Studies of postmortem or biopsy tissue, obtained near to the time of diagnosis of type 1 diabetes, indicate that the disease is characterized by an islet infiltrate predominantly composed of T-cells (1–5). A more florid version of this insulitic lesion is observed in animal models such as the nonobese diabetic (NOD) mouse (6). The infiltrating cells observed in man have yet to become available in sufficient quantity to be examined functionally. In contrast, it has been demonstrated in several studies that T-cells infiltrating NOD mouse islets are enriched for those with specificity for islet autoantigens. Most notably, Wegmann and coworkers (7–9) have demonstrated a high frequency of islet-infiltrating CD4 T-cells reactive with insulin, and Wong et al. (10) has shown a high frequency of insulin-specific CD8 T-cells. As a result of these reports, it is considered probable that the T-cells that infiltrate human islets in type 1 diabetes are also enriched for those recognizing islet autoantigens, including proinsulin, GAD65, and the islet tyrosine phosphatase IA-2.

In recent years there have been considerable advances in the understanding of the transmigration of cells across the endothelial barrier from the blood into the tissues (11,12). Transmigration is a complex, sequential process initially involving selectins and their counter ligands that induce rolling of cells along the luminal surface of endothelial cells (ECs). This is followed by firm adhesion and diapedesis, during which cells transmigrate across the EC barrier. Adhesion and diapedesis are dependent upon interactions between cell-expressed integrins, such as leukocyte function-associated antigen-1 (LFA-1) and their counter-ligands the intercellular adhesion molecules (ICAMs) and junctional adhesion molecules (13,14). The relevance of these mechanisms to islet infiltration by T-cells is illustrated by immunohistochemical studies of islets obtained near to the time of type 1 diabetes diagnosis that show abundant adhesion molecule expression on vessels and immune cells (3–5). However, these processes alone may not fully account for the observed enrichment of islets with infiltrating autoantigen-specific T-cells.

In the present study, we set out to examine whether the endothelium could have a role in the selective recruitment of antigen-specific T-cells. Our study was prompted by the frequent and consistent observation that ECs are one of...
ENDOTHELIAL CELL PRESENTATION OF GAD65

the major cell types expressing class II HLA molecules in postmortem studies on human islets from type 1 diabetic patients. Because the only known function of class II HLA molecules is presentation of antigen to CD4 T-cells, we examined whether ECs are capable of internalizing, processing, and presenting disease-relevant epitopes of the islet autoantigen GAD65. In addition, we studied the effect of GAD65 presentation on the transmigration of GAD65-specific T-cells in vitro.

RESEARCH DESIGN AND METHODS

Antigen peptides. Peptides representing sequences of GAD65 and IA-2 (GAD65115–127, LAFTESEHFSFLKLVPYVFLPLAVAD; GAD65115–127, MNLLQYVVKFSFD; and IA-277–275, KLKVSSFRSDYNPIE HDK) were synthesized by standard F-moc chemistry (Thermo Hybaid, Uberlingen, Germany) and purity established as >98% by reverse-phase high-pressure liquid chromatography and mass spectrometry. Whole recombinant human GAD65 and IA-2 generated in insect cells using the baculovirus expression system were kind gifts of Dr. Peter van Essent (INSERM U725, Paris, France) (15).

Monoclonal antibodies. For the flow cytometric analyses of ECs the following monoclonal antibodies (mAbs) were used: anti–HLA-DR (clone L243), anti–HLA-DP (clone H143), and IgG2a isotype-matched control antibodies (all supplied by BD Pharmingen, San Diego, CA); anti–HLA-DQ (clone SPV-L3), anti–CD62E (clone L2B6), anti–CD54/ICAM-1 (clone C06934), anti–CD106 (vascular cell adhesion molecule) (VCAM-1) (clone 127FP), anti–CD31 (clone 300), anti–CD105 (clone 8N6); and isotype-matched control antibodies (all supplied by Serotec, Oxford, U.K.) and anti–CD146 (clone P1H12) (Chemicon, Temecula, CA). A phycoerythrin (PE)-labeled goat anti-mouse Fab immunoglobulin-specific polyclonal antibody (BD Pharmingen) was used as the secondary antibody.

Murine anti-human LFA-1 CD11a (clone 38, a kind donation of Dr. Nancy Hogg, Cancer U.K., London) (16) and rat anti-murine LFA-1 CD11a (clone H68, donated by Dr. Nancy Hogg) (17) were used as blocking reagents in the migration assays. Both of these mAbs have been defined as function blocking, binding to activation-dependent sites on the LFA-1–a-subunit. In addition, we used murine anti–HLA-DR (clone L243) and isotype- and species-matched control mAbs; all mAbs were used as purified immunoglobulin from ascites.

