Immunology, 1958, 1, 217.

The Application of the Fluorescent-Antibody Technique to Haemagglutinating Systems*

W. R. M. ALEXANDER

Rheumatic Unit, Northern General Hospital, Edinburgh, 5

Summary. The Coons' technique has been modified for the detection of antibodies on erythrocytes agglutinated by fluorescein-conjugated antisera. The results of experiments with several haemagglutinating systems suggested that specific fluorescence was found only on erythrocytes which had been agglutinated by species-specific antibodies. The significance of these findings is discussed.

INTRODUCTION

COONS, Creech, Jones and Berliner (1942) reported that antibodies conjugated with fluorescein *iso*cyanate acquired fluorescence without loss of immunological properties. They described a technique whereby foreign antigens could be detected in tissues by exposure to specific antisera labelled with fluorescein. Marshall (1951) successfully applied the method to the study of antigens native to tissue. Coons (1956) has reviewed further developments of the technique and discussed its application to a variety of biological problems.

The possibility of adapting the fluorescein-labelling technique to systems involving erythrocytes was considered during investigations of the sensitized sheep cell test. This diagnostic test depends on the presence in human serum of a factor which has the property of agglutinating sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte serum (haemolysin). The factor occurs almost exclusively in rheumatoid arthritis and many attempts have been made to determine its physical and chemical properties. Information available suggested that the factor was a protein, in which case it would become fluorescent on conjugation of the rheumatoid serum with fluorescein. It was decided, therefore, to expose sensitized sheep erythrocytes to conjugated serum of high titre and examine the agglutinates for specific fluorescence.

The results of this experiment and of others designed to provide an explanation of the findings are reported here.

EXPERIMENTAL METHODS

FLUORESCEIN-CONJUGATED SERA

Sera were heated at 56° C. for 30 minutes to inactivate complement.

Sera were conjugated with fluorescein by the method of Coons and Kaplan (1950).

The conjugated sera were absorbed with erythrocytes of appropriate origin when it was necessary to establish specificity of agglutination or fluorescence. One-fifth volume of packed erythrocytes was added to the serum and incubated at 37° C. for 60 minutes.

* The substance of this report was briefly presented to the British Society for Immunology on November 9, 1956.

The erythrocytes were separated by centrifugation and the conjugate was absorbed for a second time.

ERYTHROCYTES

Blood was collected in an equal volume of 3·8 per cent sodium citrate, stored at 4° C. and used within 3 days of withdrawal.

Suspensions of erythrocytes were prepared by washing whole blood three times in 0·89 per cent sodium chloride. The washed erythrocytes were used on the day of preparation.

Sensitized sheep erythrocytes were prepared by exposure of a 1 per cent suspension of washed erythrocytes to an equal volume of rabbit haemolysin diluted to contain one-fifth of the minimum agglutinating dose (M.A.D.). Control material was prepared by similar treatment of sheep erythrocytes with non-immune rabbit serum.

'Tanned' sheep erythrocytes were prepared and sensitized with human gamma-globulin (Cohn Fraction II) as described by Heller, Jacobson, Kolodny and Kammerer (1954).

Sensitized human erythrocytes were prepared by exposure of a 1 per cent suspension of Rh-positive erythrocytes to an equal volume of serum containing incomplete antibody. The highest dilution of this serum capable of rendering Rh-positive erythrocytes agglutinable by anti-human globulin serum was 1 in 800. It was diluted 1 in 100. The erythrocytes were incubated with the diluted serum at 37° C. for 60 minutes and thereafter washed three times in 0·89 per cent sodium chloride. Control material was prepared by similar treatment of Rh-negative erythrocytes.

TITRATION OF CONJUGATED SERA

Serial dilutions of serum were made in haemagglutination plates (M.R.C. pattern) using 0·89 per cent sodium chloride as diluent. An equal volume of a 0·5 per cent suspension of appropriate erythrocytes was added to each dilution of serum and incubated at 37° C. for 60 minutes. The plates were transferred to a refrigerator at 4° C. and read after 18–24 hours. The titre was recorded as the highest dilution of serum in which agglutination was visible to the naked eye.

