THE AGGLUTINATING AND SENSITIZING CAPACITY OF ANTISERA TO SHEEP RED CELLS AFTER VARYING DEGREES OF PHOTO-OXIDATION

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Tyler[1] showed that photo-oxidation could cause antisera to become nonprecipitating and nonagglutinating without destroying the ability of the antibodies to combine with the specific antigen. This was shown by the fact that treated antiserum could inhibit specifically agglutination or precipitation by corresponding untreated serum. On the basis of the mutual multivalence theory of Marrack,[2] Heidelberger,[3] and Pauling[4] it was considered that multivalent antibody had been made functionally univalent by the treatment, the term "univalent antibody" being used to designate an antibody that is incapable of causing precipitation or agglutination but which is still able to combine with the antigen.

A method more sensitive and direct than that of specific inhibition for demonstrating the presence of specifically combining but nonagglutinating antibody is the antiglobulin sensitization test of Coombs, Mourant, and Race.[5] However, this method was not available when Tyler's original experiments were performed. The present experiments show that it can be used with photo-oxidized antisera, and thus one can measure more directly the sensitizing capacity of sera rendered nonagglutinating by this method. There was also a more urgent reason for undertaking the present experiments on the photo-oxidation of rabbit anti-sheep red cell sera. Coombs, Howard, and Mynors[6] reported a serological procedure theoretically capable of detecting incomplete or nonprecipitating antibodies to soluble protein antigens. The method using ox red cells, although simple in theory, is rather lengthy to perform. A much shorter method, employing sheep red cells, was envisaged if a nonagglutinating rabbit sheep cell antibody were available.[7] Tyler's work showed the possibility of producing this type of antibody by photo-oxidation. Chemically linked to such an antibody, a soluble protein antigen, such as egg albumin, could be fixed to the surface of sheep cells, which would nevertheless remain stable in suspension. This cell system could then be used for the specific absorption of incomplete antibody, in human serum, to the conjugated protein—the absorption being detected finally by an agglutination reaction using a rabbit anti-human globulin serum. Preliminary studies in this direction have been promising, and a shortened serological procedure for detecting incomplete antibodies to soluble protein antigens has been developed by Coombs and Fiset.[8] In the present paper various sheep cell antisera have been examined, before and after varying degrees of photo-oxidation, for their direct agglutinating action and for their combining capacity, as demonstrated by the indirect antiglobulin sensitization test.

MATERIALS AND METHODS

The anti-red cell sera were produced by intraperitoneal injection of rabbits with a 10 per cent suspension of washed cells. Each injection was composed of 1–1$^1/_2$

ml., and a course consisted of 6–8 injections. The rabbits were bled 8–10 days after the last injection

Anti-Sheep Red Cell Serum.—A pool was made of the second course from two rabbits.

Anti-Fowl Red Cell Serum.—The sera after the first course from four rabbits were pooled—likewise after the second course. A third-course pool was made from a different set of four rabbits. Preinoculation sera served as controls.

All the above sera were heated for $^1/_2$ hour at 56° C. to inactivate complement.

Preparation of Globulin Fraction.—The total globulin fraction was precipitated from unheated serum by adding to the serum an equal volume of saturated ammonium sulfate. The precipitating mixture was left overnight in the cold. The following day the material was centrifuged and the precipitate washed once in a 50 per cent saturated ammonium sulfate solution. The precipitate was recentrifuged and dissolved in saline buffered at pH 7.2 with phosphate buffer. The globulin solution was dialyzed against buffered saline until free of NH_4^+ and then stored at −20° C. until required. The protein concentration was determined by the micro-Kjeldahl method.

Photo-oxidation of Serum or Globulin Solutions.—The method used followed in all main respects that described by Tyler.[1] The serum in the presence of the photosensitizer, eosin Y, was exposed to a powerful source of light in one of the flasks of an enlarged modification of the constant-pressure manometer devised by Dr. M. Dixon and illustrated in Figure 2 of his book.[9] The flasks had a capacity of about 100 ml., and the sidearm pipette a capacity of about 12 ml. Ground-glass taps (including one between the two arms of the U tube) were built in, so that, once the flasks had been mounted, oxygen could be flushed through the apparatus. The apparatus was run in a Warburg bath at 23° C. Light was focused obliquely on the flask from above. Mirrors were placed in the water both underneath and beside the flask to reflect as much of the light as possible onto the flask. The light consisted of a special 12-volt 239-watt bulb set in a powerful reflector. It had previously served as an airplane landing light. The distance between the bulb and the flask containing the serum was 33 inches, and the light intensity at this distance was 300 foot-candles.