Human and murine T-cell lines and clones. The human GAD65-reactive T-cell clone PM1#11 was generated from a patient with Type 1 diabetes who subsequently developed type 1 diabetes (18). PM1#11 recognizes the GAD65274–286 epitope presented by HLA-DR4 (20). The hybridomas were generated in a HLA-DR4 (termed VEC1) or VEC2) cell lines cultured alone or in the presence of interferon (IFN)–γ 100 IU/ml (Genzyme, Cambridge, MA) for 48 h. Cells were removed using trypsin/EDTA, washed twice in HBSS, resuspended at a concentration of 1 × 106 cells/ml, and stained with anti–HLA-DR, -DP, and -DQ. To examine GAD65 presentation of GAD65, VEC1 cells were cultured alone or in the presence of IFN–γ 100 IU/ml in MCDB 131/20% FCS, and after a 4-h culture on well-96 flat-bottomed well plates (Greiner Labortechnik, Stonehouse, Gloucestershire, U.K.) in MCDB 131/20% FCS, antigens were added for 18 h at 37°C. Antigens comprised whole GAD65 or IA-2 (control) both at final concentrations in the range of 0.1–25 μg/ml and synthetic peptides representing GAD65274–286 or IA-275–77, both tested at concentrations in the range of 0.1–25 μmol/l. T33.1 hybridoma cells (1 × 105) were then added to each well, and after 18 h at 37°C, cell culture supernatants were harvested, concentrated to 3,000 μl to remove contaminating cells, and IL-2 secretion measured using a europium-streptavidin enzyme-linked immunosorbent assay system. Antigen concentrations were selected on the basis of published data on T33.1 (20) and our own preliminary results. Briefly, 96-well flat-bottomed plates (Maxisorp; Nunc Nalgene, Rochester, NY) were coated with 50 μg/ml mouse anti-mouse IL-2 (2 mg/ml (BD Pharmingen) and then washed with PBS/0.1% Tween (Tris-buffered saline with Tween (TBST)). The cells were then blocked with PBS/10% FCS for 1 h and washed with TBST. Cell culture supernatants (100 μl) were incubated overnight at 4°C and washed with TBST, and 100 μl biotinylated anti-human IL-2 (1 μg/ml (BD Pharmingen) was added for 4 h at room temperature. The plates were washed with TBST, incubated with 100 μl assay buffer (Wallac, Turku, Finland) for 45 min at room temperature, and washed again, followed by the addition of 100 μl enhancement (Wallac). Plates were read on a real-time DELFIA fluorimeter and IL-2 concentration calculated via regression analysis relative to the IL-2 standard curve. As a positive control, T33.1 cells were cocultured for 18 h at 37°C with the Priess Epstein Barr virus (EPV)-transformed B-cell line (homozgyous for HLA-DRB1*0401) preincubated for 18 h at 37°C with either GAD65274–286 peptide or whole GAD65.

Transwell migration assays. Polysacryl 4-μm pore size transwells (Corning Costar, MA) were initially prepared by coating transwell inserts with 100 μl 10% gelatin. Inserts were air dried overnight, incubated at 60°C for 2 h and then conditioned with MCDB 131/10 mmol/l L-glutamine/EC-derived growth factor (TCS Biologicals)/20% FCS for 2–4 h. Endothelium was then seeded at 2 × 105 cells transwell insert in the same medium and allowed to grow to confluence for 7–10 days, with replenishment of the media every 2–3 days. Endothelial monolayers were then cultured overnight at 4°C and washed with TBST, and then conditioned with MCDB 131/10 mmol/l L-glutamine/EC-derived growth factor (TCS Biologicals)/20% FCS for 2–4 h. Endothelium was then seeded at 2 × 105 cells transwell insert in the same medium and allowed to grow to confluence for 7–10 days, with replenishment of the media every 2–3 days. ECs were then cultured for 10 days with 100 μl biotinylated anti-human IL-2 (1 μg/ml (BD Pharmingen) was added for 4 h at room temperature. The plates were washed with TBST, incubated with 100 μl assay buffer (Wallac, Turku, Finland) for 45 min at room temperature, and washed again, followed by the addition of 100 μl enhancement media (Wallac). Plates were read on a real-time DELFIA fluorimeter and IL-2 concentration calculated via regression analysis relative to the IL-2 standard curve. As a positive control, T33.1 cells were cocultured for 18 h at 37°C with the Priess Epstein Barr virus (EPV)-transformed B-cell line (homozgyous for HLA-DRB1*0401) preincubated for 18 h at 37°C with either GAD65274–286 peptide or whole GAD65.

