The Membrane Attack Complex of Complement Alters the Membrane Integrity of Cultured Endothelial Cells: A Possible Pathophysiology for Immune Complex Vasculitis

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Recently, the possibility of direct involvement of the membrane attack complex of complement in the endothelial damage of immune complex vasculitis has been pointed out. However, no studies have so far elucidated this mechanism.

The present study investigated the effects of complement on the membrane integrity of endothelial cells, using the fluorescein diacetate and ethidium bromide staining method.

Cultured human umbilical vein endothelial cells were maintained in medium containing 10% zymosan-activated normal human serum. Cell detachment began to occur after 3 h of incubation, and the number of fluorescein diacetate-positive adherent cells decreased significantly, whereas that of ethidium bromide-positive detached cells increased significantly. Heat inactivation of the serum or replacement of the complement source with non-activated normal human serum or C5-, C7- or C9-deficient serum resulted in complete inhibition of these effects.

These results suggest that complement induces detachment of endothelial cells by altering the cell membrane integrity and support the contention that the membrane attack complex of complement plays a significant role in the mechanisms of endothelial cell damage in immune complex vasculitis. *Key words: fluorescein diacetate/ethidium bromide test; endothelial damage.*

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Conventionally, much emphasis has been laid on the role of chemotactic complement components for neutrophilic leukocytes in immune complex vasculitis (ICV). More specifically, lysozomal enzymes and reactive oxygen are released from the neutrophils that have infiltrated due to the action of complement components, such as C3a, C5a and C5b67, thereby resulting in damage of local vessels (1, 2). This concept, however, has not adequately or unequivocally explained the fact that in ICV, marked vascular destruction is often observed on the affected vessels of the skin and glomerulus, which are almost free from infiltrating neutrophils (3). Recently, we have noted the possibility of direct involvement of the membrane attack complex of complement (MAC), which is a product of terminal activation of complement, in the vascular damage of ICV, for the following reasons: i) MAC-related antigens have been frequently identified in the vessel walls of the skin lesions and renal lesions in patients with ICV (3, 4); ii) swelling, deformation and deciduation have been markedly observed in MAC-positive vascular endothelial cells (3); and iii) in patients with ICV, a significant increase has been seen in the amount of serum SC5b-9, which serves as an index of terminal complement activation (5). In addition, immunoelectron microscopic observation of marked deposition of MAC-related antigens on the endothelial cell membrane of ICV-affected vessels (6) supports the contention that the vascular endothelial cell is the initial target of the vessel-injuring action of MAC. Boom et al. (7) recently pointed out that a decay-accelerating factor, C3/C5 converting enzyme inhibitor, is absent on the surface of vascular endothelial cells in ICV lesions. This implies that late complement activation is readily induced on the autologous endothelial cell and is more likely to lead to the formation of MAC. However, no studies have elucidated the mechanism by which this so-formed MAC influences the vascular endothelial cells.

In the present study, MAC was directly reacted with cultured vascular endothelial cells to investigate the cell membrane damage occurring at an earlier stage with the use of fluorescein diacetate (FDA)/ethidium bromide (EB) stain. The results demonstrated that MAC injures the integrity of the cell membrane, resulting in marked detachment of the cells from the culture plates.

