

Immunological Reaction of UV-Induced Radiation Damage in Coliphage DNA

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Denatured DNA of coliphage T1, poly-dT and dTpT were UV-irradiated (2537 Å) and conjugated to methylated bovine serum albumin. These molecules act as haptens after injection into rabbits. Antibodies are predominantly formed against UV-induced cyclobutane-type of cis-syn thymine dimers. They belong to the IgG-type of antibody molecules. Antigen-antibody complexes were detected by means of the CsCl-density gradient method. Denaturation of the DNA strongly influences the amount of specific antibodies bound to the antigen. In native DNA thymine dimers are masked concerning their reaction with antibodies. Photoproducts of UV-irradiated dTpT induce a specific production of antibody. Our results indicate that the cyclobutane type of thymine dimers is the relevant antigenic determinant group in UV-irradiated DNA.

Introduction

Immunological reactions between radiation damaged sites of DNA or DNA subunits and specific antibodies have been of increasing interest in recent years. The general problems concerning the immunology of DNA have already been reviewed in detail^{1–3}. Specific serological reactions of biomolecules altered by UV-radiation have been observed for the DNA of bacteria⁴ as well as for mammalian cells⁵. Radiation induced single stranded regions have been recognized by means of immunological techniques after UV-treatment of human and mouse chromosomes⁶ and after X-irradiation of calf thymus DNA⁷, respectively. Specific antisera react violently with nuclei of UV-irradiated mouse kidney tissue⁸.

Thymine dimers in UV-irradiated DNA are probably the specific antigenic determinants. This was suggested by the inhibition of specific immunological reactions with UV-irradiated deoxythymidine oligo-nucleotides⁵. Recent studies^{9,10} of well defined DNA molecules of coliphage T1 indicated that these molecules are suitable to further elucidate serological reactions of radiation induced specific structures. The aim of the study is to present still more evidence that thymine dimers are the most relevant specific antigenic site of UV-irradiated DNA. Furthermore, our results demonstrate that UV-irradiated dinucleotides of the dTpT type make

up the smallest possible immunogenic site for induction of antibodies to pyrimidine dimers. The amount of antibody protein bound to DNA was calculated from the change in buoyant density using the CsCl-density gradient centrifugation technique.

Materials and Methods

Preparation of antigens

DNA from coliphage T1 and calf thymus DNA, respectively, was prepared according to our standard phenol method already described^{9,11}. For the radioimmunoassay phage DNA was labelled with [¹⁴C] thymidine. Poly-dT and the dinucleotide dTpT were supplied from Miles Lab., U.S.A, and Boehringer, Mannheim, respectively. DNA was heat-denatured by keeping it 10 min in a boiling water bath. This was followed by chilling in ice water. To avoid renaturation heat-denatured DNA was treated in some cases by addition of 0.1 ml 1 N NaOH and 0.01 ml 10% formaldehyde per 20 µg DNA in 1 ml and neutralising with saturated KH₂PO₄ solution.

UV-sources

The incident UV-dose delivered to the T1 phage particles or to their infectious DNA¹² was determined by plotting survival curves for each preparation. The intensity of the UV-sources was determined from the inactivation dose for phage T4 ($D_{1\%} = 23 \text{ J m}^{-2}$) as a standard reference measured by Setlow and Boyce¹³. While stirring suspension in borate buffer contained in small Petri dishes (6 cm diameter) we exposed small volumes of radioactively labelled DNA as a thin layer (1 mm) to a 6 W Hanau UV-lamp (mercury-vapor

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low-pressure, model NK 6/20) for use in the immuno-assay. A dose rate of $0.16 \text{ Jm}^{-2} \text{ sec}^{-1}$ was applied at a distance of 45 cm. When high UV-doses had to be delivered a specially designed low-pressure mercury lamp (Grüntzel, Karlsruhe; Vycor glass) was used. At a distance of 1 cm the incident dose rate was about $11.6 \text{ Jm}^{-2} \text{ sec}^{-1}$. During irradiation the samples were kept in ice water.