Transwell migration assays. Polysacryl 4-μm pore size transwells (Corning Costar, MA) were initially prepared by coating transwell inserts with 100 μl 10% gelatin. Inserts were air dried overnight, incubated at 60°C for 2 h and then conditioned with MCDB 131/10 mmol/l L-glutamine/EC-derived growth factor (TCS Biologicals)/20% FCS for 2–4 h. Endothelium was then seeded at 2 × 105 cells transwell insert in the same medium and allowed to grow to confluence for 7–10 days, with replenishment of the media every 2–3 days. Endothelial monolayers were then cultured overnight at 4°C and washed with TBST, and then conditioned with MCDB 131/10 mmol/l L-glutamine/EC-derived growth factor (TCS Biologicals)/20% FCS for 2–4 h. Endothelium was then seeded at 2 × 105 cells transwell insert in the same medium and allowed to grow to confluence for 7–10 days, with replenishment of the media every 2–3 days. ECs were then cultured for 10 days with 100 μl biotinylated anti-human IL-2 (1 μg/ml (BD Pharmingen) was added for 4 h at room temperature. The plates were washed with TBST, incubated with 100 μl assay buffer (Wallac, Turku, Finland) for 45 min at room temperature, and washed again, followed by the addition of 100 μl enhancement media (Wallac). Plates were read on a real-time DELFIA fluorimeter and IL-2 concentration calculated via regression analysis relative to the IL-2 standard curve.
for 2 h before addition of the T-cells with anti-HLA-DR or control mAb and then washed, or T-cells were added to transwells at the same time as anti-LFA-1 or control mAb (all mAbs at a final concentration of 1.5 μg/ml). Transwell ECs were then pulsed with whole GAD65, IA-2 (10 μg/ml), or synthetic peptides representing GAD65 339-352 or control peptide IA-2 752-775 for 2 h before addition of the T-cells with anti-HLA-DR or control mAb and then washed, or T-cells were added to transwells at the same time as anti-LFA-1 or control mAb (all mAbs at a final concentration of 1.5 μg/ml). Transwell ECs were then pulsed with whole GAD65, IA-2 (10 μg/ml), or synthetic peptides representing GAD65 339-352 or control peptide IA-2 752-775 for experiments with PM1#11 clone T-cells (both peptides tested at 1 and 10 μmol/l). These concentrations were chosen on the basis of previous results with this clone (15,22). For experiments with T33.1 hybridoma cells, whole antigens were used at the same concentration as for PM1#11, and GAD65 274-286 or control peptide GAD65 115-127 were used at 0.1, 1, and 10 μmol/l for 18 h at 37°C. GAD65-specific T-cells, 5 × 10⁵ per well, were then added (clone PM1#11 or T33.1) into the upper chamber of the transwell. T33.1 hybridoma cells were used direct from growing cultures; PM1#11 clone T-cells were either used at the end of the PHA/IL-2 expansion phase or after thawing of previously expanded, cryopreserved cells, in which case cells were allowed to recover in IMDM/10% AB for 3 h before migration assays. T-cells migrating into the lower chamber were quantified by direct sampling and counting in a hemocytometer at 15, 30, and 60 min and then at hourly intervals up to 6 h and finally at 18 h. All of the above transwell studies were carried out using at least duplicate wells, and counting of cells in the lower chamber was in triplicate. Results were expressed as the cumulative migration at each time point, as a percentage of the starting cell numbers.

In similar studies designed to examine the potential effects of activated PM1#11 clone cells on EC integrity, the control T-cell clone MT-1 was washed with PBS and stained for 4 min at room temperature with 0.5 μmol/l carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Molecular Probes, Leiden, the Netherlands). Cells were then washed twice in cold PBS with 0.1% BSA and taken up in tissue culture medium for the migration experiments. Viability after CFDA-SE staining was >98%. CFDA-SE-labeled MT-1 cells were mixed with unstained PM1#11 cells at an approximate 50:50 ratio and the precise proportion of each cell established by flow cytometry. The mixed population of T-cells, 5 × 10⁵ per well, were then added into the upper chamber of transwells containing VEC2 ECs pretreated with 100 units/ml IFN-γ and prepulsed with GAD65 339-352 (1 μmol/l) or left unpulsed. T-cells migrating into the lower chamber were quantified as above and the relative proportions of PM1#11 and MT-1 cells examined by fluorescence microscopy and flow cytometry.

**T-cell proliferation assays and examination of T-cell receptor expression.** At specific time points, PM1#11 clone T-cells migrating into the lower chamber were harvested, washed twice, and resuspended either for T-cell proliferation assays in RPMI-1640/10% AB or in HBSS for examination of T-cell receptor (TCR) expression. Clone T-cells that had not been used in transwell experiments were also examined for TCR expression and proliferation. For proliferation, 2 × 10⁵ PM1#11 cells were cocultured in 96-well flat-bottom plates in triplicate with either GAD65 339-352 peptide at a final concentration of 1 μmol/l, 10 units/ml rhIL-2, 0.4 μg/ml PHA-L, or medium alone. After 3 days, wells were washed with 0.5 μCi [3H]-thymidine (Amersham International, Amersham, U.K.) for 18 h, cells harvested onto glass fiber filters (Wallac), and [3H]-thymidine incorporation measured by liquid scintillation spectroscopy.

For examination of TCR expression, PM1#11 clone T-cells pre- or post-migration were resuspended at a concentration of 1 × 10⁶/ml and stained with fluorescein isothiocyanate–conjugated mAb directed against TCR-αβ (clone BD Pharmingen, San Diego, CA) for analysis by flow cytometry.