MATERIALS AND METHODS

Preparation of human umbilical vein endothelial cells

Human umbilical cords were obtained immediately after normal vaginal deliveries or Caesarean sections and kept in Hanks' balanced salt solution (HBSS, calcium and magnesium free, GIBCO, Grand Island, NY, USA). The human umbilical vein endothelial cells (HUV-EC) were obtained from human umbilical cord veins by 0.1% collagenase (*Clostridium histolyticum* type I, 315 units/mg, Sigma Chemical Co, St. Louis, MO, USA) treatment, as previously described (8). The isolated endothelial cells were gently resuspended in Eagle's minimal essential medium (MEM, Nissui Pharmaceutical Co, Tokyo, Japan) containing 20% fetal bovine serum (FBS, Meiji, Pharmaceutical Co, Tokyo, Japan), 2 mM L-glutamine, 0.04 mg/ml of gentamycin sulfate, and 150 µg/ml of endothelial cell growth supplement (Sigma Chemical Co). The cells were plated in 1% gelatin-coated plastic culture dishes and incubated at 37°C, 5% CO_2. The medium was changed every 2 days for fresh MEM-20% FBS until the cells were confluent. For subculturing, the cells were treated with 0.25% trypsin-0.014 M ethylenediaminetetraacetic acid (EDTA). One to two passages were used for our experiments.

Source of complement

Fresh normal human serum (NHS) was obtained from 3 healthy donors with AB blood type, Rh^+. Specific complement component C5-, C7- or C9-deficient sera were purchased (Cytotech, San Diego, Calif., USA). Each serum was absorbed with HUV-EC in order to remove antibody to substances on HUV-EC surfaces. Cells were suspended in each serum at 37°C for 3 h and incubated at 4°C overnight, with gentle rocking. After centrifugation at 800 rpm for 10 min, the absorbed serum from the supernate was collected, sterilized by Millex filtration (Millipore Co, Bedford, MA, USA, pore size 0.22 µm) and stored at −70°C. By hemolytic assay, the absorbed

NHS contained approximately 50 CH₅₀ hemolytic units of complement.

Activation of complement

Zymosan (Sigma Chemical Co) was used as a complement activator, as previously described (9). Zymosan was added to VBS^{++} at the concentration of 1 mg/ml and boiled at 100°C for 30 min. The sample was centrifuged at 4°C and at 800 rpm for 5 min, resuspended and washed three times with cold VBS^{++}. The zymosan particles were incubated at the concentration of 1 mg/ml of serum for 30 min at 37°C in either absorbed AB NHS as described above, heat-inactivated AB NHS (prepared by preincubation at 56°C for 30 min) or a specific complement component-deficient serum, C5, C7 or C9. The serum sample was centrifuged at 4°C at 800 rpm for 20 min and the supernatant was used as the complement source.

Measurement of serum C5b-9 concentration

An enzyme immunoassay was conducted to detect SC5b-9 using an SC5b-9 enzyme-linked immunosorbent assay kit from Quidel (San Diego, Calif., USA). Measurements were conducted as recommended by the manufacturer. All serum samples were examined in triplicate. The results were acceptable for cases in which agreement was at least 90% or more.

Fluorescein diacetate and ethidium bromide

FDA (Nutritional Biochemicals, Irvine, Calif., USA) and EB (Sigma Chemical Co) were purchased. FDA stock solution, 5 mg/ml in acetone, and EB stock solution, 200 g/ml in HBSS, were prepared as previously described (10); these were stored at −20°C. Two microliters of FDA stock solution and 0.1 ml of EB stock solution were added to 4.9 ml of HBSS, pH 7.2, just before use.

Cytotoxicity studies

The HUV-EC were plated in duplicate at 5×10^5 cells/well in collagen-coated 2-well glass chamber slides and cultivated in MEM–20% FBS. All wells were confluent at time of use. HUV-EC cytotoxicity (cell detachment and membrane integrity) studies were carried out using various complement sources. HUV-EC monolayers were gently washed twice with MEM, then incubated in MEM containing 10, 20 and 30% zymosan-activated NHS as a complement source for 3 or 24 h. After removal of the media, the adherent monolayer cells were gently washed with PBS, pH 7.2, and immersed in 0.5 ml of FDA/EB assay solution for 3 min at room temperature. Then the solution was removed and the monolayer cells were immediately examined by both phase contrast and fluorescent microscopy. A Nikon B2 filter was utilized for FDA-stained cells, while a Nikon G filter was utilized for EB-stained cells.