Preparation of antisera

The different antigens used for immunization were conjugated to methylated bovine serum albumin (MBSA)¹⁴. Before this treatment native DNA (500 $\mu\text{g/ml}$) was denatured by heat¹⁵. The standard incident UV-dose applied to DNA for rabbit immunization was about 10^4 Jm^{-2} . The dose delivered to Poly-dT (100 $\mu\text{g/ml}$) was $2 \times 10^4 \text{ Jm}^{-2}$. DTpT (100 $\mu\text{g/ml}$) was frozen on dry ice and irradiated. After exposure to a dose of 10^4 Jm^{-2} the solution was thawed, mixed and frozen again. This procedure of irradiation was repeated until the rate of thymine dimer production (measured at OD 260 nm) nearly reached a plateau. 1 ml of antigen solution prepared as described was administered once intravenously to adult rabbits and thereafter 3 ml (1.5 ml antigen + 1.5 ml Freund's Adjuvant complete) were injected intramuscularly. The injections were given at weekly intervals. Test bleedings were done after 5 to 6 weeks. The sera were kept at -25°C after addition of Thiocid 1:5000 (Asid Institut GmbH., München-Lohhof).

Immunoassay

Equal volumes of antiserum dilution (usually 1:20 in 0.05 M borate buffer, pH 8.8) and radioactively labelled T1-DNA (10 μg per ml borate buffer) were incubated for 20 h at 4°C . Then 1 ml of the reaction mixture was overlaid on top of 11 ml of a preformed linear CsCl-density gradient in a 0.05 M borate buffer, pH 8.8 ($\rho = 1.7 - 1.3 \text{ g/ml}$). An SW41 swinging bucket rotor of a Spinco model L3-50 ultracentrifuge was used. Centrifuging at 35000 rpm ($g_{\text{max}} = 208600$) was performed for 20 h at a temperature of 15°C and subsequently the gradient was fractionated. Samples were withdrawn from fraction 2, 15, 30, and 45 in order to check their refractive indices ($n_{D_{25}}$) in an Abbé refractometer, and the corresponding density ρ of the CsCl-solution was read off from a table¹⁶. After centrifugation 10 ml Insta-Gel (Packard Instrument, Inter S.A. Zürich, Switzerland) were added to the fraction collected in 20 ml polyethylene vials. The radioactivity was measured in a liquid scintillation spectrometer (Isocap/300; Nuclear Chicago) and the data were plotted as percentage of the total input

radioactivity in the gradient versus the fraction number.

Results and Discussion

Fig. 1 shows a typical result of an antigen-antibody reaction after centrifuging the DNA-antibody complex in a preformed CsCl-density gradient. Unirradiated T1-DNA, denatured at 100°C , served as an antigen control. This sample banded at a density corresponding to $\rho = 1.715$. It can be concluded from this density and from the small and nearly symmetrical profile of this band that the antigen used in our experiments is represented by a homogeneous population of denatured DNA molecules, though not completely single stranded ($\rho = 1.725$). After incubation of UV-irradiated ^{14}C -labelled T1-DNA together with a negative serum, diluted 1:20, only a small shift to lighter fractions of the density gradient can be observed at the bottom of the sedimentation profile. However, if the serological reaction mixture contains an

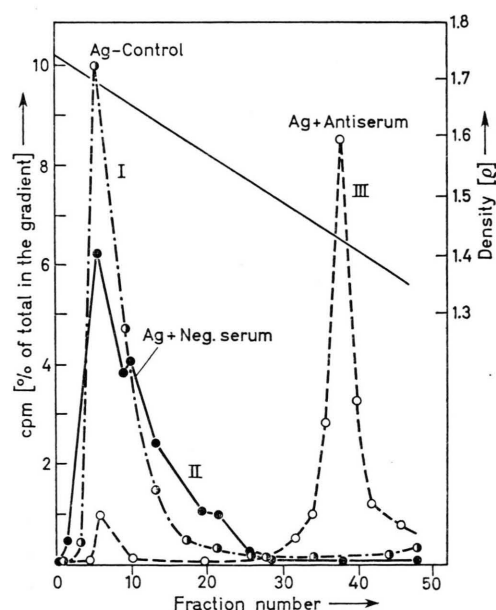


Fig. 1. Density distribution of T1-DNA (Ag-control) and of antigen-antibody complexes in a CsCl-density gradient after centrifugation in a SW41 Spinco swinging bucket rotor for 20 h at 35000 rpm and at 15°C . UV-dose applied to the DNA was 10^4 Jm^{-2} . Ag+neg. serum = density distribution of T1-DNA after incubation with a serum of a non-immunized rabbit. Ag + antiserum = density distribution of T1-DNA after incubation with a specific antiserum against UV-irradiated DNA. Direction of sedimentation is from right to left and the solid line shows the linear density gradient in the centrifugation tube.

