Brief Definitive Report

CLEARANCE OF CIRCULATING DNA-ANTI-DNA IMMUNE COMPLEXES IN MICE*

By WOODRUFF EMLEN AND MART MANNIK

From the Division of Rheumatology, University of Washington School of Medicine, Seattle, Washington 98195; and the Seattle Public Health Hospital, Seattle, Washington 98114

Immune complexes composed of DNA and antibodies to DNA contribute to tissue injury in systemic lupus erythematosus (SLE) (1, 2). High titers of antibodies to both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) are associated with the disease (2, 3), and antibodies to DNA have been eluted from the kidneys of SLE patients (4, 5). Direct demonstration of circulating DNA-anti-DNA immune complexes has been reported (6, 7), but not confirmed (8).

Whereas the fate of circulating immune complexes such as HSA-anti-HSA has been well characterized (9), the behavior of immune complexes containing DNA has not been studied. DNA is cleared from the circulation extremely rapidly, faster than large-latticed complexes (10). Furthermore, in vitro DNA binds to glomerular and skin basement membranes (11). These unique features of DNA might also influence the fate of immune complexes containing DNA as the antigen. In this study, immune complexes were prepared in vitro with ssDNA and antibodies to DNA from a patient with SLE. The clearance and organ localization of these immune complexes was studied in normal mice and compared with the clearance of heat aggregated immunoglobulin (IgG), serving as a model for immune complexes. Our results demonstrate that the clearance of ssDNA-anti-DNA immune complexes parallels the clearance of DNA alone and is much more rapid than the clearance of aggregated IgG.

Materials and Methods

Antibodies to DNA. Serum from a patient with active SLE, kindly provided by Dr. J. V. Jones of the University of Chicago, was precipitated with 50% saturated ammonium sulphate and resuspended in borate buffer (0.2 M borate, 0.15 M NaCl; pH 8.0). This material was radiolabeled with ^{125}I by the iodine monochloride method (12), and free ^{125}I was removed by dialysis (final sp act 30,000 cpm/μg). This preparation, containing IgM and IgG, was gel filtered over Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) and the included IgG peak was pooled (see Figure 1 A). Rechromatography of this peak showed no excluded protein.

ssDNA. Calf thymus DNA (Worthington Biochemical Corp., Freehold, NJ) was heat denatured and labeled with ^{131}I according to the method of Commerford (13). After dialysis to remove free ^{131}I, the DNA was passed over Sepharose 4B, and the excluded material was pooled (Fig. 1 B). Analysis of this DNA on 2% agarose gel electrophoresis revealed a mean mol wt of 640,000 with a range of 400,000–800,000.

Preparation of Immune Complexes. 10 mg of ^{125}I IgG was mixed with 20 μg of ^{131}I ssDNA and incubated at 37°C for 30 min. This mixture (2 ml) was gel filtered on a 50-ml Sepharose 4B column, and the ^{125}I and ^{131}I counts per 0.5 ml fraction were determined (Fig. 1 C). The excluded material was pooled, analyzed, and used as immune complex in animal studies.

* Supported by grant AM 24015 from the National Institutes of Arthritis, Diabetes and Digestive and Kidney Diseases.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/04/1210/06 $1.00
Volume 155 April 1982 1210–1215

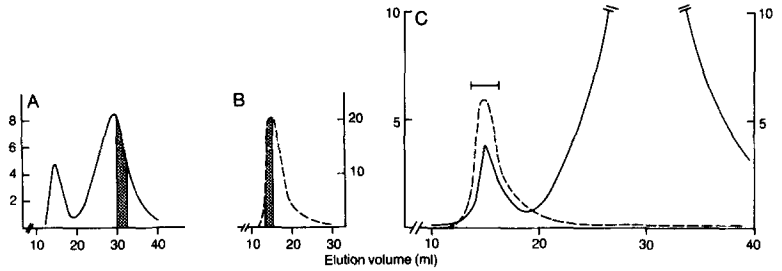

FIG. 1. Preparation of ssDNA-anti-DNA immune complexes. Serum from a patient with SLE with high DNA binding was precipitated with 50% ammonium sulfate and gel filtered on a Sepharose 4B column (A). The Sepharose 4B excluded pool of heat-denatured calf thymus DNA was obtained (B). The IgG pool (^{125}I cpm × 10^{-5} [——]) from (A) and the ssDNA pool (^{131}I cpm × 10^{-4} [- - -]) from (B) were combined and incubated at 37°C for 30 min. This mixture was then passed over Sepharose 4B (C) and the excluded material, as indicated by the bar, was characterized and used in animal experiments.