Examination was also made to determine whether MAC was present on the surface of the HUV-EC. The adherent monolayer cells treated with the complement source were incubated with monoclonal antibody to C5b-9 neoantigen, as previously described (11). C5b-9 binding was detected with FITC-labeled goat-mouse IgG Fc (Cappel Laboratories, Cochransville, Pa, USA).

Controls included non-activated NHS, heat-inactivated NHS before the addition of zymosan, and zymosan-activated C5-, C7- or C9-deficient serum.

Quantitative study of the cells stained with FDA/EB

HUV-EC monolayers were treated as outlined above. After the supernatant media had been recovered, the dishes were gently rinsed with PBS. The supernatant and rinse were combined to represent the detached cell fraction. The adherent monolayer cells were harvested from the plate by incubation in 2 ml of PBS containing 0.2% trypsin and 0.2% EDTA, pH 7.2. After 20 min at room temperature, the cells were released from the plate by gentle agitation. The detached and adherent cell fractions were centrifuged at 800 rpm for 10 min, resuspended in 1.0 ml of the FDA/EB assay solution, and incubated for 3 min at room temperature. The cells were resuspended, and a drop of the suspension was placed on a slide which was analyzed by fluorescent microscopy. One hundred cells were counted on each slide

and divided into 3 staining types: (a) cells with green fluorescent cytoplasm (FDA-positive cells); (b) cells with non-fluorescent cytoplasm and orange fluorescent nuclei (EB-positive cells); and (c) cells with intermediate green fluorescent stained cytoplasm (in speckled pattern) and orange nuclei. The total per cent of cells in each category (FDA-positive, EB-positive, intermediate) detached plus attached were compared.

Statistical analysis

Student's t-test was employed to evaluate differences between the two groups, and a p-value of less than 0.05 was taken to indicate a significant difference.

RESULTS

The SC5b-9 concentration of 3 samples of the zymosan-activated NHS was 39.12 ± 12.23 µg/ml (mean ± SD), a significant increase compared to the 0.35 ± 0.12 µg/ml of nonactivated NHS and 0.19 ± 0.06 µg/ml of heat-inactivated NHS at 56°C for 30 min before the addition of zymosan ($p < 0.01$). This increase in SC5b-9 level was also inhibited significantly when the complement source was replaced with sera deficient in any of the individual components C5, C7 and C9 (Table I). Complement activation leading to a terminal complement sequence is thus shown to occur in NHS after zymosan activation, as previously demonstrated (8).

When the HUV-EC monolayer was further incubated in MEM containing 10, 20 or 30% zymosan-activated NHS, swelling, crumbling and detachment of the cells began to occur at the margin of the monolayer after 3 h of incubation, and marked detachment extended to the center of the monolayer at 24 h (Fig. 1a). At that time, C5b-9 neoantigen deposition was observed in a dotted pattern similar to that described by Xia et al. (11) on both adherent and detached cells. Under UV illumination, the cells located at the margin of the monolayer, which were detached from the plate, demonstrated FDA-negative dark cytoplasm and EB-positive orange nuclear fluorescence at 3 h of incubation; this tendency was more striking at 24 h (Fig. 1b,c). By comparison, cells cultured in the presence of one of the heat-inactivated NHS, which had been prepared by heating at 56°C for 30 min followed by zymosan-activation, non-activated NHS, or any of the zymosan-activated C5-, C7- and C9-deficient sera did not exhibit signs of detachment (Fig. 1d). The large majority of the adherent monolayer cells demonstrated FDA-positive green fluorescence in the cytoplasm under UV illumination, except

Table I. *SC5b-9 generation in sera after zymosan activation*

Each serum was prepared by absorption with human umbilical vein endothelial cells, followed by zymosan activation (except for non-activated NHS). Values are mean ± SD.

