Specific Role of Magnesium Ion in Protecting the Anticomplementary Effect of DNA in Immune Hemolysis¹

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OKUHARA, E., IGARASHI, J., WAKIZAKA, A. and MATSUMOTO, T. *Specific Role of Magnesium Ion in Protecting the Anticomplementary Effect of DNA in Immune Hemolysis.* Tohoku J. exp. Med., 1975, 116 (1), 33-37 — When a constant quantity of denatured DNA required for C'-fixation reaction was employed as the antigen, an anticomplementary action of DNA was frequently observed. In order to cope with this difficulty in the analysis of C'-fixation, the relationship between DNA, Mg²⁺ and C' was investigated. It was found that this phenomenon was closely related to the flexible conformation of DNA which is highly influenced by Mg²⁺. Further, this effect could be eliminated in the presence of 5 mM MgCl₂. The mechanism of the elimination by Mg²⁺ of the anticomplementary effect of DNA was discussed. ——— DNA; complement fixation

The C'-fixation test has been routinely used for serological analysis of antigen and antibody in our immune system using salmon sperm DNA (ssDNA) as antigen. While examining the nature of rabbit anti-DNA antisera by the C'-fixation test, we frequently observed an anticomplementary phenomenon caused by the addition of denatured DNA or oligonucleotides from DNA to the reaction mixture. In 1963, Stollar and Levine mentioned that denatured DNA was anticomplementary for sera from patients with systemic lupus erythematoses (SLE) at concentrations greater than 2.0 μ g per reaction mixture and that a zone of excess antigen inhibition was not observed for the sera of some patients with SLE, and also that values close to 1.0 μ g of denatured DNA was optimal for the majority of sera studied. The importance of magnesium ion to the function and conformation of nucleic acids has been long recognized (Sander and Ts'o 1971) and it was conjectured that the trouble with C'-fixation might be overcome by changing the concentrations of magnesium ion in the reaction mixture. Now, we have found that a certain amount of magnesium ion in the reaction mixture

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reduced the anticomplementary action of DNA remarkably. The present paper is a report of the studies on the mode of interaction between the complement and DNA in the presence of magnesium ion.

MATERIALS AND METHODS

DNA preparation. Salmon sperm (*Oncorhynchus keda*) were collected at the Tama River, Akita. The sperm was frozen and stored at -25°C prior to the extraction of DNA. Preparation of DNA from salmon sperm was prepared by the SDS-phenol treatment (Okuhara 1970) of the frozen sperm.

Analyses of DNA preparation. The DNA preparation used had the following composition: protein, 0.5%; RNA, less than 0.5%; $\epsilon(\text{P})_{260}$, 6000. Base composition was adenine 27.97, guanine 20.77, cytosine 20.95, and thymine 30.31. T_m was 81°C .

Protein was determined by the method of Lowry et al. (1951); RNA, by that of Savitsky and Stand (1965), and phosphorus, by that of Fiske and Subbarow (1925). For the determination of base composition, a sample of DNA was hydrolyzed in a sealed tube with formic acid at 175°C for 30 min and analyzed by a modification of the method of Murakami et al. (1970) using the cation-exchange chromatography. For the measurement of T_m , 25 μg of DNA were dissolved in 1.0 ml of 0.01 M NaCl-0.05 M sodium cacodylate (pH 7.0) and T_m was determined by using a Hitachi UV-VIS spectrophotometer 139.

Antiserum. This was produced by repeated immunization of rabbits with thermally denatured ssDNA-methylated bovine serum albumin complex. The detailed procedure was described in our previous paper (Matsumoto and Okuhara 1974).

C'-fixation. The quantitative C'-fixation measurements were made by a modification of Kolmer's micromethod as previously described (Matsumoto and Okuhara 1974). Thermally denatured DNA was employed as the antigen. Hereafter, the term DNA in this paper indicates thermally denatured DNA. The veronal buffer used here as the diluent contained 0.55 mM MgCl_2 .

RESULTS

The anticomplementary effect of ssDNA in immune hemolysis is shown in Fig. 1. When freshly prepared ssDNA in excess of 4 μg was added incomplete hemolysis occurred, while with the ssDNA* 30-day-old after extraction, even as small as 0.5 μg of ssDNA caused the anticomplementary action, and this action became potent in parallel with the increase of the antigen. However, as shown in Fig. 2, the anticomplementary effect was reduced gradually with the addition of increasing amounts of MgCl_2 . The curves in Fig. 2 represent respectively the effect of Mg^{2+} added in a range of 0.5 to 5 mM on the anticomplementary action of four different concentrations of DNA. It can be seen in each curve that the hemolytic activity increased with the increase of Mg^{2+} added; at 5 mM of Mg^{2+} 100% lysis occurred, indicating that the anticomplementary action of DNA in a range of 0.5 to 10.0 μg was completely eliminated. In an experiment not reported here, 20.0 μg of NDA were added as the antigen in the presence of 5 mM Mg^{2+} , but again no anticomplementary effect was observed. Thus 5 mM should be the optimal concentration of Mg^{2+} .

