

Transient Perturbation of Endothelial Integrity Induced by Natural Antibodies and Complement

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Summary

The barrier function of blood vessels is thought to be regulated at least in part by endothelium. This concept is supported by the dramatic loss of barrier function occurring in the hyperacute rejection of vascularized grafts mediated by anti-endothelial cell (EC) antibodies and complement. In this process, the endothelium is not destroyed but instead loses the ability to retain blood cells and plasma proteins within capillaries. The noncytotoxic mechanism that allows this change in EC function has been unknown. Here we report that within 10 to 20 min of exposure to human xenoreactive natural antibodies and complement, porcine EC undergo alterations in cell shape and in the cytoskeleton that disrupt monolayer integrity and lead to formation of intercellular gaps. Gap formation is not associated with cell death but requires the complement complex C5b67. The gaps induced by anti-EC antibodies and complement are transient; gap closure requires formation of C5b-9 complexes on the cells and the rate of recovery depends on the release of cellular products into the medium. Preincubation of EC with dibutyl cAMP (0.5 mM) prevents gap formation and disruption of the cytoskeleton caused by antibodies and complement. These results provide evidence that the integrity of endothelium is regulated by components of the complement system and suggest a mechanism that may explain the prominent loss of endothelial integrity seen in humoral immune responses.

Vascular endothelium plays a pivotal role in regulating the movement of macromolecules, solutes, and blood cells (1) across blood vessel walls into tissues. This "barrier function" of endothelium is subject to dynamic regulation by various mechanisms. For instance, vascular permeability is increased by the action on endothelium of such inflammatory mediators as histamine, bradykinin, thrombin, IL-1 α and TNF- α (1-6), whereas vascular permeability is decreased by heparan sulfate, cyclic AMP (cAMP)¹, phosphodiesterase inhibitors, prostaglandins, atrial natriuretic peptide, catecholamines, and β_2 -adrenoreceptor stimulators such as isoproterenol and terbutaline (7-11).

The boundary created by endothelium not only confines blood cells and macromolecules to the intravascular space, it also contributes to the hemostatic balance by separating plasma coagulant factors from coagulation activators present in the underlying matrix. For instance, interruption of endothelium exposes tissue factor (elaborated by smooth muscle cells [12]) to plasma factor VIIa and von Willebrand factor (synthesized by endothelial cells [EC]; 13) to platelets. The tissue factor-VIIa complex activates factor X and/or IX (14),

In response to thrombin, platelets become activated (13), expressing surface receptors that bind von Willebrand factor/factor VIII in the subendothelium (15), thus mediating attachment and aggregation of platelets (16).

Perhaps the most dramatic evidence for the physiologic importance of the barrier function of endothelium is provided by hyperacute rejection of vascularized grafts (17-19). Hyperacute rejection, initiated by the binding of xenoreactive antibodies to donor EC and by the activation of complement, is characterized by development of interstitial edema, hemorrhage, and thrombosis within minutes of perfusion of the graft by the recipient blood. Whereas the manifestations of edema, hemorrhage, and thrombosis could reflect complement-mediated lysis of donor EC, in many cases, the endothelium remains intact early in the course of rejection and thus the pathologic changes reflect a loss of barrier function in otherwise potentially viable blood vessels (20, 21).

The goal of the studies reported here was to elucidate non-cytotoxic mechanisms by which anti-EC antibodies and complement could mediate rapid and profound changes in the endothelial barrier. Reasoning that an abrupt alteration in EC morphology might rapidly compromise the integrity of endothelium leading to hemorrhage, edema, and thrombosis, we examined the morphology of EC after exposure to xenoreactive natural antibodies and complement.

We report that incubation of cultured EC in xenogenic

¹ Abbreviations used in this paper: cAMP, cyclic AMP; dB-cAMP, dibutyl cAMP; EC, endothelial cell; MAC, membrane attack complex; PKC, protein kinase C.

serum containing natural antibodies directed against the cells and complement causes, within minutes, changes in EC morphology resulting in the formation of intercellular "gaps." These changes are noncytotoxic, reversible, and followed by an increase in the intracellular level of cAMP. The formation of gaps strictly depends on the assembly of C5b-7 complexes and can be mediated by homologous as well as heterologous complement. Formation of gaps is prevented by cAMP analogues such as dibutyryl cAMP (dB-cAMP). The restoration of endothelial integrity after exposure to anti-EC antibodies and complement requires formation of the membrane attack complex (MAC) and is hastened by some factor(s) secreted by EC. Our findings thus demonstrate that terminal complement complexes regulate the barrier property of endothelium. We postulate that in addition to allowing formation of inflammatory edema, the gaps may expose preexisting inducers of thrombosis in the underlying matrix, such as tissue factor and von Willebrand factor, to plasma coagulation factors and platelets, thus explaining the prominent evidence of thrombosis seen in humoral immune reactions. Furthermore, by allowing mitogens, released from EC or platelets stimulated by complement to gain access to vascular smooth muscle (22, 23), the gaps may contribute to the development of more chronic and proliferative disorders such as atherosclerosis.

Materials and Methods

Materials. DMEM, L-glutamine, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). FCS was from Hyclone Laboratories, Inc. (Logan, UT). Human sera each immunodepleted of C2, C3, C5, C6, C7, or C8, purified recombinant C5a, C5, C6, C7, and C8, and mouse monoclonal anti-human iC3b antibodies were purchased from Quidel (San Diego, CA). NP-40, dB-cAMP, methyl isobutyl xanthine (MIX), gelatin (type B, from bovine skin), BSA (fraction V), glutaraldehyde, *Limulus* amoebocyte lysate, osmium tetroxide, and alkaline phosphatase-conjugated antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin was supplied by Calbiochem-Novabiochem Corp. (La Jolla, CA). Rhodamine-conjugated phalloidin, calcein AM, and ethidium homodimer were obtained from Molecular Probes, Inc. (Eugene, OR). Acetylated low density lipoprotein (LDL) was obtained from Biomedical Technologies, Inc. (Stoughton, MA). Monoclonal anti- α actin antibodies were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Plastic chamber slides, [35 S]L-methionine were obtained from ICN Biomedicals, Ltd. (Costa Mesa, CA). RIA for cAMP level and 51 Cr were from DuPont-NEN (Wilmington, DE).