antiserum specific with respect to UV-irradiated T1-DNA, and diluted 1 : 20, the density profile of the DNA is shifted far to the region of lower density. We conclude from this sedimentation behaviour of the DNA-antigen that a considerable amount of antibody protein was stably bound to the antigen during the time the antigen-antibody mixture was incubated. The density of this complex results from the combined density of denatured T1-DNA and immune gamma globulin protein, respectively. The distribution profile of the DNA-antibody complex exhibits a fair symmetry, which suggests a population of complexes with the antibody protein evenly distributed among the DNA molecules. As to the small fraction of lighter molecules at the bottom of the right side of profile II, we assume that, for reasons still unknown, some normal rabbit sera contain a variable and small amount of protein binding to UV-irradiated DNA.

As shown in an earlier paper⁹ the density of the specific DNA-antibody complex depends directly on the concentration of anti-UV-DNA antibodies in the immune serum. The same should hold within certain limits for an increase in concentration of specific antigenic sites in the UV-irradiated DNA. Fig. 2 shows shifting of the band of the DNA-antibody complex to the region of lower density with increasing UV-dose delivered to the DNA in the range between 0 and $1.4 \times 10^3 \text{ Jm}^{-2}$. We conclude from this result that, proportional to the dose, photoproducts, *i.e.* specific antigenic sites, are produced by radiation. It is plausible to assume that saturation is reached at a higher dose.

Similar to the experiments just described and shown in Fig. 2, the concentration of the specific UV-induced antigenic sites can also be changed by simply varying the amount of DNA molecules UV-irradiated at a constant dose, in the reaction mixture. It is evident from the density distribution (Fig. 3) of the DNA-antibody complexes that for a fixed concentration of antibodies the DNA band gets shifted to the lighter region of the gradient with decreasing amount of DNA in the incubation mixture. It is reasonable to assume that the amount of specific gamma globulin coating UV-irradiated DNA molecules depends on the relation of the concentration of both the number of antigen sites and of antibodies. Besides banding of the complexes in different density regions it is shown in Fig. 3 that the shape of the profiles also changes depending

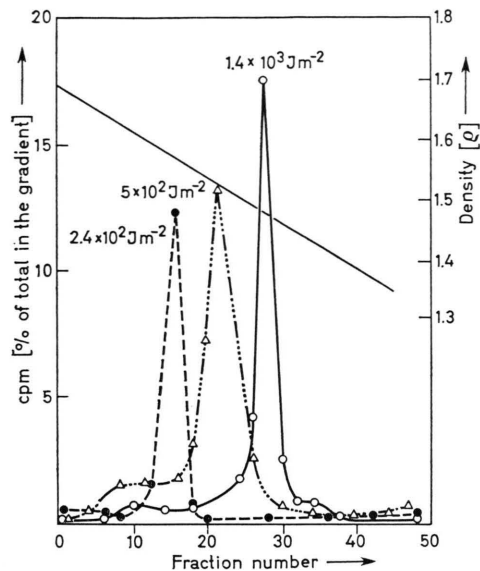


Fig. 2. Influence of the UV-dose given to the DNA on the density distribution of a specific antigen-antibody complex.

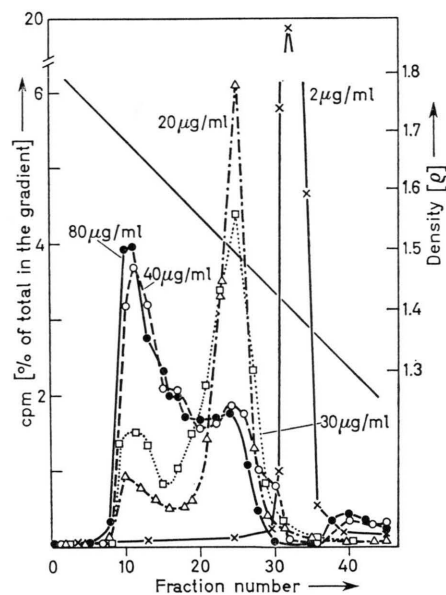


Fig. 3. Influence of the DNA (antigen) concentration on the density distribution of a specific antigen-antibody complex.

on the amount of antigen. Our interpretation of this result is that at antigen concentrations beyond $10 \mu\text{g DNA/ml}$ antibodies get bound only to a part of the population of DNA-molecules. This process results in two populations of DNA-molecules. One population bands at the density of the DNA-control and roughly corresponds to free antigen, *i.e.* with no or only a small and not detectable amount of

antibody protein bound. The other part of the DNA-population which increases with decreasing antigen concentration exhibits a density banding at $\rho = 1.5$. At DNA concentration of $10 \mu\text{g/ml}$ and less symmetrical profiles are observed (*cf.* Fig. 2). This phenomenon, *i.e.* the DNA molecules banding at the position of the antigen control, could be due to dissociation of antigen-antibody complexes. As a matter of fact, specific complexes seem to be surprisingly stable in a CsCl-gradient in our case over the period of centrifugation.