* This DNA was used as antigen in experiments shown in Fig. 3 and Table 1.

Fig. 1. Anticomplementary effect of thermally denatured ssDNA in immune hemolysis. 0.1 ml of the antiserum appropriately diluted with veronal buffer, 0.1 ml of antigen (1 to 10 μ g of ssDNA/ml of 0.15 M NaCl) and 0.2 ml of C' (2 units) were mixed, and the fixation was allowed to proceed for 18 hr at 4°C. Then, 0.2 ml of a (3 units of hemolysin)-(red cells) mixture was added and the whole mixture was incubated at 37°C for 30 min. ○, freshly prepared ssDNA; ●, ssDNA 30 days old after extraction.

Fig. 2. Influence of Mg^{2+} to the anticomplementary effect of denatured DNA (μ g).

To explore the above findings, the experiments in Fig. 3 were carried out. In Fig. 3A, the concentration of C' was kept constant (2 or 6 units) and the concentrations of DNA and Mg^{2+} were varied. The magnesium concentration required for 50% hemolysis increased in parallel with the increase of DNA added. With 2 units of C', the effect of Mg^{2+} was remarkable. Fig. 3B indicates that when

Fig. 3. Relationships between the amounts of C', Mg^{2+} and DNA.

TABLE 1. Effect of preincubation of C' with DNA and Mg^{2+} on the hemolytic action

Exp. No.	Preincubation (37°C, 10 min)		Incubation (37°C, 30 min)	Hemolysis (%)
1	C'	(-DNA, - Mg^{2+})	+EA*	100
2	C'+DNA	(- Mg^{2+})	+EA	70
3	C'+ Mg^{2+}	(-DNA)	+EA	100
4	C'+ Mg^{2+} +DNA		+EA	100
5	C'+ Mg^{2+}	(-DNA)	+EA+DNA	100
6	C'+DNA	(- Mg^{2+})	+EA+ Mg^{2+}	70

* Sensitized sheep erythrocytes.

the concentration of Mg^{2+} was relatively low (2 mM or 1 mM), the consumption of C' increased with the addition of increasing amounts of DNA. At the optimal concentration of Mg^{2+} (5 mM), 2 units of C' were sufficient to produce the usual hemolysis. Fig. 3C indicates that when the amount of DNA was kept constant (1.5 or 10 μg), the concentration of Mg^{2+} required decreased with the increase in the amount of C'. The results in Fig. 3 revealed that the extent of hemolysis largely depends on the concentration of Mg^{2+} in the projected hemolytic system. Then, the protective effect of Mg^{2+} was further investigated and the results are shown in Table 1. When C' alone was incubated with the hemolytic system, 100% hemolysis occurred (Exp. 1). When C' was preincubated only with DNA and the mixture was incubated with hemolytic system, the extent of hemolysis was 70% (Exp. 2). As seen especially in Exp. 4, when Mg^{2+} did exist with C' prior to the addition of DNA, Mg^{2+} acted protectively. However, when Mg^{2+} was added after C' and DNA were mixed together, it failed to exert a protective effect (Exp. 6). Apparently Mg^{2+} inhibited the inactivation of C' by DNA.

DISCUSSION

Our results have demonstrated that the anticomplementary action of DNA can be eliminated by the addition of more than 5 mM Mg^{2+} . The data obtained by kinetic studies showed that Mg^{2+} actually functioned as a "protective agent" and prevented non-specific binding between C' and DNA. The results can be interpreted as follows: Some change in the electrostatic state within the DNA molecule leads to the inactivation of C', whereas in the presence of Mg^{2+} , the stabilized structure which is responsible for the antigenic activity and has no anticomplementary action may be restored. We have also observed that when the DNA solution was kept at 4°C, the anticomplementary effect of DNA increased with time (data not shown). Moreover, when the DNA which was extracted from frozen salmon sperm by the method of SDS-phenol treatment (Okuhara 1970) was stored at -20°C and dissolved in 0.15 M NaCl, less than 1.0 μg of DNA was sufficient to exhibit the anticomplementary action, and this effect was retained even after the DNA was preserved for 100 days. The same phenomenon was observed when DNA in 70% ethanol was kept at -20°C. In addition, we have evidence that the difference in the extent of the anticomplementary effect of DNA was dependent on the preparation obtained and kinds of starting material employed. Actually, no anticomplementary action was observed when 20 mg of DNA prepared from mouse embryos were used as antigen in immune hemolysis. It would be of interest to investigate in what manner the DNA acts on each component of the complement system.

As the anticomplementary phenomenon caused by DNA as described in the present paper is closely related to the extent of denaturation of DNA preparations, it seems possible to examine structural changes of DNA preparations by measuring the anticomplementary effect serologically.

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