Complement Activation by Diesel Exhaust Particles (DEP)

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The effect of diesel exhaust particles (DEP) on the hemolytic activity of human serum complement was investigated. Previous treatment of human serum with DEP extracts at 37°C decreased the hemolytic activity of human serum complement dose dependently, to 20% of its original value. This decrease in complement activity by DEP extracts was observed with previous incubation at 37°C but not at 4°C. A decrease in complement activity was observed after previous incubation at 37°C in the presence of EGTA/Mg but not in the presence of EDTA, indicating that the alternative pathway of the complement system had been activated by DEP extracts. Activation of the complement system by DEP extracts was further demonstrated by observation of the cleavage of the third component of the complement (C3) in serum to C3b with immunoelectrophoresis using goat anti-human C3 antiserum. This cleavage of C3 was similarly observed in the presence of EGTA/Mg, indicating the activation of the alternative pathway of the complement system by DEP extracts. These results indicate that DEP can activate the alternative pathway of the complement system, resulting in a decrease in the hemolytic activity of complement and in the production of biologically active degradation products of complement proteins such as C3a. The biological significance of the activation of the complement by DEP in the alveoli is discussed in relation to the influx of neutrophils.

Key words diesel exhaust particle (DEP); complement activation; C3 fragmentation

Diesel exhaust particles (DEP) emitted from diesel-operated cars contain a variety of components, such as carbon nuclei, that absorb a vast number of organic compounds.1–3 It has been reported that some of these compounds are strong mutagens5 and that others are pulmonary carcinogens.5 Moreover, it has been known that DEP can produce chronic obstructive pulmonary diseases such as bronchial asthma and chronic bronchitis6 and that DEP has an adjuvant effect on increasing the production of IgE.7 However, the mechanism of DEP toxicity that causes non-neoplastic lung lesions is not fully understood.

Complement is one of the host defense systems. It is composed of about 20 plasma proteins and several membrane proteins.8 There are two cascade systems for the activation of complement: the classical pathway and the alternative pathway. The biologically important functions that are mediated by complement activation include: 1) formation of the MAC (membrane attack complex), which is responsible for target cell damage and cell lysis; 2) opsonization, the degradation products of complement such as C3b or iC3b that bind to the target cell and are recognized by the specific receptors on the surface of phagocytes and thus increase phagocytosis; and, 3) production of anaphylatoxins such as C3a, C4a and C5a, which promote smooth muscle contraction and increase vascular permeability. C5a has chemotaxis activity for neutrophils and promotes the migration of neutrophils to an inflammatory region.9 Complement usually exists in the blood but has also been reported to exist in tissues of the brain10 and lung.11 When complement is continuously activated in tissue, it can cause inflammation or tissue lesions.

It has recently been reported that neutrophils migrated into the alveoli when DEP was administered in the trachea of mice.12 We assumed that this migration of neutrophils may be correlated with activation of the complement system by DEP. The present study was undertaken to determine whether DEP affects the activity of complement in serum.

MATERIALS AND METHODS

Materials HEPES, EDTA and EGTA were purchased from Dojin Laboratories, Kumamoto, Japan. Gelatin was purchased from Wako Pure Chemicals, Osaka, Japan. LPS (lipopolysaccharide) from E. coli 026: B6 was purchased from Difco Laboratories, Detroit, MI. Goat anti-human C3 antiserum was purchased from ICN Pharmaceuticals Inc.-Cappel Products, Durham, NC.

Buffers The buffers used were isotonic veronal-buffered saline (VB), pH 7.4, and VB containing 0.15 mM CaCl₂, 0.5 mM MgCl₂ and 0.1% gelatin (GVB±).

Serum A pool of normal human serum (NHS) was obtained from young and apparently healthy volunteers.

Diesel Exhaust Particles Diesel exhaust particles (DEP) were collected from the diesel exhaust of a 4JBU1-type engine from Isuzu Automobile Company, Tokyo, as described previously.13 The particles were stored in a sealed bottle at −20°C in the dark.

Preparation of DEP Extracts DEP extracts were prepared as follows: DEP were suspended in 20 mM HEPES-buffered saline, pH 8.0, as 5 mg dry weight/ml, and then left standing for 15 h at room temperature, with stirring. The susenson was filtered with a 0.45-μm cellulose nitrate membrane filter (from Advantec Toyo, Japan). The final pH of the filtrate was adjusted to 7.4 with 4 N NaOH. This filtrate was used as DEP extracts in the experiment.