EC Culture. Porcine aortic EC were isolated and cultured as previously described (24, 25). EC identity was determined based on polygonal morphology at confluence, ability to take up acetylated LDL, and lack of reactivity with anti- α actin antibodies. EC were used between passages 5 and 10. The EC monolayers were grown to confluence on gelatin-coated 24-well, 96-well plates, glass coverslips, or plastic chamber slides and were maintained in DMEM with 1% FCS for 16–20 h before each experiment.

Source of Natural Antibodies and Complement. Well-characterized human serum samples were used as a source of xenoreactive natural anti-EC antibodies and human complement (26–28). Samples of human and porcine blood, collected in sterile and pyrogen-free containers, were allowed to clot at 4°C, and the serum fraction was then separated and stored in plastic vessels. The *Limulus* ame-

bocyte lysate test (29) was used to exclude endotoxin contamination in the sera and purified complement components. The titer of xenoreactive natural antibodies in serum was determined by ELISA as described previously using the cultured porcine EC as a target (24). To obtain xenoreactive natural antibodies, the sera were heated to 56°C for 30 min to inactivate complement. As a source of human complement, samples of human serum which were immunodepleted of IgM (30) or which contained very low or undetectable levels of xenoreactive natural antibodies (26) were used. These sera are known to have intact classical and alternative pathways. Porcine serum was used as a source of homologous complement.

Assessment of Complement Activation on EC. After incubation with xenoreactive natural antibodies for 30 min, EC grown in 96-well plates were incubated with complement at 37°C for 1 h and fixed with 0.1% glutaraldehyde containing PBS. Complement activation was determined by measuring deposition of iC3b neoantigen on ECs by ELISA using mouse monoclonal anti-human iC3b antibodies followed by alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (24). The amount of complement-depleted serum used in different experiments was based on standardization of the amount of iC3b generated. Normally, a concentration of complement-depleted serum which generates iC3b equal to that generated by 10–15% normal serum complement was used.

Morphology of Porcine Aortic EC. Unfixed EC monolayers were examined by phase contrast microscopy. For visualization of actin-based cytoskeleton, cultures were fixed with 3.5% paraformaldehyde and 0.1% NP-40 for 15 min, stained with rhodamine-conjugated phalloidin for 45 min and examined using a Zeiss fluorescence microscope. For scanning electron microscopy, EC monolayers were fixed in 2.5% glutaraldehyde for one hour, postfixed in 1% osmium tetroxide for 1 h and dehydrated through increasing concentrations of ethanol. Samples were then dried in a Ladd critical point-dryer, coated with gold palladium in a Sputter coater and were examined using a Philips scanning electron microscope.

Quantitation of Gap Formation. The number of gaps observed in the EC monolayer stained with rhodamine phalloidin, was quantitated with the aid of an ocular grid. In repeated experiments, random fields of 100 blocks (0.4 mm²) were examined. In an individual block, an area where two EC had lost contact was counted as one gap. Each test condition shown here is representative of multiple experiments; the values depict the mean of three quantitations from a single experiment.

Viability of Cultured EC. The viability of EC exposed to xenoreactive natural antibodies and complement was evaluated using calcein AM (31), ethidium homodimer (32), 51 Cr release (25), [35 S]-methionine incorporation, and staining with 0.4% trypan blue.

For staining with calcein AM and ethidium homodimer, EC after exposure to xenoreactive natural antibodies and complement, were washed with PBS to remove residual serum. They were then covered with a solution containing 2.0 μ M calcein AM and 4.0 μ M ethidium homodimer and incubated at 37°C for 30 min. The cells were rinsed with fresh solution, mounted, and evaluated by fluorescence microscopy.

Cytotoxicity of antibodies and complement was measured by 51 Cr release. EC were labeled with 51 Cr (2 μ Ci/well) for 3 h at 37°C, washed and incubated with DMEM containing antibodies and complement. The percent 51 Cr release was a measure of cytotoxicity of human serum on porcine EC and was determined as described (25).

The metabolic activity of EC was also measured by incorporation of [35 S]methionine. After exposure to antibodies and complement for 1 h, EC were washed and incubated in [35 S]methionine (100 μ Ci/ml) for 4 h. At that time the EC were washed and ex-

tracted with 1 N NaOH. Samples were precipitated in 10% TCA and incorporation of ^{35}S was determined in a liquid scintillation counter.

Measurement of cAMP Levels. The intracellular level of cAMP was measured by RIA (9). In preparation for cAMP measurement, EC were grown to confluence in 24-well plates and incubated with DMEM containing 25% xenoreactive natural antibodies and complement. After treatment with natural antibodies and complement, EC were washed with PBS containing 1 mM MIX to inhibit phosphodiesterase and to prevent subsequent breakdown of cAMP. EC monolayers were lysed by addition of 5% ice-cold TCA at 4°C for a period of 1 h. The TCA-soluble supernatant was removed and extracted three times with 5 ml of ethyl ether saturated with H_2O , then dried and resuspended in 400 ml of sodium acetate buffer (pH 6.2). Samples were added to the tracer [^{125}I]cAMP and cAMP antiserum complex. The mixture was incubated at 4°C for 18 h, spun at 1,500 g for 15 min at 4°C, decanted, and the radioactivity of the pellets was determined using a gamma scintillation counter.

Results

Binding of Xenoreactive Natural Antibodies to Porcine EC and Activation of Complement. Fig. 1 A shows binding to cultured EC of xenoreactive IgM present in two sera. The serum containing xenoreactive IgM is depicted by Ab^+ and the serum free of xenoreactive natural antibodies is represented by Ab^- and is used as the source of complement. After the exposure of EC to various concentrations of Ab^+ , there was a linear relationship between binding of xenoreactive IgM antibodies and the concentration of serum up to 30% (Fig. 1 A). Activation of complement measured by the amount of iC3b deposited on EC was a linear function of IgM binding up to 25% and complement concentrations up to 10% (Fig. 1, B and C).