Analysis of Immune Complexes. IgG/DNA molar ratios in the immune complexes were calculated using the specific activity of each preparation and a mol wt of 145,000 for IgG and 640,000 for ssDNA. In these calculations, all the IgG in the excluded peak was assumed to be in the form of immune complexes. To rule out the possibility that IgG was simply aggregated or coeluting with DNA, rather than actually binding to DNA, a modified Farr assay was performed on the immune complexes. 50 μl of immune complexes were mixed with 50 μl of Cohn Fraction II (Miles Laboratories, Elkhardt, IN) as carrier protein and 100 μl of saturated ammonium sulfate. After 1 h at 4°C, the samples were spun and 100 μl of the supernatant was removed. The precipitate (P) and supernatant (S) were counted, and the percent of ^{131}I DNA in the complexes bound to IgG was calculated by the formula: P − S/P + S.

Preparation of IgG. Monomeric IgG was prepared from Cohn Fraction II by gel filtration over Sephadex G200 (Pharmacia Fine Chemicals) and labeled with ^{125}I (12). Aggregated human IgG (AHGG) was kindly prepared by Dr. Richard Jimenez from Cohn Fraction II by ion exchange chromatography on DEAE cellulose (Pharmacia Fine Chemicals), followed by heating at 63°C for 10 min in 0.1 M Tris-HCL, 0.15 M NaCl, pH 7.55 (14). Aggregates were separated by size on Sepharose 4B, and the intermediate molecular weight pool was concentrated and labeled. After passage over Sephadex G200 to remove monomer, the aggregates were dialyzed against borate and used in animal experiments. The size of these aggregates was 22–25 S by linear 10–40% sucrose density gradients.

Animal Studies. All experiments were performed on 3–4-mo-old female C57Bl/6J mice weighing 20–25 g (The Jackson Laboratory, Bar Harbor, ME) without detectable antibodies to DNA. Animals were placed on iodized water 24 h before experiments. Immune complexes containing 5 μg antibody, AHGG, or monomeric IgG in 0.5 ml borate were injected into the lateral tail vein, and blood samples were obtained from the retro-orbital venous plexus at 0.5, 1, 2, 3, 5, 10, 20, and 30 min and 1, 2, 4, and 6 h. Blood samples were mixed with an equal volume of cold 10% trichloroacetic acid (TCA) and spun in a microfuge. The supernatant was removed, and the ^{125}I (IgG) and ^{131}I (DNA) counts in the TCA-precipitable fraction were determined. Organ uptake experiments were performed as above except that after the 3 min blood sample, animals were sacrificed by cervical dislocation, and the liver, spleen, kidneys, and lungs were removed and counted for ^{125}I and ^{131}I.

TCA-precipitable ^{125}I (IgG) and ^{131}I (DNA) counts remaining at each time point were plotted on semilogarithmic paper, and the slope and y intercept of the linear portion of the disappearance curves were calculated by linear regression analysis. Half-lives for each individual animal were calculated according to the formula $t_{1/2} = -0.693/2.303K$, where K is the slope of the disappearance curve. Clearance rates for groups of animals were calculated by taking the mean of the half-lives of individual animals in each group.

Organ uptake in μg IgG (or DNA) was calculated by dividing the counts per organ by the specific activity of the IgG (DNA). All statistical analyses were performed using a two-tailed Student's *t* test.

Results

Characteristics of Immune Complexes. Less than 0.5% of the IgG added to the ssDNA bound to the DNA and eluted in the excluded fraction of the Sepharose column (Fig. 1 C). Because of the low yield and limited amounts of serum, we produced only small amounts of complexes for animal studies, and consequently the dose of administered complexes was small.

Analysis of the DNA-anti-DNA immune complexes by rechromatography on Sepharose 4B at room temperature 24 h after preparation showed <5% monomeric IgG, and 50% saturated ammonium sulfate precipitated 80% of the DNA in the complexes. Thus, the immune complexes contained only small amounts of free antibody (5%) and free antigen (20%). All complexes used in animal studies were used within 12 h of preparation.

The ratio of IgG to DNA in these immune complexes was 2.5 μg IgG/μg DNA, yielding a molar ratio of 7–15 (mean 10) IgG molecules per DNA. The used DNA had an average size of 1,900 bases, corresponding to ~1 IgG molecule/190 bases.

