# Serological Detection of Desoxyribonucleic Acid (DNA) Adsorbed to Formalinized Erythrocytes.* (24024)

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During the past 3 years we have studied the immunology of the Rous sarcoma, a virus-induced tumor of chickens. A virus-containing eluate of tumors prepared by the method of Riley(11), and desoxyribonucleoprotein (DNAP) preparations from tumor and normal chicken muscle prepared by the method described for calf thymus by Mirsky and Pollister(10) have been examined. It has been found that potent precipitin serums prepared against DNAP will not precipitate with corresponding purified protein-free desoxyribonucleic acid (DNA) preparations. This has also been the experience of other workers investigating the serological reactivity of nucleoproteins(8). Cole and Farrell(3) reported the use of formalinized erythrocytes with protein antigens coupled to them, to detect antibodies against the attached proteins. We used formalinized erythrocytes coupled with sulfanilazo groups to measure formation in rabbits of antibody homologous to this hapten. It has been found that DNA will couple to formalin-treated erythrocytes to form an antigen which can be agglutinated by serums prepared against DNAP.

*Materials and methods. Phosphate-saline.* Sodium chloride, 0.145 M, containing 0.005 M sodium phosphate with final pH of 7.3-7.4 was used. *Formalin-saline.* Formaldehyde, 10 g/100 ml, in phosphate-saline with final pH of about 7.0 was employed. *Coupling solution.* McIlvaine's buffers were mixed with 0.145 M saline to give final pH 4.9. This pH was chosen as optimum after trials at pH 2.9, 3.9, 4.9, 5.9, 6.9, 7.9 and 8.9. *Formalinized erythrocytes.*‡ Human type O blood cells from an outdated blood bank unit were washed 3 times with 4 volumes of phosphate-saline containing 0.5% dextrose, made to a 50% suspension in phosphate-saline and chilled to 0-3°C. This chilled suspension was poured into 4 volumes of formalin-saline solution and stored in refrigerator at 0-3°C. The cells were resuspended once each day for the first week and then remained in refrigerator until used. The cells employed were in formalin-saline solution approximately 6 months prior to use. However, cells have been used successfully after 2-3 weeks of treatment with formalin. *Desoxyribonucleic acid.* DNA from Rous sarcoma cells (tumor DNA) and DNA from normal chicken muscle (normal DNA) were initially prepared as nucleoprotein by molar saline extraction procedure of Mirsky and Pollister(10). The method of Kay, Simmons, and Dounce(6) was then employed to remove the proteins. DNA thus recovered was a white, ropy substance with U.V. adsorption maximum at 260 mμ and an ε (P) of approximately 6600 as described by Chargaff(1). The biuret test as reported by Holden and Pirie(4) showed less than 1% protein contamination in the DNA. The N/P ratio was 2.0 and 2.1 for tumor DNA and normal DNA respectively. The antiserum against tumor DNAP and normal DNAP were prepared in rabbits by intravenous injection of antigen in 1 M saline and subcutaneous injection after incorporation into Freund adjuvant (2). *Coupling technic.* Formalinized erythrocytes were washed 5 times in 20-30 volumes of phosphate-saline to remove free formaldehyde. A 10% suspension of erythrocytes was made in the coupling solution and 0.1 mg of DNA (dry wt) was added/ml of cell suspension. This mixture was incubated in a 37°C water bath with mixing every half hour for 4 hours. Erythrocytes were then washed 3 times with phosphate-saline to remove excess DNA and the cells resuspended in phosphate-

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‡ This procedure was designed by Dr. J. S. Ingraham, Dept. of Microbiology, Indiana Univ. Med. Center.