Morphologic Changes Induced by Xenoreactive Natural Antibodies and Complement. Within 10–30 min after exposure to 25% xenoreactive natural antibodies and complement, a significant portion of EC lost polygonal appearance and cell-cell contact appeared to be disrupted (Fig. 2 B). Alteration in morphology required both natural antibodies and activation of complement since neither complement alone (serum with low or undetectable amount of xenoreactive natural an-

tibodies) nor antibodies (inactivated serum) induced morphological changes (Fig. 2, C and D). Consistent with these results, exposure of EC to xenoreactive natural antibodies followed by C2- or C3-depleted serum did not cause morphological changes (not shown).

The morphologic change in EC monolayers in response to xenoreactive natural antibodies and complement was studied by scanning electron microscopy (Fig. 2, E and F). Treated monolayers had holes or openings between individual cells and the EC appeared to protrude from the plate as if they had contracted.

Exposure of the cultured EC to a xenogeneic serum under the conditions mediating morphological change did not cause cell lysis. Our observations indicate that xenoreactive natural antibodies and complement, at the concentrations used in our studies, had no detectable cytotoxic effect on porcine aortic EC. EC exposed to natural antibodies and complement: (a) took up calcein AM; (b) excluded ethidium homodimer-1 as did controls (Fig. 3, C and D); (c) excluded trypan blue; and (d) did not release ^{51}Cr (Fig. 3 E). Furthermore, the cells remained metabolically active as they incorporated the same amount of [^{35}S]methionine during the 4 h of incubation as the controls. These results are consistent with our prior observation, as well as others, that cultured EC are relatively resistant to lysis (25).

Although morphological changes in response to stimuli such as endotoxin have been described (33), it is unlikely that “contaminants” led to the formation of gaps reported here. First, the sera used as the source of natural antibodies and/or complement were always collected under conditions that would limit contamination by environmental agents. Second, incubation of EC with sera lacking natural antibodies or components of complement did not cause alteration in cell shape as described below. Third, gap formation mediated by antibodies and complement was seen within minutes whereas endotoxin-induced EC shape changes are only noticeable at 24 h (33).

Examination of Actin-based Cytoskeleton. The disruption of the integrity of the EC monolayer has been associated with alterations in the actin-based cytoskeleton (34). To evaluate if such changes occurred in response to binding of natural antibodies and activation of complement, the microfilament-

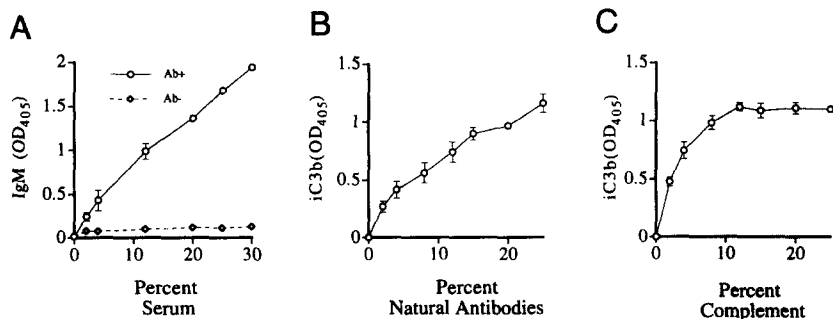


Figure 1. Binding of human natural antibodies to porcine aortic EC and activation of complement. The titer of natural antibodies in human serum was determined by ELISA using the cultured porcine EC as a target (24). (A) Titration of xenoreactive natural IgM antibodies in human serum. Ab^+ is the source of natural antibodies for B and C, and Ab^- is a serum deficient in xenoreactive natural IgM antibodies and is the source of complement for B and C. Activation of complement on EC was measured by deposition of iC3b neoantigen as described in Materials and Methods. (B) Deposition of iC3b on EC precoated with various concentrations of human natural antibodies and incubated with 25% of human complement. (C) Deposition of iC3b on EC precoated with 25% human natural antibodies and incubated with various concentrations of human complement.

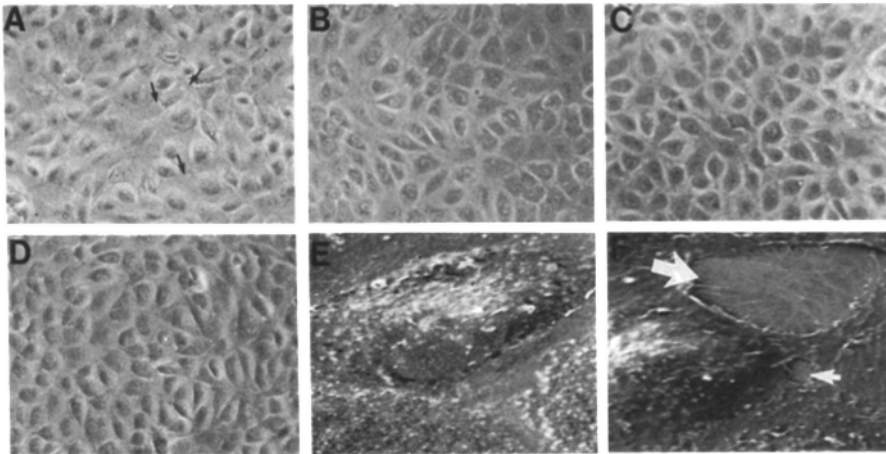


Figure 2. Interruption of EC monolayers by natural antibodies and complement. The morphology of monolayers of porcine EC treated with human natural antibodies and complement for a period of 30 min was examined by phase contrast microscopy (A-D) and scanning electron microscopy (E and F). (A) EC monolayer treated with 20% natural antibodies and complement has intercellular gaps (arrows). (B) EC monolayer treated with 20% natural antibodies remains intact. (C) EC monolayer treated with 20% human complement remains intact. (D) EC monolayer treated with medium remains intact. (E) EC monolayer incubated in tissue culture medium reveals no discontinuities by scanning electron microscopy. (F) EC monolayer incubated with 10% natural antibodies and complement shows large gaps by scanning electron microscopy. (Arrows) Disrupted areas. $\times 100$ (A-D) and $\times 1000$ (E and F).

