies (data not shown). This dose of IL-1β also did not alter the binding or clearance of either IgM (not shown), C3b/iC3b or C3d (Fig. 4b,d). There was no effect of the IgM antibody on ICAM-1 and VCAM-1 expression for either HIHS or NHS samples, with or without IL-1β (Fig. 6). Specifically for VCAM-1 in the presence of IL-1β, there was a significant difference between H/R, NHS and H/R, HIHS; however, this was present either in the presence or absence of the IgM antibody (Fig. 6d).

Discussion

The major finding in these experiments is that adherent vascular ECs can clear the covalently bound C3 split products C3b/iC3b and C3d. It is known that leukocytes, unlike erythrocytes, can shed proteins from their plasma membranes. Leukocytes in suspension can shed molecules cross-linked with antibodies from their surface [7]. Similarly, leukocytes can eliminate MAC from their plasma membrane by both endocytic and exocytic processes [8–10]. This finding is relevant to pathological processes involving ECs both on a mechanistic and diagnostic level. Mechanistically, factors determining the turnover of C3b

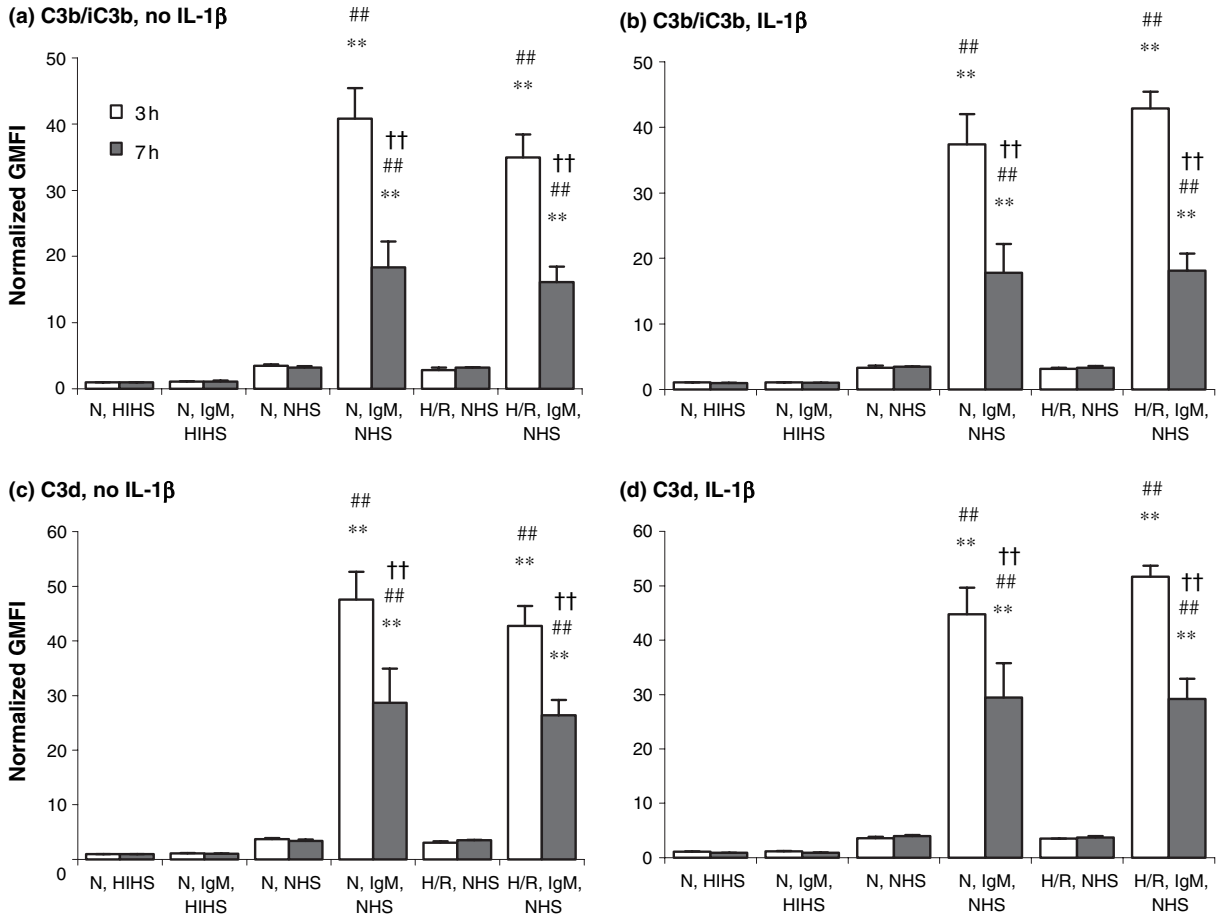

Figure 4 Staining of HUVECs for C3b/iC3b and C3d. (a) and (b) Normalized GMFI for C3b/iC3b bound to ECs with and without IL-1β, respectively. (c) and (d) Normalized GMFI for C3d bound to ECs with and without IL-1β, respectively. Symbols have the same meaning as in Fig. 3 with the addition of *, which corresponds to a comparison with the same condition with HIHS. H/R treatment had no effect on HIHS samples, therefore the results were omitted.