EXAMINATION OF ERYTHROCYTES FOR FLUORESCENCE

After determination of the titre, the erythrocytes in the highest concentration of conjugated serum (1/8 or 1/10) were pipetted from the plate to a 15 ml. centrifuge tube. The erythrocytes were washed three times in 0·89 per cent sodium chloride. The packed erythrocytes were resuspended in 0·25 ml. of 0·89 per cent sodium chloride and one drop of the suspension was transferred to a special slide (described below) for examination in the fluorescence microscope.

Two types of fluorescence were observed: (1) The erythrocytes exhibited very pale green fluorescence which was noted even when erythrocytes were exposed to conjugated serum from which specific antibody had been absorbed. This weak fluorescence, distinguishable from the faint red-brown fluorescence of erythrocytes exposed to non-conjugated serum, was attributed to non-specific transfer of fluorescent protein and was taken as a negative result. (2) The result was considered positive where brilliant green fluorescence

of agglutinated and individual erythrocytes was present. The appearances are illustrated in Fig. 1. This bright fluorescence was never observed on erythrocytes exposed to conjugates from which specific antibody had been absorbed.

FLUORESCENCE MICROSCOPY

Experience in the early stages of the present work indicated that an intense source of ultraviolet light and an efficient optical system were essential in examination of erythrocyte systems. The microscope which was finally evolved has been very satisfactory in the more

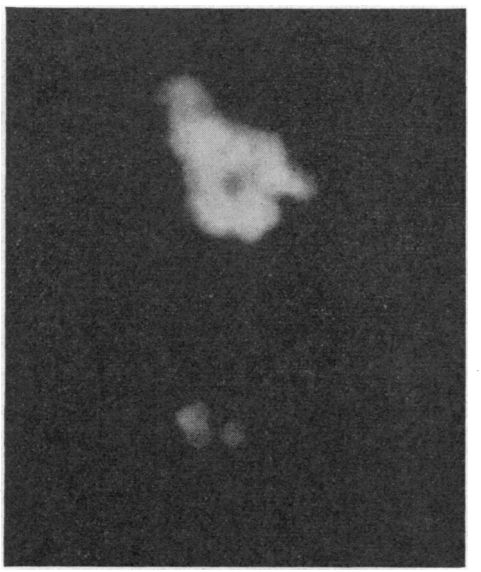

FIG. 1. Fluorescence of sheep erythrocytes exposed to conjugated rabbit anti-sheep erythrocyte serum. An agglutinate and a few individual cells are shown. ×500.

widely used applications of the Coons' technique and will therefore be described in some detail.

The source of ultraviolet light is a compact-source mercury vapour lamp of 1000 watt rating (Type ME/D/F, B.T.H. Co. Ltd., Rugby). The lamp is mounted in a protective metal housing and cooled from below by a small electric fan. The light is collected by a quartz bi-convex lens, 2·5 inches diameter placed (at its focal distance) 2·5 inches from the source. The beam is passed through a cuvette containing 15 per cent copper sulphate to remove red and infra-red radiation. The cuvette is constructed from a 'Perspex' cylinder 3 inches in diameter and is fitted with quartz end-plates spaced 1·5 inches apart. The copper sulphate is cooled by circulation of water through coils of polythene tubing attached to the inner surface of the cylinder. In the absence of cooling the copper sulphate boils. The filtered beam is focused on an aluminized mirror (clipped over the standard substage mirror of the microscope) by a quartz lens, identical with the collecting lens, and then enters a dark-ground illuminator (Cooke, Troughton and Simms, type M.1396). The specimen is mounted on a slide cut from Chance glass (Type OX7, thickness 1·03 mm.).