based cytoskeleton was visualized by staining with rhodamine phalloidin which specifically binds to F-actin (Fig. 4). In untreated monolayers, postconfluent EC were spread out; the microfilament bundles at the periphery of cells appeared as one dense band shared by adjacent cells and their central microfilament bundles (stress fibers) were short and randomly located (Fig. 4 A). Within a few minutes of treating the EC with natural antibodies and complement, peripheral bands of some cells became more distinct from their neighbors and their central microfilament bundles condensed, shortened, and appeared to fuse (Fig. 4 B). The EC appeared to have contracted and become smaller resulting in the formation of intercellular gaps and interruption of monolayer. Whereas neither natural antibodies nor complement alone induced gap formation in EC, EC monolayers treated with natural antibodies did reveal actin polymerization manifested by prominent peripheral bands and elongated central stress fibers in the absence of cell contraction and gap formation (Fig. 4, C and D). In addition to the formation of gaps, exposure of the endothelial monolayer to xenoreactive natural antibodies

caused some EC to become detached from plates creating an occasional "window" in the monolayer.

The association between the formation of gaps in EC monolayers and the activation of complement was not restricted to the heterologous systems used in most experiments. As Fig. 5 shows, porcine EC exposed to human xenoreactive natural antibodies and then to porcine serum as a source of complement, developed gaps that were morphologically indistinguishable from those mediated by human complement. Although the number of gaps caused by porcine complement was less than the number caused by human complement, the results demonstrate that interruption of monolayer can be mediated by homologous complement components.

To evaluate changes in morphology, the number of gaps per 0.4 mm^2 in EC monolayer was determined in experiments where the concentration of natural antibodies and complement were varied independently. The number of gaps was a linear function of antibody concentration between 2.5 and 25% of levels in serum (Fig. 6 A). After exposure to natural antibodies, the number of gaps was a function of comple-

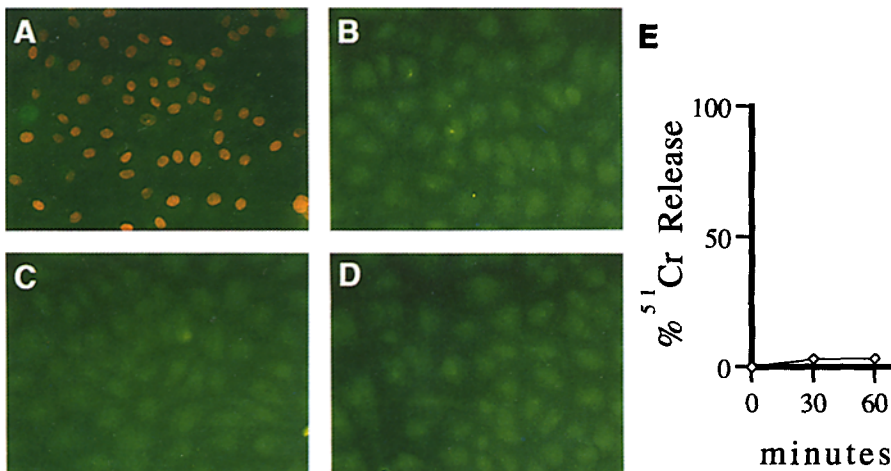


Figure 3. Viability of EC treated with natural antibodies and complement. The viability of porcine EC exposed to human natural antibodies and complement was evaluated using calcein AM, ethidium homodimer (A-D) and ^{51}Cr release (E) as described in Materials and Methods. (A) EC monolayer treated with 75% human serum. (B) EC monolayer treated with medium. (C) EC monolayer treated with 25% human serum for 30 min, antibodies, and complement was replaced by fresh tissue culture medium and cells were stained 210 min later. (D) EC monolayer treated with 25% human serum for 30 min. (E) Cytotoxicity of 25% human natural antibodies and complement assessed by release of ^{51}Cr at 30 and 60 min.

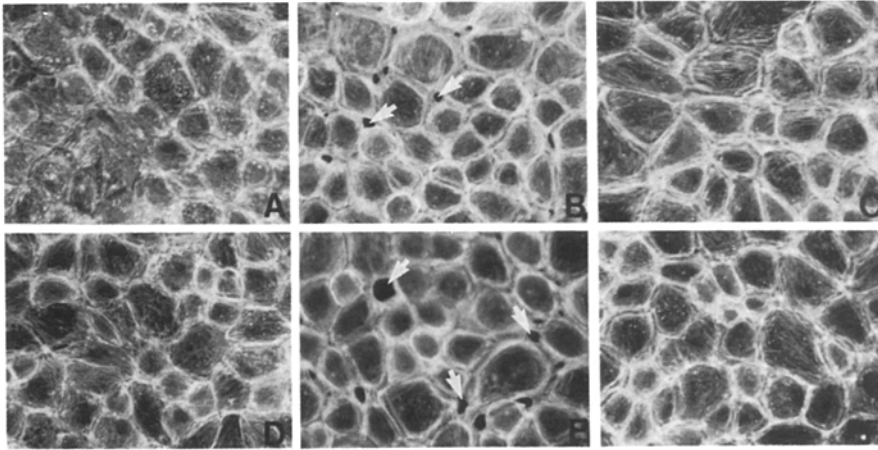


Figure 4. Gap formation in monolayers of porcine EC induced by human natural antibodies and complement. Porcine EC monolayers were treated for 30 min with human natural antibodies and complement, fixed, and stained with rhodamine-conjugated phalloidin as described in Materials and Methods. Gap formation was induced only in monolayers treated with antibodies plus complement or antibodies plus C8-depleted serum. (A) EC monolayer untreated. (B) EC monolayer treated with 25% natural antibodies and complement. (C) EC monolayer treated with 25% natural antibodies. (D) EC monolayer treated with 25% complement. (E) EC monolayer treated with 25% natural antibodies and complement depleted of C8. (F) EC monolayer treated with 25% natural antibodies and complement depleted of C5. Arrows denote gaps. $\times 400$.

ment concentration (Fig. 6 B). It should be pointed out that because we defined a gap as an area where EC have lost contact, larger gaps, possibly resulting from the fusion of two or three smaller gaps, were counted as one. This factor probably limited the increase in the numbers of gaps seen with higher concentrations of complement (Fig. 6 A). The size of gaps appeared to be larger at higher concentrations of complement whereas the actual number of gaps did not increase.