**RESULTS**

**Characterization of primary cultures of human aortic ECs.** Primary cultures of aortic EC lines were obtained from several cadaveric organ donors as described, of which VEC1 and VEC2, which carried suitable HLA genotypes for subsequent T-cell studies, were studied in greater detail. The cultured cells were identified as endothelial in origin by their typical morphology, resting surface expression of CD31 (platelet-EC adhesion molecule-1), CD105 (endoglin), and the endothelial marker P1H12 (23) giving an estimated purity of >95% (Fig. 1), and expression of CD62E after stimulation with TNF-α. Resting VEC1 and VEC2 cells had basal expression of ICAM-1 but not of VCAM-1 or CD62E (24–27), which were both inducible with TNF-α (Fig. 1).

**Inducible expression of class II HLA molecules on ECs.** Resting human ECs do not express detectable levels of class II HLA molecules, and this was confirmed for VEC1 and VEC2. After 72 h in the presence of human recombinant IFN-γ, class II HLA molecule expression was
The ability of VEC1 to process and present the islet Vascular ECs process and present GAD65 to T-cells. (J.E.G., K.T.K., and N.J.K., unpublished data). IFN-γ expression class II HLA molecules following induction and -DP was observed in the presence of IFN-γ and the control antigens whole IA-2 or GAD65115-127 peptide. These data indicate that ECs are capable of processing and presentation of a known naturally processed epitope (20,28) of GAD65 to responder T-cells and that presentation is dependent upon induction of class II HLA molecule expression.

Presentation of GAD65 promotes transendothelial migration of T-cells. To assess the effect of EC presentation of GAD65 on transmigration, we established transwell cultures of VEC1 and VEC2 with or without IFN-γ pretreatment and peptide antigens and examined migrating T33.1 hybridoma cells and PM1#11 clone T-cells, respectively, in the lower chamber.

In experiments using the human T-cell clone PM1#11 and VEC2 preincubated with IFN-γ, detectable numbers of cells incubated in the presence of cognate antigen (GAD65123-352 peptide) began to appear in the lower chamber after 15–30 min. In control wells either not preincubated with IFN-γ (no class II HLA molecule expression) or preincubated with IFN-γ and irrelevant peptide, no, or very few, clone T-cells were seen at this early stage (Fig. 4A). A rapid increase in antigen-specific migration of PM1#11 cells (APCs). In contrast, VEC1 cells failed to stimulate IL-2 production by T33.1 hybridoma cells in the presence of GAD65274-286 peptide but in the absence of pretreatment with IFN-γ, or in the presence of pretreatment with IFN-γ and the control antigens whole IA-2 or GAD65115-127 peptide. This dose-response relation is similar to that seen with Priess EBV B-cells as antigen-presenting cells (APCs). In contrast, VEC1 cells failed to stimulate IL-2 production by T33.1 hybridoma cells in the presence of GAD65274-286 peptide but in the absence of pretreatment with IFN-γ, or in the presence of pretreatment with IFN-γ and the control antigens whole IA-2 or GAD65115-127 peptide. These data indicate that ECs are capable of processing and presentation of a known naturally processed epitope (20,28) of GAD65 to responder T-cells and that presentation is dependent upon induction of class II HLA molecule expression.

Presentation of GAD65 promotes transendothelial migration of T-cells. To assess the effect of EC presentation of GAD65 on transmigration, we established transwell cultures of VEC1 and VEC2 with or without IFN-γ pretreatment and peptide antigens and examined migrating T33.1 hybridoma cells and PM1#11 clone T-cells, respectively, in the lower chamber.

In experiments using the human T-cell clone PM1#11 and VEC2 preincubated with IFN-γ, detectable numbers of cells incubated in the presence of cognate antigen (GAD65123-352 peptide) began to appear in the lower chamber after 15–30 min. In control wells either not preincubated with IFN-γ (no class II HLA molecule expression) or preincubated with IFN-γ and irrelevant peptide, no, or very few, clone T-cells were seen at this early stage (Fig. 4A). A rapid increase in antigen-specific migration of PM1#11 cells (APCs). In contrast, VEC1 cells failed to stimulate IL-2 production by T33.1 hybridoma cells in the presence of GAD65274-286 peptide but in the absence of pretreatment with IFN-γ, or in the presence of pretreatment with IFN-γ and the control antigens whole IA-2 or GAD65115-127 peptide. These data indicate that ECs are capable of processing and presentation of a known naturally processed epitope (20,28) of GAD65 to responder T-cells and that presentation is dependent upon induction of class II HLA molecule expression.

Presentation of GAD65 promotes transendothelial migration of T-cells. To assess the effect of EC presentation of GAD65 on transmigration, we established transwell cultures of VEC1 and VEC2 with or without IFN-γ pretreatment and peptide antigens and examined migrating T33.1 hybridoma cells and PM1#11 clone T-cells, respectively, in the lower chamber.