Contact between the lower surface of the slide and dark-ground illuminator is made with glycerol. The Chance glass removes all but ultraviolet and violet radiation from the beam. Visible light produced by fluorescence of cement in the illuminator lenses and of the glycerol is therefore excluded from the specimen and a very dark background is thus obtained. Cover slips cut from Chance glass (Type OY12, thickness 0·007 inch) are used. This glass transmits visible light and prevents ultraviolet radiation from reaching the objective lens of the microscope, where fluorescence of the cement might occur. The rest of the optical system is conventional, but it is an advantage to use objectives with good 'light-grasp'. It is not necessary to include an ultraviolet-absorbing filter in the eyepiece when OY12 cover slips are employed.

RESULTS

It is apparent from Table 1 that the original experiment, in which it was hoped to demonstrate specific fluorescence on sensitized sheep erythrocytes agglutinated by conjugated rheumatoid serum, was unsuccessful. Although the conjugate retained the capacity of the original serum to agglutinate sensitized sheep erythrocytes, fluorescence was not detected on the agglutinates. Nor was fluorescence observed on 'tanned' sheep erythrocytes

TABLE 1

TITRATION OF FLUORESCEIN-CONJUGATED SERA WITH SENSITIZED ERYTHROCYTES

Conjugated serum	*Erythrocytes*	*Titre*	*Fluorescence*
Rheumatoid arthritis ..	Sheep+1/5 M.A.D. rabbit haemolysin	1/640	Negative
	Sheep+1/40,000 tannic acid+Cohn Fraction II	1/1280	Negative
Normal human	Sheep+1/5 M.A.D. rabbit haemolysin	<1/8	Negative
	Sheep+1/40,000 tannic acid+Cohn Fraction II	<1/8	Negative
Goat anti-rabbit globulin ..	Sheep+1/5 M.A.D. rabbit haemolysin	1/1280	Negative
	Sheep+non-immune rabbit serum	1/10	Negative
Rabbit anti-human globulin (Coombs' serum)	Rh. positive+incomplete anti-D	1/4096	Negative
	Rh. negative+incomplete anti-D	1/16	Negative

(sensitized with Cohn Fraction II) which had been agglutinated by exposure to conjugated rheumatoid serum. It was established, by inclusion of a conjugated serum from a healthy individual, that the agglutination in these experiments was attributable to the rheumatoid agglutinating factor and not to some non-specific property peculiar to conjugated serum. As the conjugate had not lost its agglutinating properties, the absence of fluorescence on the erythrocytes suggested that the quantity of agglutinating factor involved in the reaction was less than could be detected in the fluorescence microscope or that the factor did not conjugate with fluorescein. It was decided, therefore, to examine other systems involving agglutination of sensitized erythrocytes and the results of the experiments are shown in

Table 1. It is seen that sensitized sheep erythrocytes and sensitized Rh-positive erythrocytes were specifically agglutinated by appropriate conjugated antisera, but did not fluoresce. The conjugated antihuman globulin used in the experiment with sensitized Rh-positive erythrocytes had been successfully employed to demonstrate human globulin in tissues and was therefore known to contain fluorescent antibody. For this reason it seemed probable that the present experiments had failed because the amount of fluorescent protein transferred to the erythrocytes was extremely small.

The investigation had, to this point, involved only sensitized erythrocytes and it was decided then to examine erythrocytes which had been agglutinated by conjugated antiserum reacting directly with the erythrocytes. The sheep erythrocyte/anti-sheep erythrocyte system was chosen. The antiserum was found to cross-react with human Group A erythrocytes which were therefore included in the experiments shown in Table 2. The unabsorbed conjugate produced brilliant fluorescence of sheep erythrocytes, which could be detected even when the erythrocytes had been exposed to dilutions as high as 1/320. The conjugate retained these properties after absorption with human Group A or B erythrocytes, but no fluorescence was observed on erythrocytes exposed to conjugate which had been absorbed with sheep erythrocytes.