Fig. 4 shows by a double logarithmic plot for various configurations of DNA molecules the number of antibody molecules bound per DNA molecule in the incubation mixture as a function of the incident UV-dose on the DNA. The number of antibody molecules bound to the DNA can be calculated from the mean buoyant density of the profiles observed in a CsCl-density gradient by a simple method published recently⁹. This calculation holds for symmetrical profiles only and uses the density of $\rho = 1.705$ for native T1-DNA, $\rho = 1.715$ for heat denatured, $\rho = 1.725$ for formaldehyde denatured DNA, and $\rho = 1.3$ for gamma globulin. The molecular weights of 30×10^6 daltons for native T1-DNA and 0.14×10^6 daltons for rabbit IgG-molecules, respectively, are included in the calculation. Data not yet published, which were obtained in an immuno-electrophoretic assay allow to assume that this type of antibodies is relevant to the immuno-reaction described here.

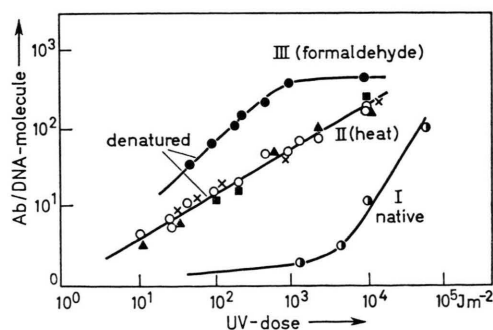


Fig. 4. Influence of the molecular configuration of T1-DNA (I = native; II = heat-denatured; III = alkali-denatured + formaldehyde) and of the UV-dose given to the DNA before incubation with antiserum on the number of specific antibodies (Ab) bound per DNA molecule (*cf.* section 3). (\blacktriangle) Reaction of specific antiserum after absorption with denatured DNA (unirradiated) for removal of unspecific antibodies. (\blacksquare) Reaction of antiserum against UV-irradiated dinucleotide dTpT. (\times) DNA extracted after UV-irradiation of T1 phage particles. (\circ) *cf.* in the text.

The data presented in Fig. 4 clearly demonstrate that the degree of denaturation of the DNA-antigen is of influence for the amount of antibodies which can be bound. Native DNA (curve I) does not exhibit any pronounced serological reaction with anti-UV-DNA antiserum up to a dose of about 10^3 Jm^{-2} given to native DNA.

The reaction of specific antiserum with heat denatured UV-irradiated DNA is shown by curve II. At variance with the experimental data of curve I, the points fit a straight line in a double-logarithmic plot. An even steeper curve is obtained when the DNA is denatured and treated with formaldehyde to avoid partial renaturation (curve III). This curve reaches a plateau at a dose of about 10^3 Jm^{-2} . The plateau region corresponds to about 400 antibody molecules bound to a single strand of T1 DNA. It is evident from this family of dose-effect curves that the number of antibody molecules coating the DNA antigen depends on the UV-dose delivered to the DNA and also on the effect of different denaturation techniques employed which lead to a more or less pronounced separation of the DNA strands. Our explanation of the experimental results given in Fig. 4 is consistent with the hypothesis that the configuration of the DNA during irradiation does not influence the number of specific antigenic determinants, *i.e.* pyrimidine dimers, formed by UV-irradiation.

This view is also supported by our experiments in which the immunogenic reactivity was compared of DNA molecules, UV-irradiated *in vitro* and *in vivo*, respectively, *i.e.* UV-irradiated in the phage head followed by extraction. The results of these additional experiments are represented by points (\circ) shown in Fig. 4, which fit curve II. Phage T1 and its infectious DNA exhibit the same UV-inactivation dose¹². It seems plausible to assume that the yield of UV-induced serologically active thymine dimers is not reduced by the compact configuration of the DNA inside the phage protein head.

