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Binding and internalization of human IgG by living cultured endothelial cells

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SUMMARY

Interactions between circulating IgG and endothelial cells (EC) in humans have been described only in conditions associated with pathologic immunoglobulins and/or activated or damaged EC. In this study we provide evidence that normal human IgG includes one/some antibody species that bind to and are internalized by living EC in culture. This novel function of EC and natural autoantibodies is of potential importance for the understanding of physiologic interactions between vessels and the immune system and for the clarification of pathogenesis of vasculitis and mechanisms of action of pooled IgG used in the therapy of such conditions.

Keywords endothelium autoantibodies IgG fragments vasculitis IgG

INTRODUCTION

It is currently thought that no interaction occurs between human circulating IgG and endothelium under physiologic conditions. According to the literature, only during infectious or inflammatory diseases do endothelial cells (EC) become activated and express Fc receptors for IgG [1–3] or neoantigens able to interact with antibodies [4,5]. Endothelial cell damage has been reported to result from circulating pathologic autoantibodies against EC [6,7]. Passive accumulation of normal IgG has been found only in irreversibly damaged EC [8].

Natural autoantibodies directed against endothelial cells (AECA) are present in the serum of healthy individuals and in therapeutic preparations of normal polyspecific IgG (IVIG). The production of these autoantibodies is strictly controlled in terms of antigen specificity in genetically diverse individuals, and the expression of AECA activity in the serum is further controlled by other serum factors. Such strict control is lost in autoimmune disorders like systemic lupus erythematosus (SLE) [9].

However, the methods used in previous studies required the use of fixed cells or cellular extracts, and an unresolved issue was the possible *in vivo* significance of naturally occurring AECA.

In the present study we visualized directly the interactions between normal human IgG and living, non-damaged EC using an entirely original procedure based on the observation of living EC exposed to FITC-conjugated human IgG (IgG-FITC) by confocal laser scanning microscopy (CLSM). This approach substantially differs from previous studies, which required the use of fixed cells and indirect immunofluorescence.

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MATERIALS AND METHODS

IgG-FITC preparation

Pooled normal human IgG (Sandoglobulin; Sandoz, Basel, Switzerland) was coupled to FITC [10] and purified by chromatography on a Sephadex G25 column and extensive dialysis against PBS and finally Dulbecco's modified Eagle's medium (DMEM) using a 3500-kD cut-off membrane (Spectra/por; Spectrum Medical Industries, Inc., Los Angeles, CA) to allow total elimination of free FITC. Aliquots of the dialysate were saved to incubate with the EC at least 2 h before the beginning of the experiments to exclude contamination of the IgG-FITC solutions by free FITC.

Single-donor IgG was prepared by precipitation of serum immunoglobulins with saturated ammonium persulphate and chromatography on protein A-Sepharose (Supelchem, Milano, Italy). IgG was then coupled to FITC as described.

Human myeloma IgG1λ (Sigma Chemical Co., Division of Sigma Aldrich Srl, Milano, Italy), $F(ab')_2$ [9] and Fc [11] fragments of IVIG were also coupled to FITC as described.

EC culture

Endothelial cells obtained from umbilical cord veins (HUVEC) [12] were grown on glass coverslips to subconfluence without attachment factors. Intact proximal segments of saphenous veins, normally discarded after therapeutic stripping for varices, were incubated for 20 min with collagenase type II 0·1% to collect EC.

All EC cultures were tested for the presence of cytoplasmic Von Willebrand factor [9].

Fibroblast and proximal tubular epithelial cell culture

Human fibroblasts were obtained as described [13]. Proximal tubular epithelial cells (PTEC) were obtained from intact human kidney tissue after surgical nephrectomy for renal cell polar carcinoma, as already described [11].

Confocal laser scanning microscopy

The confocal scanning laser inverted microscope was a Molecular Dynamics Multiprobe 2001 equipped with an Argon-ion laser. Samples were observed through an oil immersion ×100 objective (Nikon PlanApo, NA 1.4), which allows a vertical resolution of about 0·7 μm; the step size was set accordingly. Confocal aperture (pinhole) was set at 100 μm. In all experiments care was taken to keep the laser excitation power as low as possible, and in any case below 1 mW. Coarse focus was achieved in bright field observation, thus laser beam use was restricted to acute image acquisition. Image processing and fluorescence measurements were performed on a Silicon Graphics Personal Iris workstation (Image Space Software Version 3.1; Molecular Dynamics, Kensing, UK).

