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Physical and biological studies on DNA/anti-DNA immune complex formed *in vitro*

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Abstract

In vitro preparation of DNA/anti-DNA immune complex using monoclonal antibody and low molecular weight homogeneous DNA was described and the characteristics of the immune complex were identified. The immune complex was formed by the monoclonal antibody with DNA effectively at high ratios of antibody/DNA. The activation and binding ability of the immune complex to the complement was considerably high, whereas the capacity to bind red blood cells via C3b complement component receptor was shown relatively low. The assay of sucrose density gradient ultracentrifugation indicated that the sedimentation coefficient of the immune complex was between 10S and 23S. Studies of organ distribution and uptake of IC demonstrated a particular affinity to the kidney tissue. The significance of these results with respect to the latent pathogenicity of the immune complex was discussed.

Abbreviations: Ab – antibody; bp – base pairs; C3b – degradation product of the 3rd component of complement; cpm – count per minute; CR1 – complement receptor type 1; gvb – gelatin-veronal buffer; gvb²⁺ – gvb containing Ca and Mg ions; IC – immune complex; PBS(–) – Ca²⁺-Mg²⁺-free phosphate-buffered saline; RBC – red blood cells; S – sedimentation coefficient; SDGC – sucrose density gradient ultracentrifugation; SLE – systemic lupus erythematosus

Introduction

Circulating immune complex is believed to be responsible for many different symptoms and tissue damage in the autoimmune diseases such as systemic lupus erythematosus (SLE) (Koffler *et al.*, 1967; Koyama *et al.*, 1991; Mohan C and Datta SK 1995). It is known that immune complex (IC) consists of DNA and anti-DNA antibody and in some cases, immune complex is large enough to activate complement through the classical complement activation pathway (Lennek *et al.*, 1981). Generally, complement conjugated IC could be bound to erythrocyte through the complement receptor type 1 (CR1), thereafter be transported to the reticuloendothelial system (RES) and finally be degraded (Cornacoff *et al.*, 1983; Davies *et al.*, 1990; Emlen *et al.*, 1992). IC

that is not removed by RES will deposit in the kidney as well as other tissues, causing inflammation.

Recently, many attempts have been done to remove the IC from blood circulation by using an immuno-adsorbent device. Sera obtained from patients with SLE or experimental animals, such as rabbits that were immunized with DNA in advance, were used to evaluate the immuno-adsorbent device (Terman *et al.*, 1976; Takahashi *et al.*, 1987; Sagawa *et al.*, 1989). IgG fractions purified from the sera mentioned above was also used in the *in vitro* preparation of IC (Riley *et al.*, 1980; Morioka *et al.*, 1996). However, using these sera or IgG may cause following disadvantages: sera obtained from patients and animals are donor dependent, and may possess different characteristics of IC existed in patients, making the evaluation complicated. In addition, the sera from patients may occasionally be

dangerous due to the possibility of the infection by HIV and hepatitis virus (Chng *et al.*, 1993; Lu *et al.*, 1995; Cimmino *et al.*, 1996).

In this study, a simple preparation of IC using monoclonal anti-DNA antibodies and low molecular weight homogeneous DNA was developed and the characteristics were investigated both *in vitro* and *in vivo*. Our results showed the properties of the IC prepared *in vitro* in this study are likely similar to those of the IC found in patient with autoimmune diseases, indicating the possibility to apply the prepared IC for the studies both *in vivo* and *in vitro*.

Materials and methods

Preparation of immune complex

The anti-DNA antibody (IgG2a, κ) producing hybridoma was produced by fusion of the BALB/c P3U1 myeloma cells with spleen cells of unimmunized 7-month-old female NZB/W F1 mice (SLC, Japan), using the PEG method. The antibody was purified from culture medium with affinity chromatography on protein G-Sepharose (Pharmacia Biotech, Sweden).

To generate low molecular weight DNA fragments, plasmid pUC18 DNA was digested with restriction nucleases EcoR1 and ApaL1. DNA fragment of 219 base pairs (bp) was electroeluted from the agarose gel and purified with phenol and chloroform. DNA was radioactivity labeled with ^{32}P -dCTP (ICN Biomedicals Inc., U.S.A.) using DNA labeling kit (Wako Pure Chemical industries, Japan) to reach a specific activity of 0.77×10^8 cpm μg^{-1} . All the *in vitro* and *in vivo* experiments with ^{32}P -labeled DNA were performed within 5 days after the labeling reaction. In the preliminary studies, the DNA and ^{32}P -labeled-DNA were tested for the antibody binding capacity by Farr assay described in detail later. There was no significant difference in the binding capacity before and after labeling DNA with isotope.

Immune complex was formed by incubation of Ab and DNA at various Ab/DNA ratios in PBS(-) at 37°C for 1 hr. Siliconized microcentrifuge tubes were used in all assays to prevent the adhesion of IC to the wall of tube.