However, the degree of denaturation exerts a great influence on the reaction taking place in the DNA between specific antibodies and photoproducts. It is most plausible to assume that in native DNA these specific antigenic sites are masked for steric reasons. A similar conclusion was deduced by Seaman *et al.*⁵ from their results on bacterial and mammalian DNA. The best explanation of the position of curve II in between that of curve I and

curve III is a partial renaturation of heat-denatured DNA during the time consuming procedure of serological reaction and gradient centrifugation. Such renaturation is blocked by the treatment of DNA with formaldehyde. The increased number of antibodies bound to native DNA at doses beyond 10^3 Jm^{-2} is obviously caused by the effect of denaturation by UV-light occurring at high doses.

In addition to the data resulting from the antigen-antibody reaction by means of an antiserum against UV-irradiated DNA we studied the immunological reaction between this antigen and antibodies produced in rabbits after the injection of poly-dT and the dinucleotide dTpT, respectively, irradiated with high doses of UV-light. The experimental data fit curve II significantly. These experimental results give strong evidence for the hypothesis stated above that thymine dimers of the cyclobutane-type are the very specific UV-induced antigenic sites of the DNA and lead to the reaction discussed here. Regarding the size of the antigenic determinants, we conclude from our results that it roughly corresponds to the area of two dimerized thymine molecules, *i.e.* it is smaller than stated recently⁵. In connection with the hypothesis stated above it is interesting to mention the observation reported⁴ that in an inhibition test other pyrimidine dimers poorly inhibit specific immunoreactions of UV-irradiated DNA.

As to the conjugation of UV-photoproducts from dTpT to the carrier protein (MBSA) used in our experiments, it seems safe to assume that 5'-phosphate groups are not necessary for unspecific binding. Several reports deal with the successful conjugation of DNA bases and nucleosides (*cf.* ref. 1).

Fig. 5 presents a comparison of the number of antibody molecules bound per antigen, *i.e.* per denatured DNA molecule, as a function of the UV-dose delivered to the DNA in the reaction mixture and of the number of thymine dimers induced per unit UV-dose. The number of dimers is calculated according to the results of Wulff¹⁷ which are valid for the DNA of *E. coli*. For our T1-DNA in the denatured state a molecular weight of 15×10^6 Daltons and a fraction of 27 per cent of thymine are used. In the low dose range up to 10^3 Jm^{-2} the ratio of specific antibodies to thymine dimers is about unity. Beyond this dose value, saturation of UV-induced thymine dimers is reached and about 6 per cent of the thymine molecules are in a dimerized state, *i.e.* less than 2 per cent of all

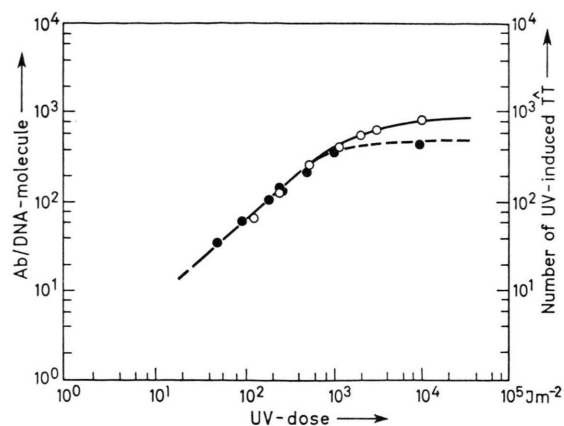


Fig. 5. Number of specific antibodies bound per denatured DNA molecule (●) and number of UV-induced thymine dimers (○), respectively, as a function of the UV-dose given to the DNA (*cf.* section 3).

nucleotides are thymine dimers. Considering the low concentration of DNA in our reaction mixture and the fixed position of the dimers within a random coiled macromolecule of $15 \mu\text{m}$ length it is justified to assume that one antibody molecule gets in touch with one thymine dimer only. The difference of both curves at the plateau region is probably due to the low titer of antibodies in our antisera.

We conclude from our results that a direct relation exists between the amount of specific antibodies bound to UV-irradiated DNA and the number of radiation induced thymine dimers. This relationship allows to measure the number of these photoproducts using a serological technique. The advantages of the technique described above, compared to a standard radioimmunoassay, are: (1) Direct and quantitative assay of antibody protein bound to DNA. (2) Convenient fractionation of the components, *i.e.* free antigen, free antibody, and specific antigen-antibody complex, during centrifugation in a one-step technique without loss of any material by further treatment of the specific complex, *e.g.* removal of unbound material by washing procedures. (3) Detection of precipitating as well as of soluble antigen-antibody complexes.

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