Flow chamber

A flow chamber was designed and built to allow the observation of cells for a prolonged time span at constant pH, temperature and atmospheric conditions and to permit the substitution of culture medium without changes in the plane of focus along *x*, *y*, or *z*-axes; thus a predetermined equatorial observational plane devoid of out of focus blurring was kept constant throughout the experimental procedure [14].

The flow chamber was a clear polyacetate block (5 × 2·5 × 1·4 cm) with a 0·5 mm deep groove milled on its bottom. The chamber was completed by the cover-slide bearing the EC monolayer, sealed at the bottom of the block by a thin layer of silicon vacuum grease. The final volume of the flow chamber was about 300 μl. Two oblique tunnels opening on the upper face of the block allowed the introduction of small sylastic catheters for medium in- and out-flow.

The flow chamber was lodged in a perfectly fitting niche formed within a cylindrical aluminium block (diameter 14 × 2 cm) whose base was appropriately mill finished so as to give a tight adhesion to the microscope stage. On the bottom of the niche an ellipsoidal slit (3 × 1 cm) allowed objective apposition and the observation of a large area of the culture monolayer by stage translation. Once the flow chamber was in place, the niche in the stand was tightly closed by an aluminium lid with three holes: two of them corresponded to the flow chamber tunnels for medium in- and outlet and the third was for influx of CO_2 5%. A small plexiglas window in the centre of the lid allowed light transmission for bright field observation of the cells. The temperature in the flow chamber was controlled by a resistance-coil embodied in the aluminium stand. A thermorelay probe was located at the inner surface of the stand niche, providing the widest surface of contact between the radiating element and the flow chamber.

Cell counterstain

Whenever appropriate to obtain an accurate visualization of the cell profile after IgG-FITC signal acquisition, EC were couterstained with 1 μM calcein-AM. This neutral dye is converted by intracellular estherases in a fluorescent anionic compound providing a diffuse uniform signal from the cytoplasm and a slightly more intense one from the nucleus. Negatively charged cell compartments result in low-to-no signal areas. Once in anionic form the probe cannot leave the cell except in case of loss of membrane integrity; in presence of membrane damage the fluorescence drops immediately to zero even with residual cell esterase activity.

Immunoflurescence on fixed EC

Living EC were incubated with non-fluoresceinated IgG 2 mg/ml in DMEM for 20 min at 30°C, washed with PBS at 37°C and fixed with methanol at 4°C followed by PBS Triton X-100 0·2% for 5 min at room temperature. Cells were then incubated at room temperature for 30 min with three anti-human IgG MoAbs recognizing two epitopes on the Fc portion and one epitope on the Fab portion, respectively (clone HP-6017, Sigma Chemical Co., St Louis, MO; clone A57H, Dakopatts A/S, Glostrup, Denmark; CloneSG-16, Sigma). After washing with PBS, cells were incubated with a fluoresceinated anti-mouse immunoglobulin antibody (Dakopatts A/S) and observed in CLSM.

RESULTS

IgG-FITC incubation of HUVEC

No fluorescence was detectable by CLSM in living HUVEC incubated in the DMEM used to dialyse the IgG-FITC. After 20 min incubation with IVIG-FITC at 2 mg/ml followed by extensive washing with DMEM, the intracellular compartment was positively stained in a fibrillar pattern (Fig. 1A). Fluorescent fibrils were distributed throughout the entire cytoplasm, but were particularly abundant in peripheral protrusions that apparently connected adjacent cells. Observation of the cells after 1 h showed that the fibrillar images had become faint and a granular aspect had become prevalent. After 2 h the intracellular staining was greatly reduced. At each time of observation calcein uptake was preserved (as in Fig. 1A), proving complete integrity of the cell membrane [15]. A wide vacuolar, iuxtanuclear, unstained area was almost constantly present in each HUVEC. Calcein counterstain, together with the stability of the pre-chosen equatorial plane of section, proved the actual intracellular localization of IVIG-FITC. The described intracellular fluorescent signals were detectable using IVIG diluted to 250 μg/ml.

To exclude the possibility that the fluorescent image was due to constituents of IVIG other than IgG, living HUVEC were incubated with IVIG for 20 min, washed and fixed; intracellular IgG were demonstrated by direct immunofluorescence using two different MoAbs to the human Fc portion of IgG and one MoAb to the Fab portion of IgG. The same fluorescent pattern as in previous experiments was observed in all cases (Fig. 2).