Kinetic Analysis of Gap Formation in EC Monolayers in Response to Natural Antibodies and Complement. The time course for alteration in EC morphology after exposure to xenoreactive natural antibodies and complement is depicted in Fig. 7. Gaps were present as early as 10 min and the maximum number of gaps was seen by 30 min after treatment.

Role of Complement Components in Mediating Change in EC Morphology. To gain insight into the role of complement components in mediating gap formation, pooled sera immunodepleted of individual complement components were used as the source of complement. In repeated experiments,

sera depleted of C8 mediated gap formation in monolayers of EC pretreated with antiendothelial antibodies (Fig. 4 E and Fig. 8), indicating that neither C8 nor C9 is needed for the formation of gaps. In contrast, serum depleted of C5 did not cause gap formation (Fig. 4 F and Fig. 8). The functional activity of C5-depleted serum was restored by addition of purified C5. Therefore, the complement component which stimulates gap formation is derived from C5.

The role of C5a in gap induction was examined by exposing EC monolayers to either recombinant C5a, in the presence and absence of natural antibodies, or C5a generated by the addition of cobra venom factor to whole serum (Fig. 8). Gap formation was not induced in either case, suggesting C5b, the initiator of terminal complement complex formation has a major role in this event.

The requirement for C5b was substantiated by the inability of sera depleted of C6 or C7 to cause EC contraction. In both cases, gap formation was restored by addition of purified C6 or C7, respectively. Thus, gap formation in EC monolayers

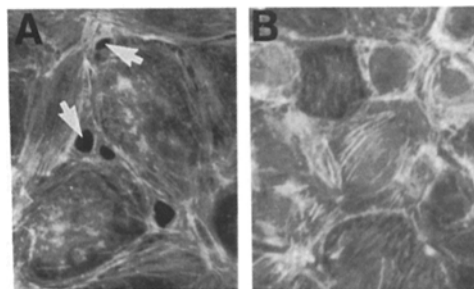


Figure 5. Gap formation in monolayers of porcine EC induced by human natural antibodies and porcine complement. Monolayers of porcine EC were incubated for 30 min with 25% heat-inactivated human serum as source of EC antibodies and then with 25% porcine serum as a source of homologous complement. The cells were then fixed and stained as described in Materials and Methods. Gaps were observed in monolayers treated with human anti-porcine EC antibodies plus porcine complement but not in controls treated with porcine serum alone. (A) EC monolayer treated with human natural antibodies plus porcine complement. (B) EC monolayer treated with porcine serum alone. Arrows denote gaps. $\times 400$.

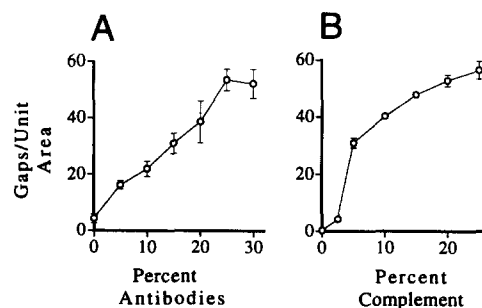


Figure 6. Dose-response for gap formation induced by natural antibodies and complement. Monolayers of porcine aortic EC treated with human natural antibodies and complement were fixed, stained, and quantitated as described in Materials and Methods. (A) EC were incubated with various concentrations of natural antibodies for 30 min after which they were exposed to 25% complement for 30 min. (B) EC were incubated with 25% natural antibodies for 30 min after which they were exposed to various concentrations of complement. The values depict the mean of three quantitations for a single experiment.

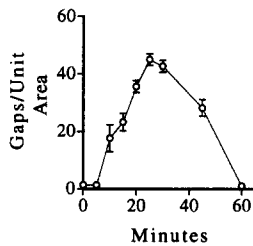


Figure 7. Kinetics of gap formation induced by natural antibodies and complement. Monolayers of porcine aortic EC pretreated with 25% human natural antibodies for 30 min were exposed to 25% complement for various periods of time at 37°C. Gaps were quantitated as described in Fig. 6.

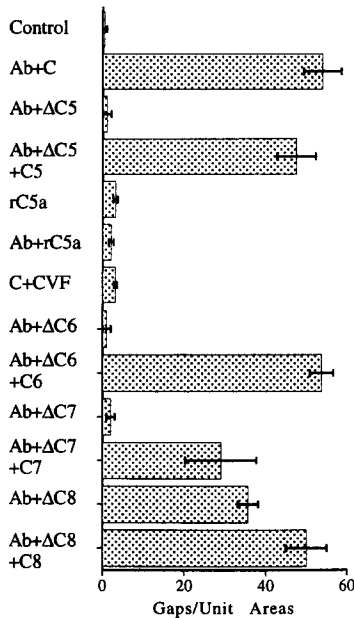


Figure 8. Role of complement components in formation of gaps. Monolayers of porcine aortic EC precoated with 25% human natural antibodies were treated with serum depleted of C5, C6, C7, or C8 and then fixed. Gaps in monolayers were counted as in Fig. 6. Only natural antibodies and complement ($Ab+C$) and serum depleted of C8 (20%, $Ab+\Delta C8$) induced gap formation. Gaps were not formed in monolayers exposed to natural antibodies and serum depleted of C5 (25%, $Ab+\Delta C5$), C6 (30%, $Ab+\Delta C6$), or C7 (30%, $Ab+\Delta C7$). Neither recombinant C5a (75 $\mu\text{g}/\text{ml}$, in absence or presence of antibodies) nor C5a generated by CVF (4 U/ml) caused gap formation. Sera depleted of C5, C6, or C7 were reconstituted with 150 $\mu\text{g}/\text{ml}$ of purified C5, C6, or C7, serum depleted of C8 with 30 $\mu\text{g}/\text{ml}$ of C8, and are indicated by +C5, +C6, +C7, and +C8.

is induced by C5b-7. Addition of purified C8 to C5b-7 almost doubled the number of gaps (in one representative experiment it increased from 23 to 47) suggesting that C8 augments the effect of C5b-7.