saline. The supernatant from third washing was negative for DNA as shown by the diphenylamine reaction. The coupled-cells could be stored at 4°C for approximately 4 weeks without loss of reactivity. *Agglutination pattern test.* Selected tubes 14.5 x 75 mm were used in racks having holes of approximately 12 mm diameter in bottom shelf. Each tube contained 0.5 ml of serum dilution in phosphate-saline containing 1:100 normal rabbit serum and 0.1 ml of a 1% cell suspension in phosphate-saline. After 24 hours the test was read by observing the bottoms of tubes in a mirror while the tubes were illuminated from above with a fluorescent lamp. The highest serum dilution which gave a pattern of agglutination clearly distinguishable from normal serum controls was taken as end point. Cole and Farrell(3) and Stavitsky(12) have discussed the problem of interpreting agglutination patterns similar to those observed in this work. The above procedure was repeated 6 times for each antiserum, and the observed end point was the same in each experiment. *Inhibition study.* To obtain further evidence that agglutination depended upon a specific combination of antibody with DNA attached to cells, an inhibition experiment was done with either homologous or heterologous DNA as the inhibiting substance. Before addition of cells, 0.1 ml of 0.5 mg/ml solution of either normal DNA or tumor DNA was added to each tube. After 2 hours incubation at room temperature the coupled-cells were added, and the test completed as described above. Various concentrations of inhibiting DNA were tried, and it was observed that inhibition increased with concentration using 0.01-0.025 mg DNA, but no further increase was observed at concentrations up to 0.1 mg DNA. Therefore, 0.05 mg DNA was employed to insure an excess of inhibiting DNA. *Optical density study of agglutination.* The method of Liener(9) was employed to obtain a semi-quantitative measure of agglutinating capacities of the serums. One ml of a 2% cell suspension was added to 1 ml of a 1:20 dilution of antiserum or a 1:10 dilution of normal rabbit serum. A 1:20 dilution of normal rabbit serum had shown no effect upon coupled or uncoupled cells, and it was felt that employment of a 1:10 dilution would demonstrate any non-specific action, which might be present but not demonstrable at a 1:20 dilution of normal serum. Optical density was read at 620 mμ using a Coleman Jr. spectrophotometer, model 6A. A reading was taken 10 minutes after mixing and every 50 minutes thereafter to 200 minutes. *Enzyme activity.* Once recrystallized desoxyribonuclease (DNA-ase) prepared according to the method of Kunitz(7) was obtained from Worthington Biochemical Corp. (Lot #D564). Trypsin (1:250) was obtained from Difco Laboratories and prepared at concentration of 5 mg/ml in phosphate buffer at pH 8.0. Aliquots of formalinized cells coupled with either normal DNA, tumor DNA, bovine serum globulin (BSG), or sulfanilic acid (SA), as well as uncoupled formalinized cells were exposed to either DNA-ase (0.1 mg/ml) for 24 hours at room temperature or trypsin (0.1 mg/ml) for 1 hour at 37°C. Controls included coupled and uncoupled cells exposed to enzyme diluents under the above conditions. Cells were washed 5 times following treatment and resuspended in phosphate-saline. The resultant cell suspensions were tested as described above for agglutination pattern and optical density change.

*Results.* From data in Table I it can be seen that after formalinized erythrocytes are exposed to either tumor or normal DNA they are agglutinated specifically by antiserum prepared against homologous DNAP. Both anti-tumor DNAP and anti-normal DNAP serums cross react in lower titer with heterologous DNA-cells. The settling pattern results in Table I agree with those from rate-of-settling experiments shown in Fig. 1. In this figure antiserum causes cells coupled with the DNA to settle more rapidly than controls. The results, which are free of subjective judgment encountered in interpreting patterns, are in complete agreement with those obtained in the agglutination pattern study.

Specific inhibition obtained in tumor DNA-anti-tumor DNAP, and normal DNA-anti-normal DNAP reactions as shown in Table I, supports the conclusion that there is at least

TABLE I. Titers Observed by the Agglutination Pattern Technic.

| Anti-serum | Cells | Inhibiting DNA* | Hemagglutinating titer |
|---|---|---|---|
| Tumor-DNAP | Tumor-DNA | | 1:16,000 |
| | " | Tumor-DNA | 1:500 |
| | " | Normal- " | 1:8000 |
| | Normal-DNA | | 1:1000 |
| | " | Tumor-DNA | <1:2 |
| | " | Normal- " | " |
| | Uncoupled | | " |
| Normal-DNAP | Normal-DNA | | 1:8000 |
| | " | Normal-DNA | 1:200 |
| | " | Tumor- " | 1:4000 |
| | Tumor-DNA | | 1:2000 |
| | " | Normal-DNA | <1:2 |
| | " | Tumor- " | " |
| | Uncoupled | | " |
| NRS† | Tumor-DNA | | <1:2 |
| | Normal- " | | " |
| | Uncoupled | | " |

* DNA preparation and antiserum were incubated 2 hr prior to addition of cells (.05 mg DNA).

† Normal rabbit serum.

a considerable quantitative difference between tumor and normal DNA preparations. Complete inhibition of cross-reactions by either DNA preparation might be expected to occur even if there were qualitative differences between the 2 DNA preparations. The fact that inhibition was obtained suggests that antibody reacts with the DNA in the absence of a carrier protein. However, it has not been possible to precipitate anti-DNAP serum with purified DNA.