Direct Agglutination Test.—The serum or globulin fraction was serially diluted in 0.1-ml. volumes. An equal volume of a 2 per cent suspension of washed sheep cells was then added to each tube, and the contents mixed. Two drops were then removed from each tube and placed in small Rh tubes (rimless glass test tubes, 7 × 50 mm.), in which the cells were allowed to settle at 37° C. After two hours the deposited cells were examined for agglutination in three ways. First, the deposit was observed with the naked eye. Second, some of the sedimented cells were removed with a Pasteur pipette and examined on a glass slide under a microscope using the $^2/_3$-inch objective. Finally, this was repeated after the tube containing the cells had been centrifuged for 10 seconds. Only if this latter procedure demonstrated no agglutination was the serum considered to be without agglutinating action.

Indirect Antiglobulin Sensitization Test.—The main titration tubes of the direct agglutination test (0.2 ml. minus 2 drops) were incubated for 30 minutes at 37° C. Each tube was then centrifuged, and the deposited cells washed twice with saline to remove free rabbit serum. After the second washing, the deposited cells were resuspended in 2 drops of saline. One drop from each tube was then added to

1 drop of a 1:10 dilution of a goat anti-rabbit globulin serum in a Rh tube. The contents of the tubes were mixed, and the test allowed to incubate at 37° C. for $1^1/_2$ hours, while the cells sedimented. The deposited cells were then read for agglutination.

The anti-rabbit globulin serum was from a goat which had received five courses of injections of rabbit serum. The serum after heat inactivation possessed no antibodies for sheep cells and therefore required no absorptions previous to its use in the test.

EXPERIMENTAL RESULTS

Rabbit antisera produced against sheep red cells and fowl red cells were photo-oxidized. Fowl red cells possess the Forssman antigen, which is also present on sheep red cells. Thus rabbit anti-fowl red cell sera may be considered as Forssman antisera. These sera possess a high titer of hemolysin for sheep cells but only a relatively low titer of agglutinin. This divergence in the titer of the hemolysin and agglutinin is due, in the authors' opinion, to the Forssman antigen sites on the sheep cells being situated slightly below the outer periphery of the cell envelope, making it difficult for antibodies to link the cells together. As one of the purposes of this investigation was to produce a nonagglutinating rabbit antibody to sheep cells, it was considered that a Forssman antiserum had much to recommend it as starting material.

The agglutinating and sensitizing capacity of the different sera for sheep cells after varying degrees of photo-oxidation are shown in Table 1. To rule out the possibility that photo-oxidation results in the production of some factor which is

TABLE 1

AGGLUTINATING AND SENSITIZING CAPACITY OF SERA AGAINST SHEEP RED CELLS

Antiserum	Treatment (Mm^3 O_2/Ml)	Titer Producing Agglutination	Titer Producing Sensitization
R.2221-2 (pool); anti-sheep cells; 2d course	Untreated	5,120	20,480
	P.O.* 372	20–1,280	20,480
	657	(20–320)†	20,480
	813	(20–320)	20,480
	1,000	0	10,240
R.2371-4 (pool); anti-fowl red cells; 1st course	Untreated	80	5,120
	P.O. 370	40	640
	420	10	640
	470	(5)	320
	520	0	80
	640	0	5
R.2371-4 (pool); anti-fowl red cells; 2d course	Untreated	160	5,120
	P.O. 350	5 (10)	2,560
	400	0	640
	450	0	160
	500	0	40
R.2281-4 (pool); anti-fowl red cells; 3d course	Untreated	320	5,120
	P.O. 500	10	2,560
	650	0	640
	766	0	320
R.2371-4 (pool); anti-fowl cells; 2d course (Total globulin fraction, $2^1/_2$ per cent)	Untreated	160	10,240
	P.O. 100	5	5,120
	200	0	640
	275	0	320
	325	0	320
	400	0	160

* P.O. = Photo-oxidation.
† Parentheses indicate weak agglutination.

nonspecifically adsorbed onto the red cells and detectable by the antiglobulin technique, preinoculation sera were photo-oxidized and tested in a control series.