Recovery of EC Monolayers after Disruption by Natural Antibodies and Complement. Two lines of evidence indicate that the formation of gaps in monolayers of EC mediated by natural antibodies and complement is a transient phenomenon. First, in repeated experiments, scrutiny of a single disrupted area, visible by phase contrast microscopy over a period of 5 h revealed that the integrity of the monolayer was restored (not shown). Second, analysis of morphology at various times after exposure to natural antibodies and complement showed that the gaps disappeared within 6 h (Fig. 9 E). In all cases, concurrent with the closure of gaps, cells spread out and became morphologically indistinguishable from cells that had never been exposed to xenoreactive natural antibodies and complement.

The events leading to gap closure appear to require additional complement components that are not involved in gap induction. This was suggested by the observation that after exposure of EC to natural antibodies and C8-depleted serum, gaps did not spontaneously close (Fig. 9 F). Progressively, these cells developed larger gaps and many of the cells appeared rounded and completely detached from their neighbors. Addition of purified C8 or forskolin (see below) to the C8-depleted serum restored gap closure. These results suggest that MAC and cAMP may have a role in restoration of the integrity of the EC monolayer.

In repeated experiments, the closure of EC gaps in monolayers after treatment with xenoreactive natural antibodies and complement was delayed when the treatment medium containing antibodies and complement was replaced after 60 min by fresh culture medium. Ultimately, the endothelial gaps closed after removal of the treatment medium and replacement with fresh culture medium. However, 2 h after exposure to antibodies and complement, the number of gaps was far higher in these monolayers than in monolayers remaining in the original treatment medium (Fig. 10). This observation suggests that the recovery of monolayers might require some factor(s) released from the EC.

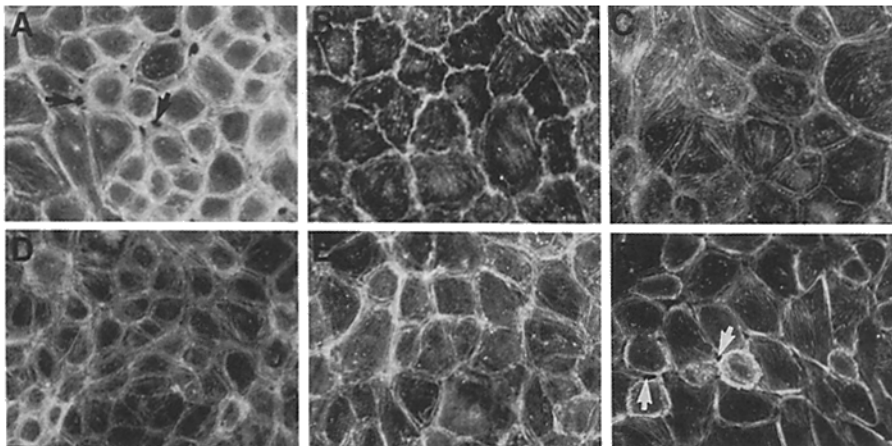


Figure 9. Cellular mechanism regulating gap formation and restoration of integrity of porcine EC monolayers. Porcine EC monolayers treated with forskolin, MIX, or dB-cAMP for 1 h or untreated were then exposed to 25% whole human serum for 30 min and stained for F-actin. Formation of gaps mediated by natural antibodies and complement was inhibited by forskolin, MIX, or dB-cAMP (A-D). (A) Untreated EC monolayer. (B) EC monolayer treated with 10 μM forskolin. (C) EC monolayer treated with 100 μM MIX. (D) EC monolayer treated with 1 μM dB-cAMP. Restoration of EC monolayer integrity in 6 h required MAC (E and F). (E) EC monolayer treated with whole serum. (F) EC monolayer treated with serum depleted of C8. Arrows denote gaps. $\times 400$.

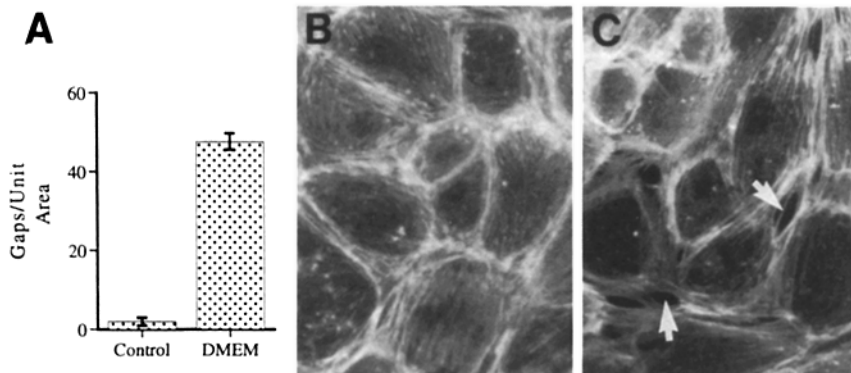


Figure 10. Role of treatment medium in restoration of integrity of porcine EC monolayers. EC monolayers were treated with 25% natural antibodies and complement for 1 h, the treatment medium was then replaced by fresh tissue culture medium, and incubation was continued for 1 h at 37°C. Gaps in monolayers were counted as described in Fig. 6. Substitution of treatment medium containing natural antibodies and complement by fresh tissue culture medium delayed gap closure. (A) Numbers of gaps in EC monolayers either maintained in treatment medium containing natural antibodies and complement (*Control*) or the treatment medium was replaced by fresh tissue culture medium (*DMEM*). (B) EC monolayer treated as control in A. (C) EC monolayer incubated in DMEM, as in A. $\times 400$.