Assays of Hemolytic Activity The hemolytic activity of complement was measured by the method of Mayer et al.14 with slight modification. Sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibody (EA) were prepared according to established methods.15 The effect of DEP ex-

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tracts on the activity of complement was estimated as follows. Various amounts of DEP extracts were added to 0.1 ml of NHS, and the final volume of the mixture was adjusted to 0.2 ml with 20 mM HEPES-buffered saline, pH 7.4. After 60 min of incubation at 37°C, the reaction mixtures were diluted to 4.5 ml with GVB222. To determine the remaining hemolytic activity of complement in serum, 0.2 ml of EA (5 x 10^6/ml) and 0.7 ml of GVB222 were added to 0.6 ml of the diluted samples of NHS, and the mixtures were incubated for 60 min at 37°C. After incubation, to eliminate the remaining EA cells, the reaction mixtures were centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was isolated and the percentage of hemolysis was estimated by measuring the absorbance at 541 nm of free hemoglobin. Thus, the effect of pre-incubation with DEP extracts on the hemolytic activity of complement was evaluated. For estimation of 100 or 0% hemolysis, 0.2 ml of EA was incubated for 60 min with 1.3 ml distilled water or GVB222 and the absorbance of the supernatant was measured at 541 nm. The absorbance value by DEP extracts was subtracted from the DEP extract-treated experimental sample as a background.

Evaluation of Modification of C3 in DEP Extract-Treated Serum To examine the modification of the structure of C3 in DEP extract-treated serum, immunoelectrophoresis was performed in a 1% agarose gel containing 5 mM barbital, 25 mM barbital sodium, and 0.5 mM EDTA. Electrophoresis was carried out at 0.5 mA/cm with 10 mM barbital, 50 mM barbital sodium, and 1 mM EDTA at 4°C. After electrophoresis, goat anti-human C3 antisera was added to the trough, and C3 precipitates were visualized after an immunodiffusion period of 24-48 h. The gel was soaked in 0.9% NaCl for 3 d to remove nonprecipitated proteins and was stained with Coomassie blue.

RESULTS

Effect of DEP Extracts on the Hemolytic Activity of Complement NHS (0.1 ml) was treated with various volumes of DEP extracts in a volume of 0.2 ml, for 60 min at 37°C, then the remaining hemolytic activity of serum complement was estimated. It was found that DEP extracts dose dependently decreased the hemolytic activity of complement in NHS (Fig. 1). We then examined the effect of time of incubation with DEP extracts on the hemolytic activity of complement. As shown in Fig. 2, the hemolytic activity of NHS decreased time-dependently and attained 20% of its original value by incubation with DEP extracts for about 30 min at 37°C. On the other hand, in the absence of DEP extracts, a decrease in hemolytic activity was not observed up to 120 min of incubation at 37°C. These results indicate that a decrease in the hemolytic activity of complement by previous incubation with DEP extracts is not due to the direct effects of DEP extracts on EA, because the decrease in hemolytic activity is dependent on the time of incubation of NHS with DEP extracts, but is related to the consumption of complement by activation of the complement system.

Effect of Temperature on the Decrease in the Hemolytic Activity of Complement by DEP Extracts We examined the effect of previous incubation of NHS with DEP extracts on the hemolytic activity of complement at 37°C and at 4°C. As shown in Fig. 3, when NHS was incubated with DEP ex-

![Fig. 1. Depletion of Hemolytic Activity of Complement in Serum by DEP Extracts](image1)

![Fig. 2. Effect of Time of Incubation with DEP Extracts on the Hemolytic Activity of Complement](image2)

![Fig. 3. Effect of Temperature of Incubation with DEP Extracts on the Hemolytic Activity of NHS](image3)
activate the alternative pathway. When NHS was incubated with LPS at 37°C, the complement system was activated through the alternative pathway and the hemolytic activity was almost exhausted. In contrast, after incubation at 4°C, the hemolytic activity of complement remained, without any consumption. It has been reported that FUT-175 (from Torii & Co., Ltd.) is a serine-protease inhibitor and inhibits the activity of the first component of complement (C1). When NHS was previously incubated with FUT-175 at 37 and 4°C, the hemolytic activity decreased to about 10% of its original value. It was presumed that, in the presence of FUT-175, the activation of C1 was inhibited directly and therefore activation of the classical pathway did not proceed. These results indicate that the effect of DEP extracts on the complement activity is similar to the effect of LPS, rather than to that of FUT-175. Accordingly, it was thought that the inhibition of complement activity by DEP extracts was induced not by direct inhibition of a component of the complement cascade in the hemolytic reaction but by a reaction correlated with an enzyme reaction in the previous incubation of NHS with DEP extracts.

**Effect of DEP Extracts on the Hemolytic Activity of Complement in the Presence of EDTA and EGTA/Mg**

From the results shown in Fig. 3, we assumed that DEP extracts could activate the complement system in NHS in a manner similar to that of LPS and that, as a result, hemolytic activity would decrease. It is known that Ca²⁺ and Mg²⁺ are essential for the activation of the classical pathway, while only Mg²⁺ is essential for activation of the alternative pathway. Therefore, in order to examine which pathway of the complement is activated by DEP extracts, the DEP extracts were incubated with NHS in the presence or absence of EDTA or EGTA/Mg, and the remaining complement activity was estimated. As shown in Fig. 4, after 60-min incubation of NHS with DEP extracts in the presence of EDTA, about 75% of the hemolytic activity remained. On the other hand, in the presence of EGTA/Mg, the activity decreased to about 40% of its original value. These results indicate that DEP extracts activate the alternative pathway of complement and decrease the subsequent hemolytic activity of complement.