In experiments using the human T-cell clone PM1#11 and VEC2 preincubated with IFN-γ, detectable numbers of cells incubated in the presence of cognate antigen (GAD65123-352 peptide) began to appear in the lower chamber after 15–30 min. In control wells either not preincubated with IFN-γ (no class II HLA molecule expression) or preincubated with IFN-γ and irrelevant peptide, no, or very few, clone T-cells were seen at this early stage (Fig. 4A). A rapid increase in antigen-specific migration of PM1#11 cells (APCs). In contrast, VEC1 cells failed to stimulate IL-2 production by T33.1 hybridoma cells in the presence of GAD65274-286 peptide but in the absence of pretreatment with IFN-γ, or in the presence of pretreatment with IFN-γ and the control antigens whole IA-2 or GAD65115-127 peptide. These data indicate that ECs are capable of processing and presentation of a known naturally processed epitope (20,28) of GAD65 to responder T-cells and that presentation is dependent upon induction of class II HLA molecule expression.

Presentation of GAD65 promotes transendothelial migration of T-cells. To assess the effect of EC presentation of GAD65 on transmigration, we established transwell cultures of VEC1 and VEC2 with or without IFN-γ pretreatment and peptide antigens and examined migrating T33.1 hybridoma cells and PM1#11 clone T-cells, respectively, in the lower chamber.

In experiments using the human T-cell clone PM1#11 and VEC2 preincubated with IFN-γ, detectable numbers of cells incubated in the presence of cognate antigen (GAD65123-352 peptide) began to appear in the lower chamber after 15–30 min. In control wells either not preincubated with IFN-γ (no class II HLA molecule expression) or preincubated with IFN-γ and irrelevant peptide, no, or very few, clone T-cells were seen at this early stage (Fig. 4A). A rapid increase in antigen-specific migration of PM1#11 cells (APCs). In contrast, VEC1 cells failed to stimulate IL-2 production by T33.1 hybridoma cells in the presence of GAD65274-286 peptide but in the absence of pretreatment with IFN-γ, or in the presence of pretreatment with IFN-γ and the control antigens whole IA-2 or GAD65115-127 peptide. These data indicate that ECs are capable of processing and presentation of a known naturally processed epitope (20,28) of GAD65 to responder T-cells and that presentation is dependent upon induction of class II HLA molecule expression.
cells was seen between 1 and 2 h, by which time ~60% of the clone T-cells had migrated across the VEC2 barrier. Maximal transmigration (typically >70%) was reached at 3 h and was similar at 1 and 10 \( \mu \)g/ml peptide. In addition, PM1#11 cells showed a similar level of markedly enhanced transmigration across IFN-\( \gamma \) treated VEC2-coated transwells pulsed with whole GAD65 at 10 \( \mu \)g/ml (Fig. 4A).

In contrast, migration across VEC2 cells pulsed with IA-2 \(^{752-775}\) peptide, whole IA-2, or in the presence of GAD65 \(^{339-352}\) without IFN-\( \gamma \) pretreatment was much lower, at ~15–20% at 3 h, similar to migration in the absence of antigen. The rate of antigen-specific migration showed a tendency to level off after peaking at 2 h. By 3 h ~80% of PM1#11 cells had migrated through the VEC2 layer in the presence of GAD65 \(^{339-352}\) and IFN-\( \gamma \). In contrast, migration of cells in control wells was ~40% at 18 h. In a further control experiment, ECs mismatched (VEC1, HLA-DR4) for the class II HLA molecules required for presentation to PM1#11 clone T-cells were prepulsed with IFN-\( \gamma \) and GAD65 \(^{339-352}\) peptide. Migration of PM1#11 clone T-cells across the VEC1 monolayer was low and similar to non-antigen-specific migration (data not shown).

Blocking studies using mAbs directed against HLA-DR and LFA-1 demonstrated that antigen-specific migration of PM1#11 cells is both dependent on antigen presentation by HLA-DR and functional activation of LFA-1, with migration reduced to background levels or below in the presence of these mAbs (Fig. 4B). The data shown in Fig. 4 is representative of four separate migration experiments.