Successive experiments were performed including 5 μg/ml polymixin-B in all incubation media to exclude that HUVEC were activated by contaminant lipopolysaccharide (LPS), and the same results were obtained.

In order to exclude a 'pooling effect' or the presence of abnormal autoantibody species in IVIG, single healthy donor IgG was used to incubate HUVEC at the same concentrations of IVIG-FITC. A pattern of fluorescence identical to that relative to IVIG-FITC was observed.

IgG-FITC incubation of saphenous vein EC

We excluded that our findings were specific for cells of fetal origin by incubating EC obtained from adult saphenous vein (HSVEC) with IVIG-FITC, and the images obtained are shown in Fig. 3A,B. As in the field shown, HSVEC in culture often encircle a large central cell which detaches after a few days; this element does not internalize IgG.

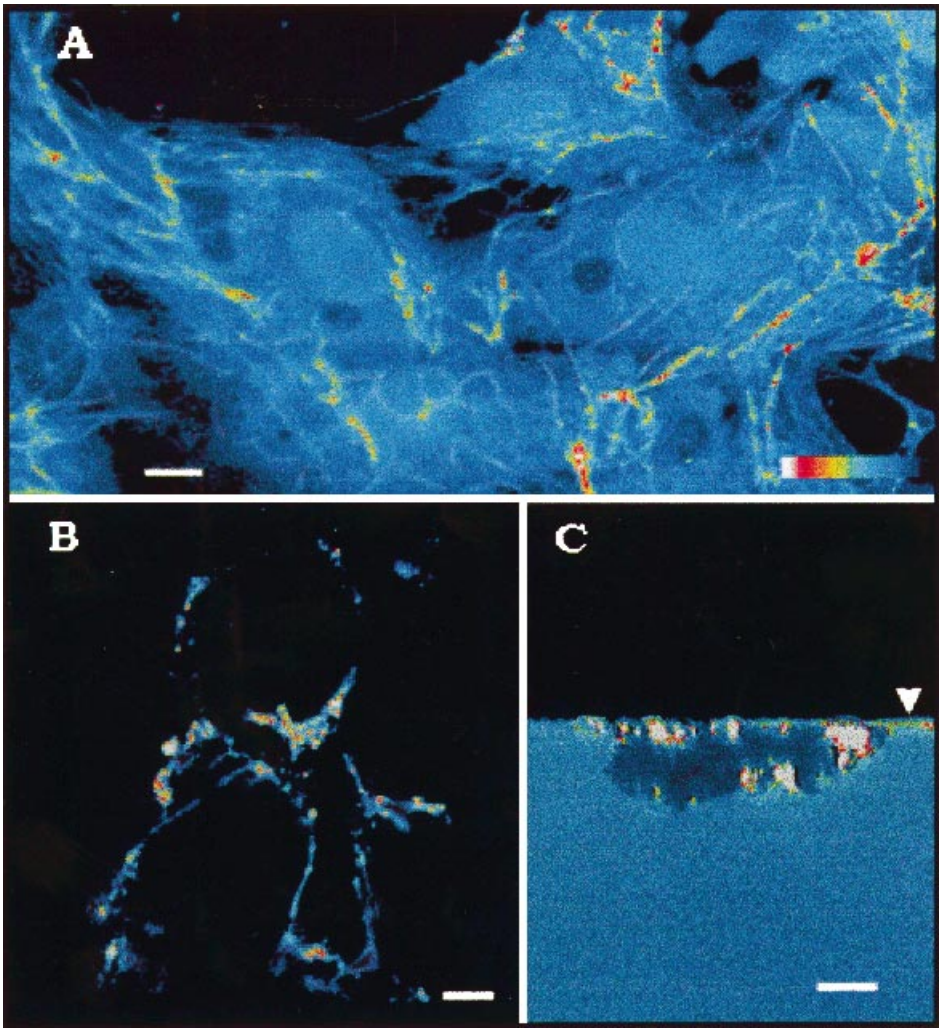

Fig. 1. IVIG-FITC interactions with living human umbilical vein endothelial cells (HUVEC). (A) Living HUVEC have incorporated IVIG-FITC, which appears as high-signal fibrils and filaments. Background couterstain for cytoplasm and nuclei is obtained by successive loading of calcein AM, which also demonstrates membrane integrity (laser beam energy 3% and photomultiplier gain ×1 as in all experiments if not otherwise stated). (B) Membrane binding of IVIG-FITC detected after 20 min incubation at 27°C. Image not couterstained. (C) Vertical section of a HUVEC. Remnants of extracellular IVIG-FITC in culture medium (blue area of the image) provide contrast for the cell, which appears as low-to-no signal volume with fluorescent spots corresponding to intracellular sites of IVIG-FITC accumulation. Arrowhead = coverslip. The colour palette applies to all the images. Bar = 5 μm.