Prevention of Gap Formation by cAMP Analogues. Elevation of intracellular concentration of cAMP enhances the permeability barrier (9). To determine if cAMP protects the EC monolayer against xenoreactive natural antibodies and complement, we tested the effect of reagents that increase the intracellular concentration of cAMP. As shown in Fig. 9, preincubation of EC in 2 mM dB-cAMP inhibited gap formation in response to xenoreactive natural antibodies and complement when examined 30 min after the treatment (Fig. 9). Concurrent with the appearance of distinct peripheral bands and stress fibers, EC became flatter and assumed their polygonal shape. As little as 0.5 mM dB-cAMP inhibited gap formation (Fig. 11 A). In contrast, 2 mM dB-cGMP did not inhibit gap formation induced by xenoreactive natural antibodies and complement (Fig. 11 B). The effect of dB-cAMP was mimicked by the adenylate cyclase activator forskolin and by the cyclic nucleotide phosphodiesterase inhibitor MIX. Forskolin and MIX, at concentrations of 10 μ M and 100 μ M respectively, protected EC against natural antibodies and complement. Increased intracellular levels of cAMP induced by forskolin or MIX caused EC to spread out, to have more organized stress fibers, and sharper and more defined periph-

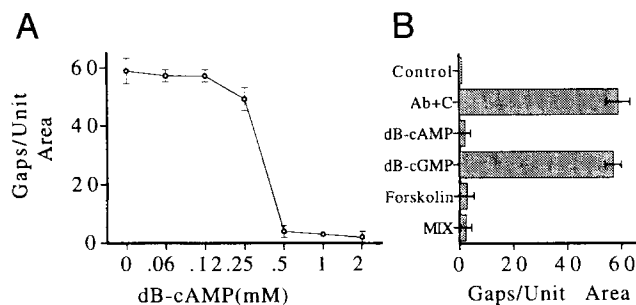


Figure 11. Effect of cAMP on gap formation induced by natural antibodies and complement. Monolayers of porcine aortic EC exposed to cAMP and cGMP analogues for 30 min were then treated with 25% antibodies and complement. Gaps were quantitated as described in Fig. 6. Gap formation induced by antibodies and complement was inhibited by cAMP analogues but not by cGMP analogues. (A) The number of gaps in EC monolayers treated with various concentrations of dB-cAMP. (B) Number of gaps in EC monolayers treated with dB-cAMP (2 mM), dB-cGMP (2 mM), forskolin (10 μ M), and MIX (100 μ M).

eral bands (Fig. 9). Forskolin induced some changes in the cytoskeleton represented by ruffled peripheral bands.

The Effect of Xenoreactive Natural Antibodies and Complement on cAMP Level. Since elevation of intracellular cAMP by forskolin and cAMP analogues inhibited gap formation mediated by natural antibodies and complement, we inquired whether the intracellular level of cAMP was reduced by exposure to xenoreactive natural antibodies and complement. In fact, we found an increase in intracellular levels of cAMP in response to natural antibodies and complement (Fig. 12). Such a rise was both transient and more profound in cells treated with C8-depleted serum (C5b-7) than in cells treated with whole serum (C5b-9). The elevation in the intracellular level of cAMP was detected as early as 15 min and disappeared by 60 min. The results suggested that formation of MAC prevented accumulation of cAMP, perhaps due to leakage from the cells.

Variability. Although all EC were obtained from pig aorta

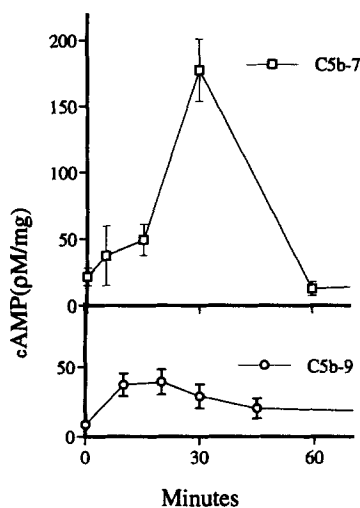


Figure 12. Induction of cAMP synthesis in EC by antibodies and complement. EC precoated with 25% antibodies were treated with 25% serum complement (C5b-9) or 25% serum depleted of C8 (C5b-7) for various periods of time. Total cAMP level was measured by RIA as described in Materials and Methods. Values depict the mean of four measurements for a single experiment.

and were used after confluence, we observed variations in the number of gaps, the kinetics of gap formation, and the recovery time with cells from different animals. This can be explained by variations in the number of binding sites for xenoreactive natural antibodies and the source of natural xenoreactive antibodies, which varied in some experiments. However, when the same cells and the same source of antibodies were used, the experimental results were highly reproducible.

Discussion

The barrier function of endothelium which controls the movement of macromolecules and cells from circulating blood to the surrounding tissues is compromised in many pathologic states. Perhaps the most dramatic alteration in barrier function is seen in hyperacute rejection of vascularized organs mediated by anti-EC antibodies and complement where interstitial hemorrhage, edema and thrombosis develop in minutes to hours (17–19). Our results suggest that the aberrant function of endothelium in hyperacute rejection and perhaps in other conditions mediated by humoral immunity may be due, in part to alteration in the structure of EC leading to the formation of intercellular gaps. The formation of gaps is a noncytotoxic event associated with alteration in the actin-based cytoskeleton and with an increase in the level of cAMP. Our results show that the formation of gaps can be induced by C5b67 complexes although it is amplified by the full complement cascade. The endothelial gaps induced by antibodies and complement are transient, restoration of endothelial integrity resulting from alterations induced by MAC and perhaps by some factor(s) released by EC.

The system used in our experiments involved exposure of EC to a heterologous serum as a model for the reaction that might occur in a xenogeneic organ graft. In homologous systems, complement activation is controlled at the level of C3/C5 convertase by decay-accelerating factor (35, 36) and membrane cofactor protein (37) and at the level of MAC formation by CD59 (38). That these molecules may function less effectively against heterologous than against homologous complement could in part explain the enhanced susceptibility of a xenograft to complement-mediated injury (39, 40). Although alteration in EC shape might be expected to be more severe in a heterologous system such as a vascularized xenograft, our experiments reveal that these changes also occur when EC are the target of homologous complement (Fig. 5). In light of this finding, it would seem appropriate to consider whether some changes in models such as Forssmann shock and the Arthus reaction might result in part from the direct action of complement on endothelium.