Before, during, and after these transmigration experiments, the integrity of the EC monolayer was inspected by microscopy. There was no evidence of cell lifting or changes in EC morphology. In addition, electrical resistance measurements across the EC monolayer before and at 18 h after introduction of antigen and T-cell clones were similar (145 and 147\( \Omega \), respectively, in one representative experiment). However, to exclude the possibility that damage to ECs by activated PM1#11 T-cell clones allowed nonspecific migration to occur, we established transmigration experiments in which PM1#11 clone cells were mixed at an approximate 50:50 ratio with MT-1 clone cells (HLA-DR3 [B1*0301] restricted T-cell clone recognizing a mycobacterial peptide) prelabeled with the fluorescent dye CFDA-SE. Cells migrating in the presence of IFN-\( \gamma \)-treated ECs and GAD65 \(^{339-352}\) peptide and in the presence of IFN-\( \gamma \)-treated ECs alone were recovered and counted by light microscopy, fluorescence microscopy, and flow cytometry. Flow cytometry was able to distinguish PM1#11 cells from MT-1 cells that had been prelabeled with CFDA-SE detectable on the FL1 fluorescence channel. The results indicated that under conditions of antigen-driven migration of PM1#11 clone cells, very few MT-1 cells appeared in the lower chamber of the transwell. Of the cells that had migrated at 3 h in the presence of IFN-\( \gamma \)-treated ECs and GAD65 \(^{339-352}\) peptide, 97% were and 44%, respectively) and of cells appearing in the lower chamber after 3 h in the presence of GAD65 \(^{339-352}\) (97 vs. 3%, respectively) or in the absence of antigen (42 vs. 58%, respectively). A portion of nonspecific migration of MT-1 T-cells cocultured with mycobacterial peptide showed full proliferative response (data not shown). Results indicate that antigen-specific transmigration of PM1#11 is not accompanied by nonspecific migration of the control clone MT-1. Peptide concentration was 1 \( \mu \)mol/L.
FIG. 5. Antigen specific transendothelial migration of GAD65 specific murine hybridoma T33.1. A similar migration profile is seen to that obtained for PM1#11. Because T33.1 migration was less efficient and data shown are normalized (100% equivalent to maximal migration). In the experiment shown, 100% migration was 1.45 × 10⁵ × 10⁶ cells (29%) at 3 h and 1.7 × 10⁶ cells (34%) at 18 h. In the experiments shown, peptide concentration was 10 μmol/l and whole antigen was used at 10 μg/ml. A: In the presence of IFN-γ and cognate antigen (synthetic peptide GAD65274-286) or whole GAD65, T33.1 cell migration was markedly enhanced at 3 h, compared with absence of antigen (○), irrelevant synthetic peptide (GAD65115-127, □) or migration in the presence of GAD65274-286 but in the absence of IFN-γ pretreatment (□). B: Migration of T33.1 was significantly reduced using relevant mAbs. Transendothelial migration of T33.1 in the presence of GAD65274-286 and IFN-γ (□) has been normalized to 100%. Migration in the presence of IgG2a control mAb (■) achieved levels comparable with the maximum at 3 and 18 h. In contrast, migration in the presence of mAb blocking murine LFA-1 (□) and HLA-DR (□) was greatly reduced, to levels of 24 and 28% at 3 h and 32 and 39% at 18 h, respectively (P < 0.05 for all). The data in Fig. 5 are representative of four separate migration experiments.

PM1#11 (Fig. 4C). In contrast, in the absence of peptide, 42% of the migrated cells at 3 h were PM1#11 and 58% were MT-1 clone cells. These results indicate that T-cell clone transmigration under these experimental conditions is promoted by EC presentation of specific antigen and that there is no evidence of toxic effects on ECs that allow nonspecific migration.

In experiments using T33.1 hybridoma cells incubated in the presence of cognate antigen GAD65274-286 or whole GAD65, transmigrated hybridoma cells began to appear at 3 h, whereas there were few or no cells seen at this stage in the absence of IFN-γ pretreatment or peptide (Fig. 5). The antigen specificity of migration was maintained through 18 h with up to 40% of cells having migrated in response to the whole GAD65 or GAD65274-286. In contrast, migration in the absence of cognate antigen or IFN-γ was <20%. Blocking studies using mAbs directed against murine LFA-1 and HLA-DR again demonstrated that antigen-specific migration is both dependent on functional activation of LFA-1 and on HLA-DR, with migration reduced by >50% (Fig. 5B). Of the concentrations of peptide used, optimal transmigration was seen at 10 μmol/l, with reduced but detectable migration at 1 μmol/l and no transmigration above background detectable at 0.1 μmol/l.

Phenotypic and functional analysis of migrated T-cells. Levels of TCR-αβ expression by PM1#11 clone T-cells were similar before and after migration. This was true irrespective of whether cells migrated actively, in the presence of HLA-DR and cognate antigen, or passively, through ECs in control wells (data not shown).

In functional terms, migrated PM1#11 T-cells showed similar levels of proliferative responsiveness to GAD65339-352, IL-2, and PHA irrespective of the times at which migrated cells were sampled (3 and 18 h) or the conditions under which they migrated (in the presence of cognate or control antigen). Levels of proliferation to each stimulus were similar to those obtained with cells tested premigration (Fig. 6). Transmigrated cells cultured in the presence of medium alone showed low background levels of proliferation, similar to those seen in nonmigrated cells, indicating that interaction with antigen-pulsed VEC2 is not of itself sufficient to induce T-cell cycling.