With the second-course anti–sheep cell serum, photo-oxidation to the degree of 900–1,000 mm^3 O_2/ml serum was required before all trace of agglutinating capacity was lost. The serum acquired a syrupy consistency as a result of this extensive treatment. When diluted 5,000 times, it was still capable of sensitizing sheep cells. After photo-oxidation to the extent of 657 mm^3 O_2/ml, the serum was no longer capable of showing macroscopic agglutination, but weak agglutination could be shown microscopically over the zone of the $^1/_{20}$–$^1/_{320}$ dilutions of the serum. The prozone is interpretable as due to inhibition by antibody rendered nonagglutinating.

The degree of photo-oxidation required to render the Forssman antisera totally nonagglutinating may be seen in Table 1. The second- and third-course sera were better than the first-course serum in retaining their sensitizing capacity after the agglutinating capacity had been destroyed. The anti-Forssman sera required less photo-oxidation than did the anti–sheep cell serum to be rendered completely nonagglutinating. This may be due to the fact that only a single antigen-antibody system is involved or to the special circumstances covering agglutination by the Forssman antibody. Photo-oxidation of the isolated globulin fraction of the Forssman antiserum also rendered the antibodies nonagglutinating.

It may be seen most clearly with the Forssman antisera that, alongside the conversion of the agglutinating antibody into sensitizing but nonagglutinating antibody, there is also a gradual destruction of the sensitizing capacity of the antibodies. This is most clearly seen with the first- and second-course antisera. For this reason the treatment given to a particular serum must be kept to the minimum compatible with the removal of all agglutinating activity.

DISCUSSION

The results obtained in these experiments show that a nonagglutinating but sensitizing rabbit anti–sheep red cell serum may be obtained with the aid of photo-oxidation. Much more work needs to be done to determine the extent of photo-oxidation required to render different sera nonagglutinating and also to study the effect of hyperimmunization on the resistance of the antibody molecules to the oxidative process.

The uptake of oxygen by the globulin fraction proceeded at a much slower rate than did that of an equivalent amount of the corresponding whole serum, but the extent of photo-oxidation (in cubic millimeters of oxygen per milliliter) required to convert agglutinating into nonagglutinating antibody appears to be roughly proportional to the protein concentration (Table 1). The stability of these photo-oxidized antibodies is another matter which must be studied. Some have been kept over six months in the dark at $-20°$ C. with little if any apparent change in serological reactivity.

The application of these results to the problem of obtaining a sheep red cell protein antigen unit for the test envisaged by Coombs, Howard, and Mynors[6] for the detection of incomplete antibodies to soluble protein antigens has been encouraging. Egg albumin has been successfully coupled by means of tetrazotized benzidine to a photo-oxidized nonagglutinating sheep cell antibody. The photo-

oxidized antibody was then able to carry the coupled egg albumin to the sheep cell surface, giving a stable sheep cell–egg albumin unit capable of detecting either precipitating or nonprecipitating antibodies to egg albumin.[8]

The action of photo-oxidation on antisera has been considered[1] to be one of splitting of the protein molecules and complexing of the different fragments. In view of this, an attempt was made to see whether a protein such as egg albumin could be made to complex with a sheep cell antibody. An approximately equimolecular mixture of egg albumin and protein from the globulin fraction of a Forssman antiserum was photo-oxidized. After an arbitrary degree of oxygen absorption the mixture was found to be capable of sensitizing sheep cells, so that, after washing, they could be powerfully agglutinated by anti–egg albumin serum. This indicated that the sheep cell antibody in the globulin fraction had complexed with the egg albumin. This phenomenon is being investigated more thoroughly in order to determine its possible application to the formation of sheep cell protein antigen units.[6]

SUMMARY

The antiglobulin sensitization test has been found to be effective in detecting the sensitizing capacity of sheep cell antibodies which have been rendered nonagglutinating by the process of photo-oxidation. Such treated antibodies evidently still retain globulin specificity. In the discussion it is mentioned that nonagglutinating rabbit antibodies to sheep cells may be conjugated with a foreign protein such as egg albumin. A sheep cell–egg albumin unit may then be formed by which it is possible to detect serologically complete or incomplete antibodies to egg albumin in sera of man or animals other than the rabbit.

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