TABLE 2

TITRATION OF FLUORESCEIN-CONJUGATED RABBIT HAEMOLYSIN WITH SHEEP AND HUMAN (GROUP A) ERYTHROCYTES

Conjugated serum	*Erythrocytes*		*Titre*	*Fluorescence*
	For absorption	*In titration*		
Rabbit anti-sheep erythrocyte (haemolysin)	Unabsorbed	Sheep	1/1280	POSITIVE
		Human 'A'	1/1280	Negative
	Sheep	Sheep	<1/10	Negative
		Human 'A'	<1/10	Negative
	Human (Group A)	Sheep	1/1280	POSITIVE
		Human 'A'	1/10	Negative
	Human (Group B)	Sheep	1/1280	POSITIVE
		Human 'A'	1/640	Negative

Attempts to inhibit fluorescence by previous treatment of erythrocytes with non-conjugated antiserum were unsuccessful. Erythrocytes coated with antibody were invariably haemolysed when subsequently incubated with the conjugated antiserum and it was observed that the stromata acquired non-specific fluorescence. (This phenomenon was also noted when erythrocyte stromata were incubated with conjugated serum from the same individual. Bright fluorescence, persisting after repeated washing, was observed.)

Although the conjugated anti-sheep erythrocyte serum agglutinated human Group A erythrocytes to the same titre as sheep erythrocytes, specific fluorescence was not detected on the human cells. This suggested that bright fluorescence occurred only when the erythrocytes were agglutinated by serum containing species-specific antibody. This possibility was strengthened by the finding that a conjugated infectious mononucleosis serum, which agglutinated sheep erythrocytes at a titre of 1/2560, failed to impart fluorescence to the cells.

The investigation was continued by experiments with human erythrocytes and three conjugated sera which had in common the property of agglutinating Group A erythrocytes. The results are summarized in Table 3. Group A erythrocytes exposed to

conjugates containing iso-agglutinin *a* or anti-A-substance were specifically agglutinated, but did not fluoresce.

The third serum was prepared against Group A erythrocytes. Human erythrocytes of

TABLE 3

TITRATION OF HUMAN ERYTHROCYTES WITH FLUORESCEIN-CONJUGATED SERA

Conjugated serum	*Erythrocytes*		*Titre*	*Fluorescence*
	For absorption	*In titration*		
Rabbit anti-human A substance	Unabsorbed	Human A	1/512	Negative
		Human B	<1/8	Negative
		Human O	<1/8	Negative
Human anti-A (iso-agglutinating)	Unabsorbed	Human A	1/128	Negative
		Human B	<1/8	Negative
		Human O	<1/8	Negative
Rabbit anti-human erythrocyte (Group A)	Sheep	Human A	1/1024	POSITIVE
		Human B	1/512	POSITIVE
		Human O	1/512	POSITIVE
	Human A	Human A	<1/8	Negative
		Human B	<1/8	Negative
		Human O	<1/8	Negative
	Human B	Human A	1/256	Negative
		Human B	<1/8	Negative
		Human O	<1/8	Negative
	Human O	Human A	1/512	Negative
		Human B	1/8	Negative
		Human O	<1/8	Negative

all groups were agglutinated by this conjugate and the agglutinates fluoresced. This fluorescence was not abolished by previous absorption of the conjugate with sheep erythrocytes. When the conjugate was absorbed with Group B or Group O erythrocytes agglutinins for Group A erythrocytes remained, but fluorescence of the agglutinates did not occur. Absorption with Group A erythrocytes removed agglutinins for all human erythrocytes and again no fluorescence of cells exposed to the absorbed serum could be detected.

These results conform with those obtained with the sheep erythrocyte system, in that specific fluorescence of agglutinates was observed only when the erythrocytes had been exposed to conjugates containing species-specific antibody.

DISCUSSION

It is considered that the latter results adequately explain the failure to demonstrate by fluorescence the transfer of rheumatoid agglutinating factor to sensitized sheep erythrocytes.

The negative results obtained with other haemagglutinating systems, where an antigen/antibody reaction was known to occur, suggest that the Coons technique is relatively insensitive in this application. The low sensitivity may have been due to several factors. The erythrocytes may themselves have absorbed ultraviolet radiation, thus reducing the amount of radiation available for excitation of the conjugated antibody. In support of this theory are the observations of Gitlin, Landing and Whipple (1953) that fluorescence was quenched in solutions containing high concentrations of haemoglobin and, in the present

work, that stromata exposed to conjugated sera fluoresced brilliantly. The positive results obtained with intact cells and species-specific antibodies show, however, that quenching of fluorescence was not wholly responsible for the insensitivity in other systems.