Modulation of barrier formation by natural antibodies and complement is not a consequence of cell lysis and does not require insertion of MAC. Although transmembrane channels formed by MAC can normally lyse erythrocytes, nucleated cells seem to be more resistant to complement-mediated killing and more effective molecules of complement are required to lyse nucleated cells than erythrocytes (41). We showed that the concentrations of natural antibodies and complement which induced gap formation were not cytolytic to EC (Fig.

2); therefore, the holes in the monolayer were neither by-products of lysis nor removal of EC. Furthermore, MAC or even C5b-8 needed for cytolysis was not required for interruption of the monolayer since depletion of C8 still induced gaps.

Modulation of barrier function by natural antibodies and complement is reversible. The existence of gaps in monolayers was transient and disappearance seemed to depend on formation of MAC. The latter was supported by the persistence of gaps in cell monolayers treated with C8-depleted serum (Fig. 9 F). Restoration of the integrity of EC monolayers may also require release of some factor(s) from endothelium, possibly stimulated through MAC. This was suggested by the observation that when the medium containing natural antibodies and complement was replaced by fresh medium that did not contain natural antibodies and complement, there was a delay in repair of monolayers (Fig. 10).

Disruption of EC monolayers by natural antibodies and complement was abrogated by cAMP. In systems where the endothelial barrier function is impaired by mediators of inflammation, barrier function can be restored by agents that increase the intracellular concentration of cAMP (8, 9, 42, 43). The exact role of cAMP in the restoration of barrier function of endothelium in these systems is still obscure; however, elevation of intracellular cAMP relaxes EC cytoskeleton and increases cell-cell contact (44). We showed that the gap formation in EC mediated by the action of natural antibodies and complement was eliminated by an increase in intracellular concentration of cAMP caused by dB-cAMP, forskolin, or MIX (Figs. 9 and 11). This suggests that activation of a cAMP-dependent system protects the barrier function in our system as well.

Clearly, natural antibodies and complement delivered signals to the cultured EC as indicated by the increase in intracellular level of cAMP (Fig. 12). However, a significantly higher level of cAMP was observed when serum depleted of C8 was used (Fig. 12 B). Given what is known about signaling by complement C5b-7 and C5b-9 complexes (for a review see reference 45), it is unlikely that C5b-7 is a more potent inducer of the cAMP synthesis. Rather, we think that cAMP is lost from EC treated with C5b-9; that loss may occur through membrane channels just as shown in Ehrlich cells (46).

One possible mechanism underlying the recovery of monolayers of endothelium treated with natural antibodies and complement may involve the synthesis of cAMP (Fig. 12) since we showed that cAMP abrogated gap formation (Fig. 9 D). When adenylate cyclase activity was inhibited by GDP-b-S which inhibits the G-protein, EC treated with natural antibodies and complement never recovered (not shown). Clearly the level of intracellular cAMP is not the only factor since gaps do not close in cells treated with C5b-7 that have increased intracellular cAMP level.

Antibodies and complement could elicit protein kinase C (PKC) activation and increase in the intracellular level of Ca^{2+} as in other systems (46). Such signaling by natural antibodies and complement may account for recovery of EC from humoral injury and changes in cytoskeleton as discussed below. Restoration of EC monolayers may be linked to or

be a consequence of elimination of MAC, a process which requires C5-9 (47) and is mediated by increase in Ca^{2+} and PKC activation (48). In systems where C8 is depleted, PKC is not activated (46) and the C5b-7 complex is eliminated more slowly than C5b-9 (47). In our experiments, depletion of C8 from serum prevented recovery (Fig. 9 F), suggesting a possible relationship between recovery and elimination of the complex.

Activation of PKC and an increase in the intracellular level of Ca^{2+} in EC induced by natural antibodies and complement may lead to rearrangement of actin cytoskeleton by the following mechanism. Upon phosphorylation by PKC, the actin binding protein MARCKS, which anchors actin structures to plasma membrane, loses its affinity for actin. In the presence of high levels of Ca^{2+} , MARCKS binds to calmodulin. Thus binding to actin is inhibited, resulting in changes in the actin cytoskeleton (49, 50). Activation of PKC and an increase in the intracellular level of Ca^{2+} have been linked to morphological changes in EC mediated by thrombin (51, 52).

The barrier function of endothelium is regulated by various mechanisms. The movement of solutes, fluid, and cells from blood to tissues because of disruption of endothelium mediated by the action of inflammatory mediators, ultimately can be hindered by activation of β -adrenergic receptors, and by the action of some prostaglandins and cAMP (1, 2, 53, 54). Our data suggest that the formation of gaps in monolayers of EC induced by antibodies and complement is another condition where disruption of endothelium is transient and can be restored, perhaps by mechanisms that require newly synthesized cellular products. Our findings further support the notion that the exchange between blood and tissues is sub-

ject to dynamic regulation in order to maintain the balance of macromolecules and cells in the vasculature.

Whereas we have shown that generation of C5b67 is essential for the formation of gaps, it may not be sufficient. The formation of gaps may depend upon other factors in serum. This concept is supported by preliminary studies suggesting that gap formation could not be induced in serum-free conditions and by our prior studies on the release of heparan sulfate from cultured EC (27).

The transient increase in permeability of endothelium mediated by natural antibodies and complement in the absence of cytolysis could play a major role in the pathology of hyperacute rejection by promoting edema, thrombosis, and extravasation of macromolecules and blood cells. Whereas we have focused on hyperacute rejection as a model, the changes in cell shape and endothelial integrity discussed here may contribute to a variety of tissue lesions. For example, loss of barrier function of endothelium might also contribute to the development of atherosclerosis, where injury to endothelium (55) and complement activation play a role (56, 57).

We have shown that heparan sulfate is released from EC in response to antibody binding and complement activation (25). EC release basic fibroblast growth factor and platelet-derived growth factor in response to MAC (22, 23). The potential interaction of these growth factors with soluble heparan sulfate has been shown to modulate their mitogenic activity (58) and such effects might be anticipated in the system we studied. Further, interruption of endothelium induced by C5b-7 or MAC may allow leakage into extravascular spaces of these mitogenic factors leading to proliferation of vascular smooth muscle cells (22) and thus to the development of more chronic changes such as atherosclerosis.

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