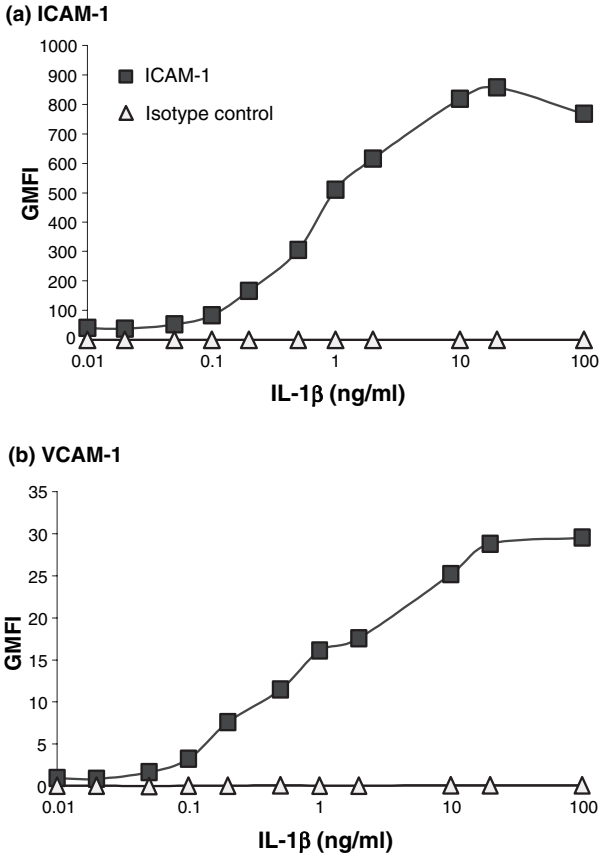

Figure 5 Dose response of surface expression of ICAM-1 and VCAM-1 to exogenous IL-1β. An isotype control antibody was included to insure detection specificity. A characteristic experiment is shown of two with similar results.

split products on ECs may determine the confines of vascular inflammation. Diagnostically, prolonged retention of C products would weaken the association between antibody-mediated pathology and assays for C3b/iC3b or C3d deposition. In transplantation, successful treatment of antibody-mediated rejection has been correlated with decreased deposition of C3d [3]. These *in vivo* correlations have significant caveats. Unlike *in vitro* experiments, therapeutic interventions to remove antibody are not immediately or completely effective. Moreover, biopsies to detect C deposition are restricted in frequency and timing relative to therapy. Finally, the infiltrating inflammatory cells can themselves produce more C or change the catabolism of the C split products [16–19].

We tested whether the proinflammatory cytokine IL-1β or transient hypoxia would increase the deposition or retention of C3b/iC3b or C3d. These two variables are especially relevant to C3 split products. Production of IL-1β is stimulated in monocytes and macrophages through CR1 or CR3 binding to C3b or iC3b, respectively [20,21]. Therefore, inflammatory responses related to deposition of C3 split products on ECs would be expected to be accompanied by production of IL-1β. In our studies, the addition of IL-1β at levels sufficient to activate the expression of ICAM-1 and VCAM-1 did not increase the deposition or retention of C3b/iC3b or C3d. Hypoxia/reoxygenation has also been reported to increase C3b/iC3b deposition on ECs in culture [13,15]. In our studies, hypoxia/reoxygenation also did not increase the deposition or retention of C3b/iC3b or C3d. This may reflect the fact that the antibody concentration studied resulted in near maximal levels of C3b/iC3b and C3d deposition and that this in turn resulted in maximal clearance. Our finding that IgM activation of C3 did not augment ICAM-1 and VCAM-1 expression by ECs is consistent with reports that additional signals from cytokines, such as tumor necrosis factor α, are required for C5a or MAC to elicit optimal expression of ICAM-1 by ECs [12,22].

The finding that iC3b cleared more rapidly than C3d is in accordance with the known regulatory pathways of C3b, in which iC3b is an intermediate product in the sequential cleavage of C3b to C3dg by factor I. C3d is the end product of C3b regulation by factor I. Clearance of C3d may by partly related to release of the IgM antibody from the ECs. It has been shown that as much as 30% of the C3 split products deposited on antibody-coated pneumococci is bound to the coating IgG [23]. Thus, some loss of C3d may result from the release or shedding of the IgM antibody. However, even 7 h after the removal of antibodies from the culture medium, enough IgM antibodies remained attached to the ECs to continue C3b/iC3b and C3d deposition.

Clearance of MAC might also account for clearance of some C3d. As a result of the highly reactive nature of C split products, they deposit in close proximity of the activation site. Therefore, MAC would be expected to deposit in the vicinity of early C components. Under these conditions, clearance of MAC by exocytosis or endocytosis could facilitate the removal of C3b, iC3b and C3d as well [8–10]. However, the clearance or iC3b and C3d is slower than the elimination of MAC reported for suspensions of tumor cells ($t_{1/2}$ of approximately 6 min) [8,9].