Clearance of Immune Complexes. ssDNA-anti-DNA immune complexes containing 5 μg antibody were cleared extremely rapidly from the circulation (Fig. 2). The anti-DNA and DNA in the immune complexes were cleared at similar rates; the half-life of anti-DNA was 2.1 ± 0.3 min, and the t½ of DNA was 1.6 ± 0.6 min. Both of these rates closely approximated the previously reported (10) clearance rate of an equal dose (2 μg) of ssDNA alone, which was 1.4 ± 0.2 minutes.

Because we administered DNA-anti-DNA immune complexes containing only 5 μg of IgG, it was possible that the rapid clearance was in part due to the small size of the dose. To directly investigate this possibility, we prepared aggregates of human IgG (AHGG) of a specific molecular weight range (22–25 S) so that each aggregate contained a comparable number of IgG molecules (9–11) as the DNA-anti-DNA immune complexes. This surrogate for immune complexes was specifically chosen because of the physical size, homogeneity, and comparable number of IgG molecules per aggregate, in contrast to other immune complexes studied in our laboratories (9). The clearance kinetics of an identical dose (5 μg) of AHGG are shown in Fig. 2. The initial clearance rate of AHGG ($t_{1/2}$ = 6.5 ± 1.8 min) was significantly slower than the clearance of the IgG in the DNA-anti-DNA immune complexes ($t_{1/2}$ = 2.1 ± 0.3, $P < 0.01$).

The late clearance component of AHGG, DNA-anti-DNA immune complexes, and monomeric IgG is shown in the Fig. 2 insert. From 2 to 6 h the disappearance curves of all three substances were parallel ($t_{1/2}$ IgG = 6.1 h, AHGG = 8.8 h, anti-DNA = 7.2 h). However, between 10 min and 2 h the disappearance of anti-DNA was strikingly different from monomeric IgG and AHGG. The amount of monomeric IgG and AHGG remaining in the circulation decreased gradually and steadily over time, but the clearance of anti-DNA virtually stopped from 10 min to 2 h. The amount of anti-DNA remaining in the circulation at 1 and 2 h was higher (although not statistically significant) than the amount remaining at 10 min. Extrapolating this curve to the y axis (time 0) indicated that this component of the clearance curve accounted for 20% of the starting preparation, and thus exceeded the amount of free antibody in the injected preparation. The ^{131}I radioactivity on DNA could not be followed beyond the initial rapid clearance because of the low counting rates.

Organ Uptake. The liver was the main organ of uptake for ssDNA, anti-DNA, and AHGG. However, at 3–5 min, significantly more anti-DNA was in the liver than

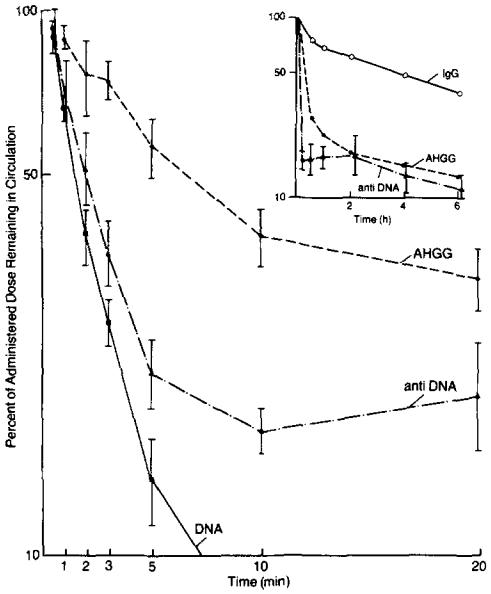

FIG. 2. Clearance of TCA-precipitable radioactivity in DNA-anti-DNA immune complexes from the circulation. Each point represents the mean from at least five mice. The initial clearance (0–5 min) of anti-DNA (▲) in the DNA-anti-DNA immune complexes is significantly faster than the clearance of IgG in AHGG (●) ($P < 0.01$). DNA (■) in the DNA-anti-DNA immune complexes is cleared at the same rate as DNA alone. Insert: clearance of monomeric IgG (○), AHGG, and anti-DNA is parallel from 2–6 h. From 10 min to 2 h, however, the anti-DNA levels did not decrease, but increased slightly, indicating that some anti-DNA returned to the circulation.

AHGG ($P < 0.05$, data not shown). No significant differences were found between AHGG uptake and DNA-anti-DNA immune complex uptake in any other organs.