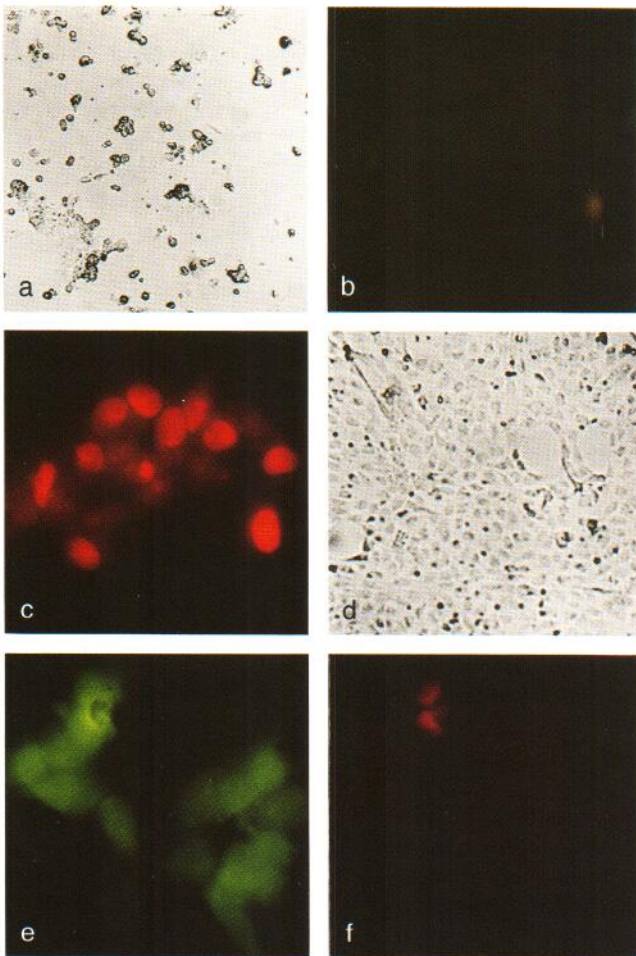| Sera	SC5b-9 (µg/ml)
Non-activated NHS ($n=3$)	0.35 ± 0.12
	]*
Activated NHS ($n=3$)	39.12 ± 12.23
	]*
Heat-inactivated NHS ($n=3$)	0.19 ± 0.06
C5-deficient serum ($n=1$)	0.32
C7-deficient serum ($n=1$)	0.10
C9-deficient serum ($n=1$)	0.15

* $p < 0.01$.

Fig. 1. HUV-EC monolayers were incubated in MEM containing 10% zymosan-activated normal human serum. Significant cell detachment was observed by phase microscopy at 24 h (*a*). A corresponding decrease in the number of adherent cells demonstrating green fluorescence in their cytoplasm (FDA-positive) under UV illumination (*b*) and an increase in the cells with orange fluorescent nuclei and dark cytoplasm (EB-positive) were observed in these cultures (*c*). When the complement source was replaced with heat-inactivated NHS, the cells appeared to be normal (*d*) and the large majority of adherent monolayer cells demonstrated FDA-positivity (*e*), except for the small number of cells with EB-positive orange nuclei and dark cytoplasm (*f*).

for a small number of the cells with EB-positive orange fluorescent nuclei and dark cytoplasm (Fig. 1e,f).

The quantitative study with FDA/EB staining demonstrated a statistically significant increase of cells with orange nuclear fluorescence ($p < 0.01$) and a decrease in the number of green fluorescent cells ($p < 0.01$) when zymosan-activated NHS was added to the medium. Heat inactivation of NHS before zymosan treatment resulted in complete inhibition of this effect ($p < 0.01$). In addition, replacement of the complement source with any of the non-activated NHS, zymosan-activated C5-, C7- and C9-deficient serum resulted in similar inhibition (Table II).