Cellular specificity of internalization of IgG

We incubated living human fibroblasts and PTEC with IVIG-FITC in the same conditions as described for HUVEC. IgG reacted with fibroblast plasma membrane, but were not internalized (Fig. 4). No interaction was detected between IgG and living PTEC.

Autologous IgG-FITC on HSVEC

We obtained purified IgG and HSVEC from the same individual in order to evaluate the possible role of natural anti-allotypic MHC antibodies in our observations. Autologous IgG-FITC reacted with and was internalized by EC, as described for IVIG.

Incubation at low temperature

In order to visualize early events in the interaction of IgG with EC we performed an experiment at 27°C (temperature at which cells did not show signs of damage, as evaluated by absence of morphology changes, trypan blue exclusion and calcein uptake) and limited the incubation of the cells with IVIG-FITC to 5 min. Linear membrane staining was observed, as shown in Fig. 1B.

Specificity of IgG–EC interactions

To ascertain whether IgG interacts with EC through its variable or constant region, we coupled $F(ab)_2$ fragments and Fc fragments of IVIG to FITC, as described earlier. Neither fragment was internalized by EC, even using fragment concentrations of 8 mg/ml. We demonstrated that interactions of IgG with EC are mediated by the variable region using 1 ml of IVIG-FITC at 500 μg/ml (concentration resulting in a positive signal) to perform sequential 30-min incubations of eight flasks (25 cm^2) of subconfluent HUVEC for a total of 8×10^6 cells. The IgG concentration in the last recovered solution of IVIG-FITC was decreased to 350 μg/ml, probably due to the progressive dilution of IgG during sequential incubations. No fluorescence was detectable in EC incubated with the adsorbed IgG-FITC (Fig. 5A); internalization did occur in the same field of