DISCUSSION

In the present study, we show for the first time that human vascular ECs are capable of processing and presenting disease-related epitopes of the islet autoantigen GAD65 to autoreactive T-cells. In addition, we demonstrate that in...
vitro transmigration across an EC monolayer by autoreactive T-cells is markedly promoted by presentation of cognate peptide/HLA complexes on the EC surface and is LFA-1 dependent. Finally, we show that this antigen-specific interaction between ECs and autoreactive T-cells has no effect on the function of migrated T-cells, which retain full proliferative capacity. These studies support the concept that inflamed islet endothelium could have a role in promoting islet migration of autoreactive T-cells.

The EC is considered to have a "gatekeeper" role, regulating the transmigration of immune cells from blood into tissues. We wished to examine the hypothesis that one facet of this gatekeeper role is the preferential recruitment of autoantigen-specific T-cells into inflamed tissues, achieved by processing and presentation of autoantigens. Our study was prompted by two observations. First, in the NOD mouse model of human type 1 diabetes, in which studies of the antigen specificity of islet infiltrating T-cells are feasible, there is preferential recruitment of islet autoantigen-specific CD4 and CD8 T-cells, most notably those reactive with insulin (7–10). Second, there is ample evidence that during the development of insulinitis in human type 1 diabetes, ECs surrounding the islet show marked induction of class II HLA molecule expression (2–5). Indeed, in some studies ECs were the predominant HLA–DR–expressing cells in inflamed islets (5). The only known function of class II HLA molecules is presentation of antigenic peptides to CD4 T-cells.

We therefore reasoned that during an inflammatory process involving islets, EC expression of class II HLA molecules could lead to processing and presentation of relevant epitopes of islet autoantigens. As a tool to detect processing and presentation of disease-related epitopes of GAD65, we made use of the T33.1 murine T-cell hybridoma, generated in HLA-DR4 (DRB1*0401) transgenic mice immunized with the 274-286 peptide sequence of GAD65 and able to recognize both peptide and naturally processed whole GAD65 (19). Such T-cell hybridomas have the advantage of showing highly sensitive and reproducible IL-2 production in response to peptide/major histocompatibility complex (MHC), without the requirement for costimulation (20). Using T33.1 we were able to demonstrate unequivocally the presence GAD65\textsubscript{274-286}/HLA-DR4 complexes on human vascular ECs after pulsing with whole GAD65. The 274-286 epitope of GAD65 has been shown to be naturally processed and presented by conventional HLA-DR4 antigen-presenting cells (20,28), and the fact that T-cell clones directed against this epitope have been generated in patients with type 1 diabetes (28) suggests that it may represent a dominant GAD65 epitope in HLA-DR4 individuals. Further evidence that ECs process and present GAD65 was obtained using the PM1#11 human T-cell clone, which showed enhanced transmigration in the presence of whole GAD65 but not in the presence of control antigen.

It is unlikely that islet vascular endothelium has any role in the priming of autoreactive T-cells, given the established importance of professional antigen-presenting cells in this process. We therefore postulated that the role of EC antigen presentation could be to modulate leukocyte traffic into the islets. We examined the effect of GAD65 presentation on autoreactive T-cell transmigration across EC monolayers and showed that transmigration occurs more rapidly and to a greater magnitude in the presence of cognate antigen and class II HLA molecules. Transmigration was comparable in the presence of either whole GAD65 or GAD\textsubscript{274-286} peptide, indicating that a sufficient density of peptide/HLA complexes to induce diapedesis can be achieved in the presence of exogenous whole antigen. The effect of antigen in promoting transmigration was greatest in the first 1–2 h after the PM1#11 clone T-cells were added. This implies that antigen-induced migration is a rapid event, capable of counteracting the shear stresses generated by blood flow across the microvascular endothelium in vivo, which would tend to prevent the slower nonspecific migration we observed in the absence of cognate antigen.

Antigen-specific enhancement of transmigration was demonstrated for the human T-cell clone PM1#11 and also for the murine T-cell hybridoma T33.1. Murine T-cell hybridomas are rarely used for functional studies, although a recent in vivo study using hybridoma T-cells specific for collagen has shown their capacity to migrate from blood into inflamed joints (30) suggesting that they may constitute an important tool in studying T-cell migration. Migration of the T33.1 murine hybridoma cells tended to be slower than for the human PM1#11 T-cells, possibly reflecting the lesser efficiency of interaction between human and murine adhesion molecules.

Previous studies have also indicated that ECs are capable of processing and presenting exogenous antigens through the class II HLA molecule pathway (31–33). However, this is the first description of antigen presentation by human aortic ECs. From this and previous studies it would appear that venous, arterial, and microvascular ECs all possess antigen processing and presenting capacity. Viewed in light of our observations concerning T-cell migration, the antigen presenting capacity of these diverse endothelia may play an important role in health and disease.