Discussion

To study the biological properties of immune complexes containing DNA, factors such as DNA size, antigen-antibody molar ratios, and DNA configuration (ss vs. ds) should be defined (15). Only by using well defined complexes can the effect of each of these parameters on the in vivo behavior and pathogenicity of DNA-anti-DNA immune complexes be ascertained. In this study, we examined the clearance kinetics in normal mice of a defined subset of immune complexes containing large molecular weight ssDNA (mol wt 6×10^5) with 1 IgG molecular bound per 190–200 bases. Both the antigen and antibody in these immune complexes were cleared extremely rapidly by the liver, at a rate approaching the clearance of antigen (DNA) alone.

The administered DNA-anti-DNA complexes contained a comparable number of IgG molecules as the aggregated IgG probe. Therefore, the fast clearance of DNA-anti-DNA complexes that approached the clearance of DNA alone indicated that the DNA in the complexes altered the clearance of these immune complexes. Recent studies have demonstrated that antigens can alter the clearance of immune complexes independent of their lattice (16, 17). The presented data indicate that the rapid clearance of the ssDNA also leads to enhanced clearance of immune complexes containing ssDNA.

Analysis of the disappearance of DNA-anti-DNA complexes beyond the initial clearance revealed highly interesting events. The TCA-precipitable counts of ^{125}I-anti-DNA actually increased beyond 10 min samples, and after 2 h declined parallel with monomeric IgG. Previous studies on the removal of immune complexes from circulation did not show increased TCA precipitable counts as seen with DNA-anti-DNA (18). The y intercept of this slow removal phase of anti-DNA was 20%, indicating that >20% of the injected anti-DNA was removed with a t½ of 7.2 h, because no

allowance was made for equilibration between the intravascular and extravascular spaces. It was unlikely that this fraction represented noncomplexed anti-DNA, because even stored aliquots of the injected complexes contained <5% of unbound anti-DNA on gel filtration. Therefore, either free ^{125}I-anti-DNA or ^{125}I-anti-DNA bound to small DNA fragments returned to the circulation. This would occur if the large ssDNA in some complexes reacted with surface nucleases in the liver, as previously postulated (10), and upon degradation free anti-DNA or anti-DNA bound to small DNA fragment were released into circulation.

A number of investigators have shown in vitro that free antibody is released from DNA-anti-DNA immune complexes by digestion with deoxyribonucleases (DNAse) (19, 20). Recently, Sano and Morimoto (21) showed that immune complexes from the sera of SLE patients contained small molecular weight DNA, and other investigators (22) found that, whereas DNAse digestion decreased the size of complexes, some IgG remained bound to small DNA fragments. We postulate that the second component of the observed anti-DNA clearance curve represents the release and subsequent clearance of either free anti-DNA or small immune complexes containing anti-DNA bound to small DNA fragments. This latter possibility could not be excluded with certainty because the radioactivity of the ^{131}I-DNA became too low to be followed.

This study represents the first characterization of the in vivo fate of DNA-anti-DNA immune complexes. We have shown that the clearance of well defined ssDNA-anti-DNA immune complexes is influenced by the antigen in the complexes and is extremely rapid. Whether or not this clearance mechanism applies to all DNA-anti-DNA complexes is not known. Examination of the influence of antigen size and antigen/antibody ratios on the clearance, tissue deposition and in vivo degradation of DNA-anti-DNA immune complexes awaits further study.

Summary

DNA-anti-DNA immune complexes, produced from single-stranded DNA and IgG from a patient with systemic lupus erythematosus were cleared from the circulation of normal mice extremely rapidly, at a rate similar to the clearance of DNA alone. The initial clearance of these complexes was more rapid than the clearance of aggregated IgG, a surrogate immune complex containing a comparable number of IgG molecules, suggesting that the antigen (DNA) in the complexes significantly altered the clearance kinetics of the complexes. Analysis of the late clearance component of these complexes showed that anti-DNA is released back into the circulation after initial removal, and is then cleared at a rate similar to monomeric IgG. Whether this anti-DNA represents free antibody, or antibody bound to small nuclease digested DNA fragments, awaits further study.

We acknowledge the excellent technical assistance of Greg Burdick and Jon Jaffe, and the secretarial assistance of Janet Bobo and Julie Paulsen.

Received for publication 4 January 1982.

References

1. Winfield, J. B., D., Koffler, and H. G. Kunkel. 1975. Role of DNA-antiDNA complexes in the immunopathogenesis of tissue injury in systemic lupus erythematosus. *Scand. J. Rheumatol.* 11Suppl:59.