Binding assays

DNA binding by Ab was measured by the Farr assay described elsewhere (Taylor *et al.*, 1980) with the fol-

lowing modifications. The initial reaction volume of DNA and Ab at various ratios was $96\ \mu\text{l}$ totally. Then $96\ \mu\text{l}$ of a 50% ammonium sulfate solution was added, and the mixture was incubated at 4°C for 1 hr. Subsequently, the sample tubes were spun at $10\ 000\ g$ at 4°C for 10 min and the radioactivity (^{32}P -labeled-DNA) was measured by scintillation counter. The total radioactivity of supernatant and pellet was regarded as a total cpm. Finally, $96\ \mu\text{l}$ (1/2 total volume) of the supernatant was transferred to a new tube and the radioactivity was counted and compared to the total cpm per assay. The DNA binding activity by Ab was expressed as follows: percentage DNA bound = $100 \times (\text{total cpm} - \text{cpm of supernatant}) / (\text{total cpm})^{-1}$.

The RBC binding assay was performed as follows. $96\ \mu\text{l}$ of IC was mixed with $2\ \mu\text{l}$ of $0.1\ \text{M}$ CaCl_2 , $2\ \mu\text{l}$ of $0.1\ \text{M}$ MgCl_2 and $50\ \mu\text{l}$ guinea-pig complement solution (prepared from lyophilized guinea-pig complement, Alfa Biotech, Italy) that was 4 times diluted in gvb (gelatin-veronal buffer: 0.1% gelatin, 5 mm sodium barbitol), and then added with $100\ \mu\text{l}$ of washed normal human erythrocyte at a concentration of $1.25 \times 10^9\ \text{ml}^{-1}$ in gvb $^{2+}$ (gvb containing 0.5 mm MgCl_2 and 0.15 mm CaCl_2). The mixture was incubated at 37°C for 5 min and spun at $8000\ g$ for 1 min. After the total cpm was counted the supernatant was removed. Cell pellet was washed twice with gvb $^{2+}$ and the radioactivity was counted. The IC binding results were expressed as the percentage of radioactivity bound to RBC as follows: percentage of IC bound to RBC = $100 \times (\text{cpm in cell pellet}) / (\text{total cpm})^{-1}$.

Complement activation capacity of IC was investigated by the C3b incorporation assay. Complement-treated IC was incubated with rabbit anti-C3b antibody (Nordic Immunological Laboratories, U.S.A.) at 37°C for 2 hr. Then sheep anti-rabbit immunoglobulin (The Binding Site Ltd., England) was added and incubated at 37°C for one more hour and then at 4°C for 2 hr. The samples were spun at $8000\ g$ for 15 min. The total cpm and the cpm of 1/2 volume of supernatant were counted. Complement activation capacity was expressed as the percentage radioactivity of the precipitation as follows: percentage of IC that incorporated C3b = $100 \times (\text{total cpm} - \text{cpm in supernatant}) / (\text{total cpm})^{-1}$.

Sucrose density gradient ultracentrifugation

The sizes of IC were measured by sucrose density gradient ultracentrifugation (SDGC). Samples were run at $40\ 000\ \text{rpm}$ for 16 hr. Human serum albumin, IgG and

Figure 1. Percentage of DNA bound to anti-DNA antibody at various Ab/DNA ratios. ^{32}P -labeled DNA and anti-DNA antibody were incubated at Ab/DNA ratios of 0.1, 1 and 20 mg of IgG per μg of DNA. DNA that bound to Ab was precipitated by ammonium sulfate and the radioactivity was counted.

IgM were used as standard markers of 4.6S, 7S, 19S, respectively. Fractions were harvested from the bottom of the tubes and the radioactivity was counted. The data were expressed as cpm per milliliter of fraction.

Complex uptake by organs

Three to five months old female BALB/c mice (SLC, Japan), of no autoimmune disease and no demonstrable anti-DNA antibody activity in blood circulation, were used for the study of organ distribution. Mice were injected with IC at a dose of 40 to 60 μg ^{32}P -labeled DNA in 0.2–0.3 ml of PBS(-) per body, via the lateral tail vein, and were sacrificed by cervical dislocation at 40 min after injection. The liver, spleen, kidney and lung were dissected, weighed and the radioactivity was counted to estimate the IC uptake by soft tissue. Total body content of radioactivity was measured by summing the content of the radioactivity in all parts of body. The radioactivity measurements were normalized to give cpm counts mg^{-1} of tissue.

Results and discussion

Immune complex formation

It was described by several investigators that the double stranded DNA of 30–240 bp in size had been found mostly existing in the IC isolated from SLE sera (Raptis and Menard, 1980; Ikebe *et al.*, 1983). IC containing DNA less than 100 bp was too small to activate enough complement (Lennek *et al.*, 1981). Therefore, IC was prepared *in vitro* with low molecular weight of DNA. Double stranded DNA is considered suitable for the experiment, and double stranded DNA of 219 bp was used in this study.