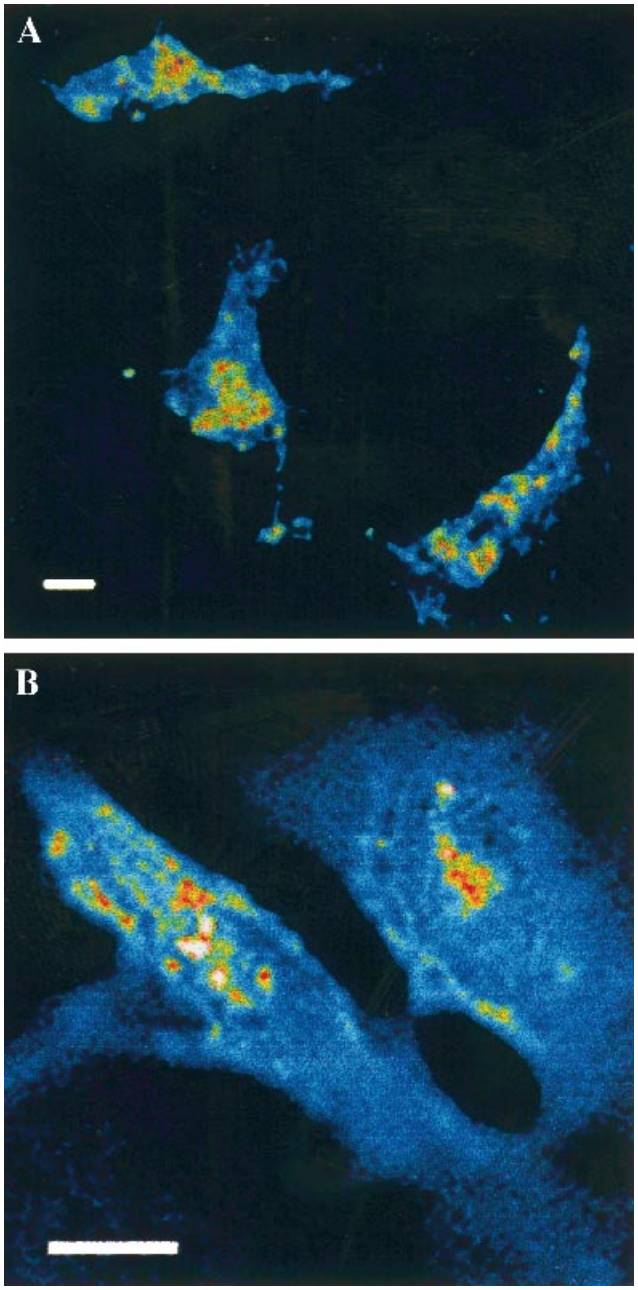

Fig. 2. Positive immunocytochemical reaction for IgG on fixed human umbilical vein endothelial cells (HUVEC) after 20 min preincubation with IVIG. Monoclonal antibodies raised against both IgG Fc (A) and Fab (B) fragments recognize intracellular IgG previously internalized by living cells. Bar = 10 μm.

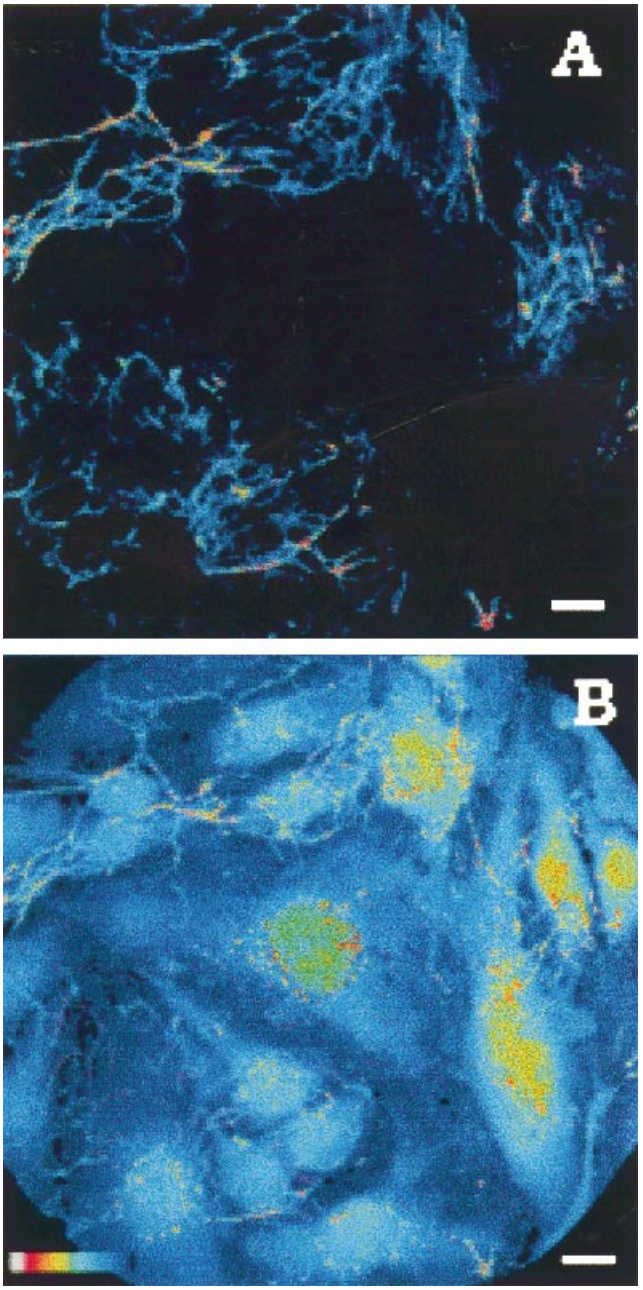

Fig. 3. IVIG-FITC interactions with living human saphenous vein endothelial cells (HSVEC). (A) HSVEC in culture after 20 min incubation with IVIG-FITC and extensive washing with Dulbecco's modified Eagle's medium (DMEM). (B) As in the case of HUVEC, subsequent incubation with calcein-AM provides evidence of IgG internalization. Note that the cell in the centre of the field does not appear in A (see text). Bar = 5 μm.

cells after washing and addition of non-adsorbed IVIG-FITC at 350 μg/ml (Fig. 5B).