2. Tan, E. M., P. H. Schur, R. I. Carr, and H. G. Kunkel. 1966. Deoxyribonucleic Acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J. Clin. Invest.* **45:**1732.
3. Koffler, D., R. I. Carr, V. Agnello, R. Thoburn, and H. G. Kunkel. 1971. Antibodies to polynucleotides in human sera: antigenic specificity and relation to disease. *J. Exp. Med.* **134:**294.
4. Koffler, D., F. Agnello, and H. G. Kunkel. 1974. Polynucleotide immune complexes in serum and glomeruli of patients with systemic lupus erythematosus. *Am. J. Pathol.* **74:**109.
5. Krishnan, C., and M. H. Kaplan. 1967. Immunopathologic studies of systemic lupus erythematosus. II. Antinuclear reaction of gamma-globulin eluted from homogenates and isolated glomeruli of kidneys from patients with lupus nephritis. *J. Clin. Invest.* **46:**569.
6. Bruneau, C., and J. Benveniste. 1979. Circulating DNA: antiDNA Complexes in systemic lupus erythematosus. *J. Clin. Invest.* **64:**191.
7. Adu, D., J. Dobson, and D. G. Williams. 1981. DNA-antiDNA circulating complexes in the nephritis of systemic lupus erythematosus. *Clin. Exp. Immunol.* **43:**605.
8. Izui, S., P. H. Lambert, and P. A. Miescher. 1977. Failure to detect circulating DNA-antiDNA complexes by four radioimmunological methods in patients with systemic lupus erythematosus. *Clin. Exp. Immunol.* **30:**384.
9. Mannik, M., A. O. Haakenstad, and W. P. Arend. 1974. The fate and detection of circulating immune complexes. *In* Progress in Immunology II. L. Brent and J. Holborrow, editors. Elsevier/North-Holland, New York. **5:**91.
10. Emlen, W., and M. Mannik. 1978. Kinetics and mechanisms for removal of circulating single-stranded DNA in mice. *J. Exp. Med.* **147:**684.
11. Izui, S., P.-H. Lambert, and P. A. Miescher. 1976. In vitro demonstration of a particular affinity of glomerular basement membrane and collagen for DNA. A possible basis for a local formation of DNA-antiDNA complexes in systemic lupus erythematosus. *J. Exp. Med.* **144:**428.
12. Helmkamp, R. W., R. L. Goodland, W. F. Bale, I. L. Spar, and L. E. Mutschler. 1960. High specific activity iodination of gamma-globulin with iodine-131 monochloride. *Cancer Res.* **20:**1495.
13. Commerford, S. L. 1971. Iodination of nucleic acids *in vitro*. *Biochemistry.* **10:**1993.
14. Jimenez, R. A. H., and M. Mannik. 1982. Evaluation of aggregated IgG in mice as an Fc receptor specific probe of the hepatic mononuclear phagocyte system. *Clin. Exp. Immunol.* In press.
15. Papalian, M., E. Lafer, R. Wong, and B. D. Stollar. 1980. Reaction of systemic lupus erythematosus antinative DNA antibodies with native DNA fragments from 20 to 1200 base pairs. *J. Clin. Invest.* **65:**469.
16. Mannik, M., and K. A. David. 1981. Covalently cross-linked immune complexes prepared with multivalent cross-linking antigens. *J. Immunol.* **127:**1999.
17. Finbloom, D. S., D. B. Magilavy, J. B. Harford, A. Rifai, and P. H. Plotz. 1981. Influence of antigen on immune complex behavior in mice. *J. Clin. Invest.* **68:**214.
18. Haakenstad, A. O., J. B. Case, and M. Mannik. 1975. Effect of cortisone on the disappearance kinetics and tissue localization of soluble immune complexes. *J. Immunol.* **114:**1153.
19. Harbeck, R. J., E. J. Bardana, P. F. Kohler, and R. I. Carr. 1973. DNA-antiDNA complexes: their detection in systemic lupus erythematosus sera. *J. Clin. Invest.* **52:**789.
20. Aarden, L. A., F. Lakmaker, and E. R. DeGroot. 1976. Immunology of DNA. IV. Quantitative aspects of the Farr Assay. *J. Immunol. Methods.* **11:**153.
21. Sano, H., and C. Morimoto. 1981. Isolation of DNA from DNA-antiDNA antibody immune complexes in systemic lupus erythematosus. *J. Immunol.* **128:**538–539.
22. Fidelus, R. K., and R. W. Lightfoot. 1981. Role of antiDNA in protection of immune complexes against DNAse digestion in systemic lupus eryethematosus. *Arthitis Rheum.* **24:**S111.