There have been considerable advances in recent years in our understanding of the processes that lead to transmigration of T-cells into inflammatory sites. In particular, it is now apparent that upregulated expression of T-cell integrins is insufficient to drive cells across the endothelium. There is an additional activation-dependent regulation of T-cell integrin functional activity (34) that induces a change in LFA-1 conformation, or its surface distribution, markedly enhancing its functional affinity (34,35). Activation-dependent upregulation of integrin activity is a required step in the successful adhesion of a cell in the blood stream to vascular ECs (36). The signaling mechanisms that achieve functional upregulation of LFA-1 have been studied in vitro (37–39) and, importantly, include T-cell receptor ligation, which has been shown to be a potent mechanism of inside-out signaling (34). Although upregulation of integrin activity is rapidly achieved in seconds or minutes (40), its effects are transient, and integrin-mediated binding decays rapidly to preactivation levels within 1 h (40). It is likely that by the time a T-cell activated in the regional lymph node has migrated to the site of inflammation, it requires additional signals to reacquire full integrin function. Our data support the proposal that TCR ligation, achieved through peptide antigen presentation by ECs, is an important component.
of integrin functional upregulation, and therefore of recruitment of cells into inflammatory sites. Such a mechanism would provide an additional level of antigen specificity in T-cell migration, safeguarding the immune system from wastefully and dangerously dispatching cells to the wrong site when more than one area of inflammation exists.

The importance of LFA-1 in antigen-directed T-cell transmigration is highlighted by our ability to block migration with anti-LFA-1 antibodies directed against activation-dependent sites on the molecule’s α-subunit. It is of particular interest that transmigration of mouse hybridomas was also mediated through an LFA-1-dependent mechanism, despite the reported inability of mouse LFA-1 to ligate human ICAM-1 (41). Previous work has shown ligation of mouse LFA-1 by human ICAM-2 and -3, implying that these receptors contribute to antigen-specific T-cell migration (41). The recently discovered family of junctional adhesion molecules (14) that also interact with integrins may be involved in antigen-directed T-cell/EC interactions, and the relative importance of these different LFA-1 counter receptors will require further scrutiny.

Our data demonstrate that the interaction of T-cells with cognate peptide/MHC displayed on ECs induces rapid transmigration. Our studies also show that such transmigrating cells, if recovered and left in culture media alone, do not go into cell cycle and proliferate, but do retain full proliferative capacity if further stimulated with mitogen or cognate peptide. The fact that PM1#11 T-cells were not induced into cell cycle by interaction with GAD65[^239-352]HLA-DR3 complexes on ECs is consistent with the fact that ECs do not express costimulatory molecules, such as B7-1 and B7-2, constitutively or after IFN-γ induction, as confirmed for VEC1 and VEC2 in our own studies (data not shown). However, it has been widely reported that the interaction between cloned T-cells and cognate peptide/MHC (signal 1), in the absence of costimulation, induces T-cell anergy (42). In physiological terms, such an outcome would render the T-cell migrating under these conditions unable to contribute to the immune response in the tissues. This was clearly not the case for PM1#11 clone T-cells following interaction with GAD65[^239-352]HLA-DR3 complexes on ECs. It remains to be established whether this is a feature of all transmigrating T-cells and whether specialized signals are exchanged between ECs and transmigrating T-cells that overcome the tendency to anergy of signal one provision alone.

It is interesting to speculate on the source of autoantigens available to vascular ECs in vivo. The islet in particular is richly supplied with EC-lined microvasculature, such that almost every islet endocrine cell is in close proximity to an EC. In physiological terms this allows rapid glucose responsiveness and insulin secretion in vivo. It also implies that islet ECs are exposed to high levels of insulin, which is likely to achieve high intracellular concentrations, and be rapidly processed and presented during class II HLA molecule induction. In addition, during islet inflammation accompanied by cellular damage and release of cell contents, islet autoantigens such as proinsulin, GAD65, and IA-2 may be taken up by activated ECs. If such inflammation is accompanied by release of cytokines such as IFN-γ, it is probable that islet ECs present epitopes of islet autoantigens in vivo, although this remains to be established experimentally. However, this proposal receives some support from the work of Savinov et al. (43), in which islet homing of an insulin-specific CD8+ T-cell clone was shown to be severely impaired when clone cells were infused into IFN-γ-knock-out mice.

In summary, in this study we show the dependency of T-cell transmigration on the availability of cognate peptide/HLA complexes. Our data suggest that autoantigen presentation by islet ECs could be an important mechanism in the development of islet infiltration and that interference in T-cell/endothelium interactions could be a useful immunotherapeutic goal.

ACKNOWLEDGMENTS

This work was supported by Diabetes U.K. J.E.G. is a Diabetes U.K. Pediatric Research Fellow, and M.P. is a Diabetes U.K. Senior Clinical Research Fellow.

We are grateful to Dr. Linda Wicker of the Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, University of Cambridge, U.K. for the gift of hybridoma cells; to Dr. Peter van Endert, INSERM U25, Paris, France, for the gift of recombinant autoantigens; and to Dr. Nancy Hogg, Cancer Research, U.K., for the gift of blocking antibodies.

REFERENCES