DNA/anti-DNA IC formed within a wide range of Ab/DNA ratios was prepared and the percentage of DNA bound to antibody was investigated. Figure 1 shows the percentage of DNA bound at different ratios of Ab/DNA. When IC was formed at high Ab/DNA ratios (≤ 10 mg IgG per μg of DNA), the percentage of DNA bound to antibody was above 80%. Control experiments using non-anti-DNA antibody showed there was no DNA bound (data not shown), which indicated that monoclonal antibody prepared in this work was high specificity to the DNA. The following exper-

Figure 2. Complement component C3b activation and incorporation of IC and RBC binding of IC. Results are expressed as percentage of IC bound or IC incorporation. Data are represented as the mean $\pm$ S.D. of three to four separate determinations.

Figure 3. Sucrose density gradient centrifugation of IC formed with DNA of 219 base pairs. Human serum albumin, IgG and IgM were used as standard marker of 4.6S, 7S and 19S respectively.

iments were performed at an Ab/DNA ratio between 10 and 20 mg IgG per μ g of DNA unless indicated.

Complement activation and binding to red blood cells

It is known that IC could be divided into three types according to their sedimentation coefficients: large,

Figure 4. Organ distribution and body uptake of IC. A representative result of five experiments in a similar design is shown. Results were represented as percentage radioactivity uptaken into each organ and the radioactivity in 1 mg of tissue. Time of sacrifice was 40 min after administration.

Table 1. Comparison of IC prepared in this study with that from patient with SLE

	IC prepared in this study	IC from SLE patient
S value	10-23	7-19
Activate complement	+	+
RBC-binding	low	- or $\pm$
Kidney affinity	high	high

small and very small. There are many evidences suggesting that large IC is cleared rapidly from the circulation by Fc mediated and complement mediated pathways in the body (Finbloom and Plotz, 1979; Cornacoff *et al.*, 1883; Davies *et al.*, 1990). The very small IC was too small to activate the complement and may therefore be non-pathogenic (Horgan and Taylor, 1984). However the small IC in the circulation can only be cleared much more slowly than the large IC, and therefore may have greater chances to deposit in tissues (Emlen and Burdick, 1985). In order to examine the complement activation ability of the IC and their binding capacity to the CR1 receptor of RBC, the C3b incorporation assay and RBC binding assay were performed. Figure 2 shows the percentage of IC bound by RBC and

the percentage of IC that activated and incorporated to the complement. The experiments were performed at an Ab/DNA ratio of 20 mg IgG/ μ g DNA, and the DNA size was 219 bp. The results revealed that the percentage of IC incorporated to C3b was about 64%. However, the IC bound by RBC was only 16 to 18% as compared with that bound by C3b. These data suggest that the IC prepared in this study was able to activate the complement and to incorporate the complement component of C3b, while the ability to bind C3b receptor of RBC was low.

Determination of the S value

Sedimentation coefficients (S value) of the IC were revealed by SDGC. The centrifugation was run at 274 000 g for 16 hr, at 5–40% w v⁻¹ linear sucrose density gradient in PBS(-). IC was fractionated and the S value was calculated corresponding to the standard marker. Figure 3 shows the distribution pattern and the S value of IC after SDGC. Results showed the S value was between 10S and 23S. These results were consistent with the findings of Horgan (1987), which demonstrated that IC of 12–22S formed *in vitro* with IgG of SLE patients was proved high complement activation and incorporation ability, but low RBC binding activity. Furthermore, small IC of 7–19S has been demonstrated in the sera of patients with SLE (Bruneau *et al.*, 1977; Pereira *et al.*, 1980; Agnello and Mitamura, 1982; Negoro *et al.*, 1985; Koyama *et al.*, 1991). Analysis of the SDGC suggested the similarity of the physical properties of the IC prepared in this study to that of the IC of SLE patients.

Organ distribution and the uptake of IC

Organ distribution and the uptake of IC were investigated in order to look more closely at the nature of the IC *in vivo*. It was observed that the radioactivity of IC remained in blood circulation dropped rapidly after injection of the IC to blood circulation (data not shown). Figure 4 shows the organ distribution and the uptake of IC at the time of 40 min after administration of IC. The fact that the liver uptook and retained about 60% radioactivity of total body content, suggested that IC was transported and degraded mainly in liver. Though kidney uptook small portion of IC, the cpm contained in one mg of kidney tissue showed relatively higher than those of other tissues except for the liver. These results suggest that the IC prepared in this study may have high affinity to the kidney.

Conclusions

As shown in Table 1, we have prepared the immune complex *in vitro* using monoclonal antibody and low molecular weight DNA. The IC was of high complement activation ability and low RBC binding capacity. The S value was between 10S and 23S. Furthermore, the IC was of high affinity with the kidney. We concluded that the IC prepared *in vitro* in this study is likely to be physically and biologically similar to those

from SLE patients, and therefore could be expected to be applied in many *in vitro* and *in vivo* studies.

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