**Evaluation of C3 Modification by Immunoelectrophoresis**

C3 is the complement component found in the highest concentration in serum and is essential for the activation of both the classical and alternative pathways. Therefore, to investigate the mechanism of complement activation by DEP extracts, we examined the modification of C3 in NHS after treatment with DEP extracts. NHS was incubated with DEP extracts for 60 min at 37°C in the presence or absence of EDTA or EGTA/Mg, and the modification of C3 was evaluated by immunoelectrophoresis with goat anti-human C3 antisera. As shown in lane B of Fig. 5, when NHS was incubated with DEP extracts, C3 was cleaved to C3b. This modification was confirmed by a comparison with the result of that using purified C3b. In the presence of EDTA, no modification of C3 was observed either in the presence or absence of DEP extracts (lanes C, D). However, in the presence of EGTA/Mg, C3 was cleaved to C3b in the presence of DEP extracts (lane F). These results indicate that DEP extracts activate the alternative pathway of complement and that C3 is fragmented to C3a and C3b.

**DISCUSSION**

The present study demonstrated that the hemolytic activity of complement in serum is reduced by pre-treatment of NHS with DEP extracts at 37°C. This decrease in hemolytic activity was dependent on the dose of DEP extracts (Fig. 1). In this experiment, NHS was treated with DEP extracts for 60 min at 37°C, and after the addition of EA, the mixture was reacted for another 60 min at 37°C. The remaining complement activity was estimated from the extent of hemolysis. Two possibilities were considered to be the cause of the decrease in hemolytic activity by DEP extracts. One possible
cause is that activation of the classical pathway was inhibited by DEP, preventing hemolysis from proceeding. Another possible cause is that the quantities of complement components were decreased when NHS was treated with DEP extracts at 37°C for 60 min.

As shown in Fig. 2, a decrease in hemolytic activity was observed in accordance with the duration of incubation with DEP extracts at 37°C, whereas no decrease was observed immediately after serum was mixed with DEP extracts. Moreover, as shown in Fig. 3, no decrease in hemolytic activity was observed when NHS was treated with DEP extracts for 60 min at 4°C. In the experiment using FUT-175, an inhibitor of C1, the hemolytic activity decreased because the classical pathway was not activated. However, in the presence of LPS, an activator of the alternative pathway, the alternative pathway was activated at 37°C and complement components such as C3 were consumed, resulting in a decrease in the hemolytic activity. In contrast, when NHS was treated with LPS at 4°C, the alternative pathway was not activated and the hemolytic activity did not decrease. It is known that Ca²⁺ and Mg²⁺ are essential for activation of the classical pathway, while only Mg²⁺ is essential for activation of the alternative pathway. When Ca²⁺ and Mg²⁺ are trapped by EDTA, activation of both the classical and alternative pathways of the complement system is stopped, while only the alternative pathway is activated when Ca²⁺ is trapped by EGTA. As shown in Fig. 4, when NHS was treated with DEP extracts in the presence of EDTA, the classical and alternative pathways were not activated in pre-incubation, and the hemolytic activity of serum complement did not decrease significantly. On the other hand, when NHS was treated with DEP extracts in the presence of EGTA/Mg, the alternative pathway was activated in pre-incubation, and the hemolytic activity decreased to 40% of its original value. Therefore, these results indicate that the decrease in hemolytic activity by DEP extracts results from activation of the alternative pathway of complement by DEP extracts.

Furthermore, the results of immunoelectrophoresis confirmed that C3 in serum was modified to C3b by DEP extracts (Fig. 5). Namely, this result supports the hypothesis that DEP extracts can activate the alternative pathway of complement in vitro and result in a decrease in complement components.

There are approximately 2000 kinds of chemicals in DEP, and many acidic compounds such as carboxylic acid or phenol are included in DEP. It has been reported that DEP can produce a superoxide anion and also cause mutation. Polysaccharides such as zymosan and LPS have been shown to be activators of the alternative pathway. Nucleophiles such as methyamine have also been shown to be activators of the alternative pathway. The present study demonstrated that DEP extracts could activate the alternative pathway of complement. However, the activator of the alternative pathway in DEP extracts and the mechanism of activation of the alternative pathway remain unclear.

Complement proteins are mainly synthesized in the liver and exist in the blood. However, expression of complement has also been confirmed in tissues. It has been reported that complement components are produced by alveolar type II epithelial cells in pulmonary alveoli. Accordingly, it is thought that the complement existing in pulmonary alveoli plays an important role as a defense system in the lung. On the other hand, it has been pointed out that the activation of complement is related to the destruction of self tissue, which is found in Alzheimer’s disease and in chronic rheumatism. Furthermore, it has been reported that C3a and C5a are related to the release of histamine from mast cells, which play an important role in allergic diseases. Therefore, it is thought that DEP inhaled in pulmonary alveoli activate the complement system, resulting in tissue injury and allergic disease in the lung.

For elucidation of the mechanism of complement activation by DEP extracts and of lung injury by DEP related to complement activation, examination of more purified samples from DEP is needed. We are currently attempting to fractionate and separate possible complement activators from DEP extracts.

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