Desoxyribonuclease at concentration employed completely removes serological reactivity of normal DNA-cells (Fig. 2). However, one observes that tumor DNA-cells retain a slight amount of serological reactivity with homologous antiserum. The DNA-ase had no apparent effect upon agglutination pattern and settling rate of BSG-cells or SA-cells in homologous antiserum. Uncoupled cells in normal and the various antiserums were unaffected by this enzyme.

Trypsin at concentration employed did not alter reactivity of DNA-cells; furthermore, it did not remove activity which remained in the homologous tumor DNA-cell system after DNA-ase treatment. However, the amount of trypsin employed was sufficient to remove completely the reactivity of BSG-cells with homologous antiserum. Trypsin did not affect either SA-cells or uncoupled cells. The trypsin diluent was also devoid of activity. The concentration of trypsin employed was sufficiently greater than that expected as a contaminant, to rule out the possibility of trypsin-like activity in the DNA-ase preparation.

One conclusion to be considered from above data, is that DNA functions in a manner analogous to haptens, *i.e.*, the DNA confers specificity, but it must be coupled with a carrier

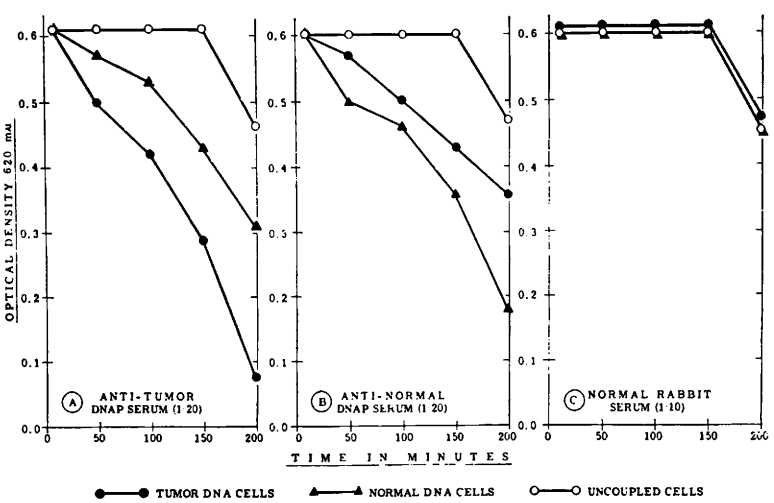


FIG. 1. Settling rate of coupled and uncoupled formalinized cells in presence of rabbit antiserums and normal rabbit serum.

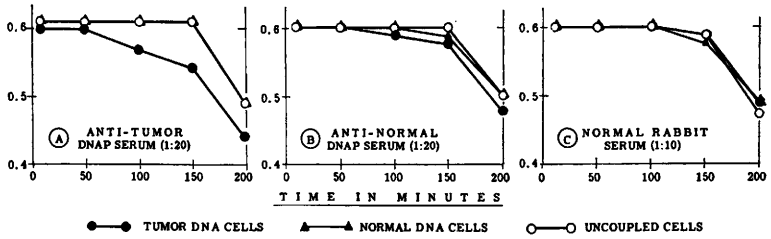


FIG. 2. Effect of DNA-ase upon settling rate of coupled and uncoupled cells in presence of rabbit antiserums and normal rabbit serum.

substance to stimulate antibody formation or to be detected by precipitin or agglutination reaction.

However, it must be admitted that it is difficult at present to understand why a hapten of the complexity of DNA would not precipitate with antiserum. This observation suggests that there might be an insufficient number of specific reactive sites per DNA molecule or that non-specific factors are involved, as observed by Ingraham(5) with respect to non-agglutination of formalin-treated red cells.

*Summary.* Formalinized erythrocytes exposed to purified DNA under the proper conditions can then be specifically agglutinated with antiserum, which has been prepared against the DNA-protein complex. Inhibition of the reaction by homologous DNA is strong evidence that agglutination is an antigen-antibody reaction and not merely a non-specific agglutination. The removal of this reactivity by desoxyribonuclease lends further support to the belief that DNA is functioning as the hapten. Trypsin does not alter the agglutination reaction.

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## Growth-Promoting Properties of Pyruvate, Oxalacetate, and α-Ketoglutarate For Isolated Walker Carcinosarcoma 256 Cells. (24025)

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Puck and his associates devised procedures for growing isolated human cells in tissue culture(1,2). The medium consisted of whole serum, amino acids, vitamins, and balanced salt solution. Media containing dialyzed serum as replacement for whole serum did not sustain growth of single cells of the S3 colony strain of HeLa cells. Supplementation of the medium with cholesterol and a variety of coenzymes, however, enabled the cells to grow (3).

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