C4d is the other split product that is used as a diagnostic marker. The mechanism by which C4d and C3d bind to tissue (i.e. via thioester groups) is identical. Therefore, under the reductionist *in vitro* conditions used (i.e. ECs, antibody and serum without leukocytes), C4d and C3d are expected to behave the same. The variable that would make a difference is the interactions with cells that bear C3d receptors. These experiments are still in the planning stages. In the current experiments, there may be an overlap between clearance of split products and ongoing C deposition. Removing C with a second wash would be

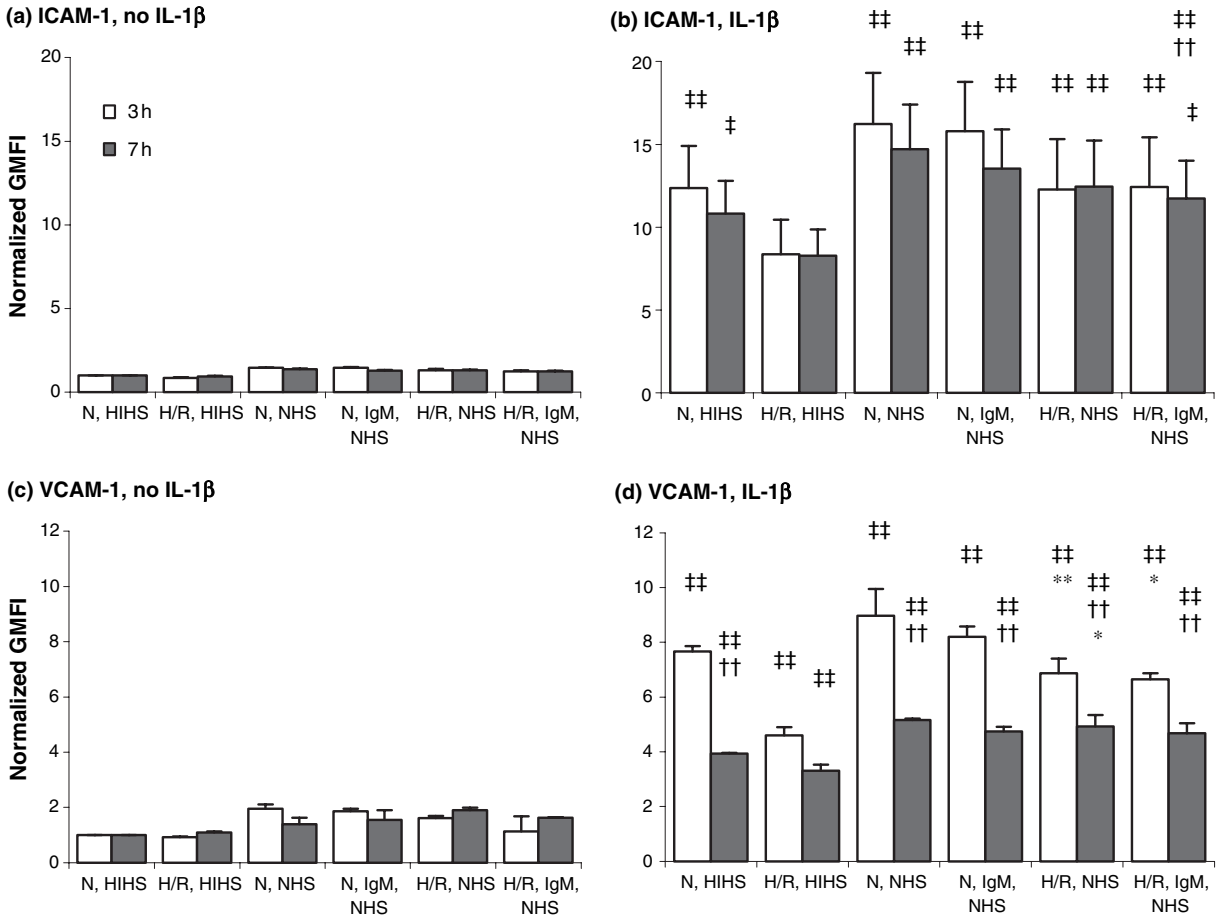

Figure 6. Staining of HUVECs for ICAM-1 and VCAM-1. (a) and (b) Normalized GMFI for endothelial ICAM-1 expression with and without IL-1β, respectively. (c) and (d) Normalized GMFI for endothelial VCAM-1 expression with and without IL-1β, respectively. Symbols have the same meaning as in Figs 3 and 4 with the addition of ‡, which corresponds to a comparison to the same condition without IL-1β. IgM antibody treatment had no effect on HIHS samples, therefore the results were omitted.

expected to accentuate the clearance of the C3 split products. However, this approach was chosen because it reflected the *in vivo* situation that we were trying to model. Last, cell proliferation is not expected to be a factor contributing to the observed decrease in fluorescence intensities, because confluent EC monolayers in buffer containing either HIHS or NHS only (not culture media) were used during the 3 and 7-h time intervals.

In summary, our data indicate that human vascular ECs clear covalently bound iC3b and C3d from their surface. This clearance occurs while a significant amount of antibodies is still present on the membrane of the ECs and C is available in the medium, which may have important diagnostic and mechanistic implications.

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