DISCUSSION

MAC has the transient ability to disrupt lipid-containing membranes by the formation of large membrane pores (trans-

membrane channels) and by structural alteration of the membrane (12–14). Red blood cells are most susceptible to membrane attack by MAC. In contrast, nucleated cells are generally known to be more resistant to complement-mediated cytotoxicity than red blood cells. There may be several reasons for the relative resistance of these cells, including inactivation or ejection of cell surface-bound complement proteins, and repair system of membrane damage. However, in the event that the defense mechanism and repair function inherent to the membrane of the nucleated cells are impaired, or acute deposition of a large quantity of MAC occurs in the cells, MAC may possibly damage the membrane of the nucleated cells. In fact, it has been reported that MAC, which is formed on the cell membrane via antibody-dependent activation or alternative complement activation, induces membrane damage in several kinds of tumor cells (15–17) and keratinocytes (18) cultured *in vitro*.

Our present study demonstrated that MAC injures the HUV-EC. When NHS, in which terminal activation of the complement had already been induced by zymosan treatment, was added to the culture medium, morphological changes such as swelling and crumbling of most of the HUV-EC and cell detachment from the culture plates occurred after 3 and 24 h. Furthermore, immunofluorescence study confirmed the deposition of MAC-related antigen on the surface of these cells. The number of detached cells increased depending on the concentration of the added serum.

To further substantiate the effect of complement on the endothelial cells, we chose to use FDA and EB staining, which is useful for observing early damage of cell membranes (10, 19). In this staining, the cytoplasm of cells with intact membranes will emit green fluorescence and the nuclei of cells with damaged membranes will emit orange fluorescence. In the present study, we found that before incubation with complement sources nearly all of the HUV-EC in the monolayers showed FDA-positive green cytoplasm and no EB-positive orange nuclei. Only a small number of the cells were EB-positive and FDA-negative. In contrast, incubation of the cells with zymosan-treated NHS for 3 and 24 h resulted in an increase in EB-positive orange nuclei and FDA-negative cytoplasm. Such an effect on fluorescence was not observed in the cells when non-activated NHS, heat-inactivated NHS or C5-, C7- or C9-deficient serum was included in the medium. These findings suggest that MAC, formed on the cell membrane of HUV-EC, altered the membrane integrity, thereby inducing increased numbers of cells with leaky membranes and detachment of the cells from the culture plates.

Complement activation via the complement-fixing IC leading to generation of MAC has been frequently observed on the affected vessel walls of skin and glomeruli of ICV patients (3–6). Boom et al. (7) recently pointed out that a decay-accelerating factor, C3/C5 converting enzyme inhibitor, is absent on the surface of vascular endothelial cells in ICV lesions. This implies that late complement activation is readily induced on the endothelial cell and is more likely to lead to the formation of MAC.

The present study, therefore, provided evidence that MAC plays an essential role in endothelial damage through its effect on membrane integration in ICV. Further studies to clarify the mechanisms of complement activation and HUV-EC-membrane injury are being conducted.

Table II. *Fluorescein diacetate, ethidium bromide test of membrane integrity (24 h)*

Each serum was prepared by absorption with human umbilical vein endothelial cells, followed by zymosan activation (except for non-activated NHS). Values are mean ± SD.

Complement treatment	FDA-positive cells (%)	EB-positive cells (%)	Intermediate cells (%)	(n)
No complement	73.77 ± 4.84	19.73 ± 1.86	6.50 ± 5.26	(3)
Non-activated NHS	71.96 ± 1.88	20.07 ± 3.71	7.97 ± 2.64	(3)
	]*	]**		
Activated NHS	27.67 ± 14.36	57.90 ± 11.74	14.43 ± 3.25	(3)
	]*	]*		
Heat-inactivated NHS	77.50 ± 6.06	17.73 ± 2.40	4.77 ± 3.74	(3)
C5-deficient serum	80.80	14.50	4.70	(1)
C7-deficient serum	77.50	17.40	5.10	(1)
C9-deficient serum	75.30	12.20	10.50	(1)

$*p < 0.01$, $**p < 0.05$.

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