The specificity of IgG–EC interaction was further confirmed by the lack of internalization by EC of monoclonal myeloma IgG1λ coupled to FITC.

Importance of microtubule integrity

When HUVEC were pre-treated with the microtubule depolymerizing agent colchicine at 100 μg/ml for 20 min before incubation with IVIG-FITC, no internalization of IgG occurred, indicating that microtubules were involved in the process.

DISCUSSION

In the present study we have directly shown that normal human IgG includes antibody species that bind to and are internalized by living, non-damaged, non-LPS-activated EC in culture. Such interaction was first demonstrated in the case of pooled normal

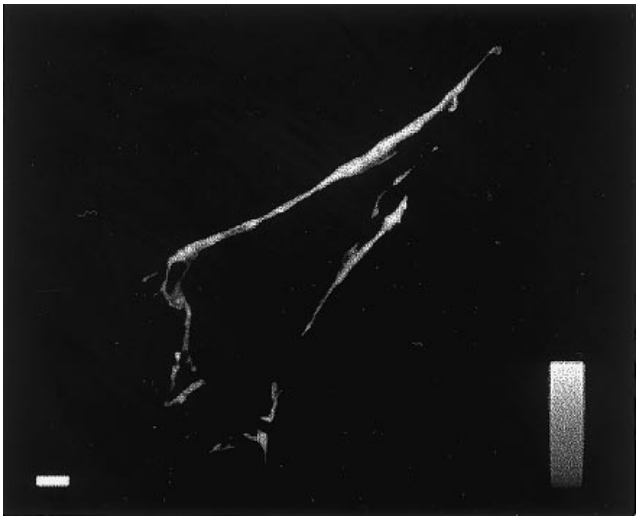

Fig. 4. Cellular specificity of internalization of IgG. Membrane staining of a living human fibroblast incubated with IVIG-FITC, for 30 min at 37°C. Bar = 10 μm.

human IgG (IVIG). Calcein counterstain of living cells proved both the actual intracellular localization of IgG and the integrity of EC membranes. The possibility that a substance copurifying with IgG could be responsible for the intracellular fluorescence was excluded by revelation of intracellular IgG with three MoAbs recognizing epitopes on both Fc and Fab fragments: this double positivity proves that the whole molecule is internalized.

Binding and internalization of IgG contained in IVIG could be due to the presence of abnormal autoantibody species in the serum of certain blood donors, or to a pooling effect that has been described capable of unmasking autoantibody activity by idiotype–antiidiotype interactions [16]. Such a possibility was excluded by the binding and internalization of single healthy donor IgG by EC.

As EC in culture are known to express class I MHC molecules constitutively [17], the role of natural anti-allotypic MHC antibodies was ruled out by experiments performed with single donor IgG on autologous HSVEC. The internalization of both autologous IgG and IVIG by saphenous vein EC also demonstrated that such an event was not limited to cells of fetal origin.

Internalization does not occur in both PTEC and human fibroblast, pointing to a cellular specificity of such endothelial function. Fibroblast membrane binding of IgG could reflect natural autoantibody activity [18].

The mechanisms of the interaction between IgG and EC were investigated first by inhibiting energy-consuming processes of the cells by low temperature and by limiting the incubation time with IgG-FITC. The linear membrane binding of IgG to EC in such conditions, together with the lack of internalization of $F(ab')_2$ and Fc fragments of IgG in a successive experiment in standard conditions, were consistent with a specific process of internalization of selected mediators rather then a non-specific process such as pinocytosis [19] or catabolism of extracellular proteins.

A possible explanation for the lack of internalization of IgG fragments is that the entire IgG molecule is required for the interaction to occur, or that the process of production of fluoresceinated fragments introduces modifications of the reactive site.