The possibility that certain antibodies failed to conjugate with fluorescein must also be considered, but seems most unlikely in the light of the experiences of other workers with the technique. The recent report of Glynn, Holborow and Johnson (1957) is particularly relevant in this connexion in that conjugated anti-A substance serum was used successfully to localize A-substance in human tissues. The antibody must therefore conjugate with fluorescein, although in the present study it could not be demonstrated on Group A erythrocytes with which it had been specifically combined. It seems probable that the apparent discrepancy in the results obtained with this antibody when applied to tissues and erythrocytes is explained by differences in the amounts of antigen and antibody involved, only very small quantities of antibody reacting with the Group A erythrocytes. In experiments with radio-iodinated sera, Boursnell, Coombs and Rizk (1953) showed that the amount of incomplete antibody adsorbed on rhesus-positive erythrocytes was very small and was difficult to measure in the presence of protein which had been non-specifically adsorbed.

If the degree of fluorescence detected on erythrocytes is accepted as an index of the amount of antibody combined, the present results suggest that species-specific antibodies combine in larger amounts than antibodies to other components of the erythrocyte. This quantitative difference was not revealed by simple titration of the sera and the results seem to provide further evidence of the limitations of titration as a measure of agglutinins in serum.

ACKNOWLEDGMENTS

The author is greatly indebted to Dr. Lynda Roy, who prepared the fluorescein amine and conjugated the sera.

Dr. E. J. Holborow, Taplow, and Dr. R. R. A. Coombs, Cambridge, kindly supplied anti-human Group A substance serum and infectious mononucleosis serum, respectively.

Throughout the investigation, Dr. J. J. R. Duthie, Physician-in-Charge, Rheumatic Unit, has offered continued encouragement and advice, which has been most helpful.

During the period when the work was done, the Rheumatic Unit was in receipt of grants from the Nuffield Foundation, the Medical Research Council and Boots Pure Drug Company Ltd.

REFERENCES

Boursnell, J. C., Coombs, R. R. A. and Rizk, V. (1953). 'Studies with marked antisera. Quantitative studies with antisera marked with Iodine ^{131}Isotope and their corresponding red-cell antigen.' *Biochem. J.*, **55**, 745–58.

Coons, A. H. (1956). 'Histochemistry with labelled antibody.' In *International Review of Cytology*, vol. 5, pp. 1–23. Ed. by Bourne, G. H. and Danielli, J. F. New York: Academic Press Inc.

Coons, A. H., Creech, H. J., Jones, R. N. and Berliner, E. (1942). 'The demonstration of pneumoccal antigen in tissues by the use of fluorescent antibody.' *J. Immunol.*, **45**, 159–70.

Coons, A. H. and Kaplan, M. H. (1950). 'Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody.' *J. exp. Med.*, **91**, 1–13.

Gitlin, D., Landing, B. H. and Whipple, A. (1953). 'The localization of homologous plasma protein in the tissue of young human beings as demonstrated with fluorescent antibodies.' *J. exp. Med.*, **97**, 163–76.

Glynn, L. E., Holborow, E. J. and Johnson, G. D. (1957). 'The distribution of blood group substances in human gastric and duodenal mucosa.' *Lancet*, **ii**, 1083–8.

Heller, G., Jacobson, A. S., Kolodny, M. H. and Kammerer, W. H. (1954). 'The Haemagglutination Test for Rheumatoid Arthritis. II. The influence of human plasma Fraction II (gamma globulin) on the reaction.' *J. Immunol.*, **72**, 66–78.

Marshall, J. M. (1951). 'Localization of adrenocorticotropic hormone by histochemical and immunochemical methods.' *J. exp. Med.*, **94**, 21–9.