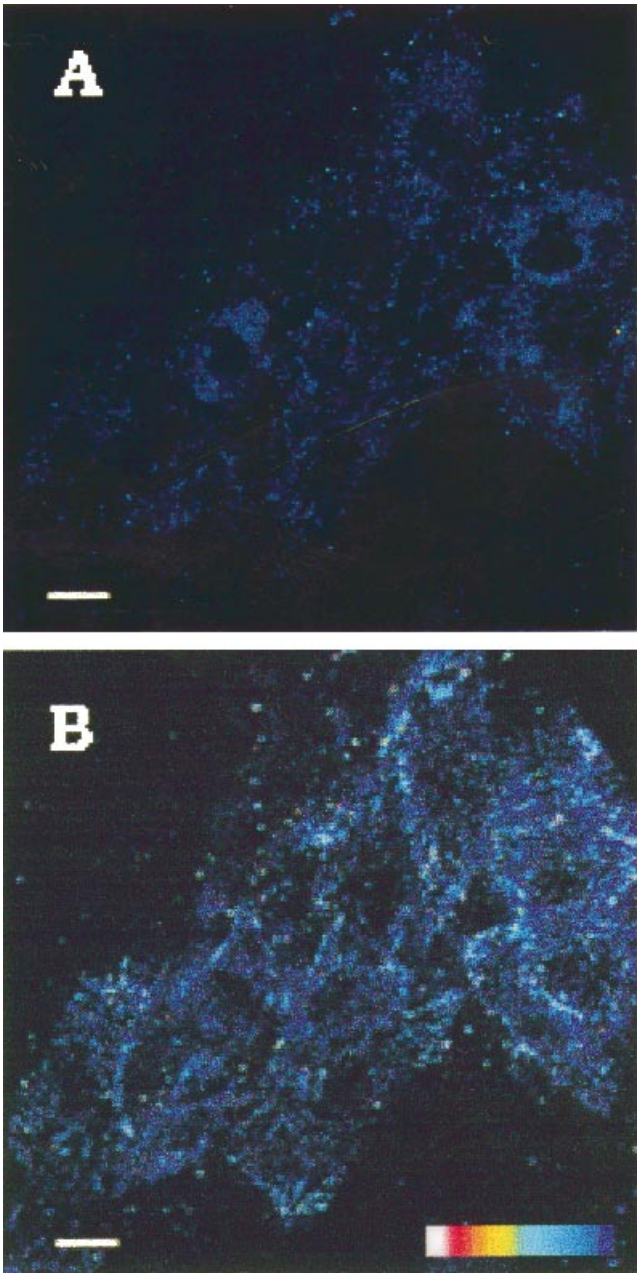

Fig. 5. Interactions between human umbilical vein endothelial cells (HUVEC) and endothelial cells (EC) pre-adsorbed IVIG (A) and standard IVIG (B). (A) HUVEC after incubation with adsorbed IVIG-FITC (through sequential incubation in eight flasks of EC) at 350 μg/ml. In order to obtain a reference 'ghost' image of the cells, autofluorescence was evoked with both high laser beam energy (100%) and photomultiplier gain (×8). (B) Medium replacement with non-adsorbed IVIG-FITC at 350 μg/ml results in a mixed (speckled and fibrillar) intracellular fluorescent pattern. The image was obtained after resetting the instrument (3% and ×1 as in other experiments). Bar = 10 μm.

The lack of internalization of myeloma IgG by living EC indicates that a specific variable region is required for internalization to occur. Such a point is strengthened by the fact that the additional experiment, showing that pre-adsorbed IgG on living EC through several sequential incubations was not able to interact with EC at the same IgG concentration, proved to be effective in

the case of non-adsorbed IgG. Although this result strictly proves only a variable behaviour amongst different antibody molecules, the presence of the whole molecule of IgG in the cytoplasm and the temperature-dependency of the transport strongly suggest a receptor-mediated, energy-dependent mechanism, as we already excluded a role for pinocytosis. As a consequence, the possibility that molecular features other than variable regions (such as glycosylation or relative charge) are involved as selectivity factors in binding and internalization is far less likely. The evidence of a specific process of internalization of IgG by EC, the importance of the microtubule system in signal-macromolecule trafficking within the cell and the fibrillar pattern of fluorescence observed in our experiments led us to investigate the possible involvement of microtubules in the internalization of IgG by EC. The inhibition of the interaction of IgG with EC pretreated with the microtubule-depolymerizing agent colchicine indicates that microtubule integrity is necessary for the internalization to occur.

In conclusion, we have demonstrated that normal human circulating IgG includes one/some autoantibody species that interact with normal, non-damaged EC *in vitro*. Such specific interaction includes membrane binding and internalization, requiring an intact microtubule system. These findings, together with a previous study [12] in which we demonstrated that IVIG modulates endothelial secretion *in vitro*, suggest that natural AECA may have a role in the regulation of endothelial physiologic functions, and that such regulation could be among the mechanisms of the therapeutic action of IVIG on vascular inflammation in several pathologic conditions.

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