AN ISOPRECIPITATION REACTION DISTINGUISHING HUMAN SERUM-PROTEIN TYPES

A. C. ALLISON
M.A., B.M., D.Phil. Oxon.
OF THE NATIONAL INSTITUTE FOR MEDICAL RESEARCH, LONDON, N.W.7

B. S. BLUMBERG
M.D. Columbia, D.Phil. Oxon.
OF THE NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

DURING the past decade it has become apparent that human beings differ in their serum-proteins as well as in their red blood-cells. Genetically controlled variations have been described in haptoglobins and transferrins (Smithies and Connell 1959), γ-globulins (Grubb 1957, Ropartz 1960), and in the Gc system definable by immuno-electrophoresis (Hirschfeld and Beckman 1960). Though the chemical differences between the alternative proteins are not yet known, they are generally assumed to be small —analogous to the single aminoacid substitutions that distinguish the different human hæmoglobin half-molecules (Hunt and Ingram 1959) and the small sugar substitutions that distinguish the blood-group substances (Morgan 1959). Nevertheless, the possibility remains that these or other serum-proteins are sufficiently different in structure to induce serum-protein isoimmunisation when blood is transfused.

Such a reaction is already known in rabbits. Oudin (1956), Dray and Young (1959), and others have shown that rabbits can be immunised with serum from other rabbits of different γ-globulin type. In the original experiments the foreign plasma was introduced with adjuvants, but recent observations (S. Dray, personal communication) have shown that adjuvants are unnecessary for immunisation. Three or four intravenous injections of serum from rabbits of another γ-globulin type led to the production in recipients of well-defined precipitating antibodies.

The experiments described here were undertaken to find out whether any similar reaction can be shown in man. Sera were obtained from subjects who had received multiple transfusions and were tested for precipitating antibodies against a panel of sera chosen to represent all the available genetically controlled serum-protein types. A serum has been found which gives strong precipitation with some, but not all, sera; and this reaction defines a polymorphism involving the α_2-globulins. Besides being of genetic interest, this finding raises the possibility that serum-protein isoimmunisation takes place in man and represents a clinical problem in persons receiving blood-transfusions.

Materials and Methods

Post-transfusion sera.—These were obtained from patients at the Clinical Center of the National Institutes of Health, Bethesda, who had received 5 or more transfusions. The indications for transfusion included open-heart surgery, hæmolytic and aplastic anæmias, pelvic tumours, and leukæmias. Wherever possible, blood-samples were drawn between 10 and 30 days after the last transfusion. Sera were stored at −20°C and were tested within 5 days of collection. Many of the subjects had received 30 or more transfusions.

Panel sera.—These were selected from the collection of the Section on Geographic Medicine and Genetics of the National Institute of Arthritis and Metabolic Diseases. The 24 sera included haptoglobin types 1-1, 2-1, 2-2, 2-1M, and O; transferrin types CC, CD, and BC; and γ-globulin types Gm a+, Gm a−, Gm b−, Gm b−, Gm x+, and Gm x−. In addition, 3 sera from patients with definite rheumatoid arthritis having high titres of rheumatoid factor, and 1 serum from a patient with well-developed lupus erythematosus (L.E.) having positive L.E. histological preparations and high titres of antinuclear antibodies, were used. The sera had been stored at −20°C for periods varying from 2 days to 2 years. Storage did not alter the reaction to be described. The sera were from different population groups, including American whites (sixteen), American Negroes (three), Micronesians (three), an Alaskan Eskimo, and a Viet-Namese.

Gel-diffusion precipitation technique.—The Ouchterlony procedure was carried out in 5 cm. petri dishes containing 8 ml. of 0·9% 'Oxoid Ionagar' (w/v) in 0·07 *M* sodium phosphate buffer, pH 7·0, with 0·001 *M* sodium ethylenediamine-tetra-acetate and 0·001 *M* sodium azide. With a die, six wells 6 mm. in diameter were cut round a centre well of the same size. The circumferences of the centre well and peripheral wells were 4 mm. apart. After removal of the agar cores from the wells, the bases of the latter were sealed with small volumes of molten agar. The post-transfusion sera were placed in the centre wells and panel sera in the peripheral wells. The plates were stored at room temperature and observed at 18 hours (by which time clear-cut precipitation was usually visible), 48 hours, and 4 days. The precipitation is best seen by oblique illumination from below against a dark background.

Immunoelectrophoresis.—This was done by the micromethod of Grabar and Williams (1955) and Scheidegger (1955) using standard lantern-slide plates coated with agar made up in barbitone buffer 0·01 *M*. pH 8·4. Antibody (or antigen) was placed in the side well and examined after reacting for 18 hours, by which time well-defined precipitates were visible. The reaction was continued to 36 hours, after which the plates were washed in saline and stained.

Starch-gel electrophoresis.—Starch gel (Connaught Laboratories, Toronto) was made up in *tris*-(hydroxymethl)-aminomethane buffer (Poulik 1957) and poured into 6 × 80 × 150 mm. trays. Samples were inserted as a starch paste (Smithies 1959) into a slot 3 mm. wide. Electrophoresis was carried out for 5 hours at 6V per cm. The position of the components was identified in a small strip of gel; the various components were eluted by the method of Gordon (1960) and were concentrated by ultrafiltration.

Preparation of γ-globulin components.—Separation of 7S and 19S γ-globulins was achieved by sucrose density-gradient centrifugation (Fudenberg and Kunkel 1957) using 40, 30, 20, and 10% solutions of sucrose.

Bentonite-flocculation test.—The method of Bozicevich et al. (1958) was used.

Latex-fixation test.—This was carried out as described by Singer and Plotz (1956), using the Hyland 'RA-test' kit (Hyland Laboratories, Los Angeles, California).

Observations

A precipitin was found in the serum of a patient who had received many transfusions. He had a long and varied history of illness.

The patient is a 64-year-old white American retired executive of Hungarian birth. At the age of 16 he had albuminuria which lasted for 4 years. In 1934, while still living in Budapest, he had an episode of sudden onset of paræsthesiæ and paralysis of the legs. This resolved spontaneously in 2 months. A second episode, involving complete paralysis up to the waist, weakness of the arms, and loss of sensation and reflexes in the legs, took place in the United States in 1958. Cerebrospinal fluid contained 73 mg. protein and 150 lymphocytes per ml. Polyneuritis of unknown cause was diagnosed, and again remission was spontaneous.

The patient also had symptoms of peptic ulceration dating from 1921, with gastrointestinal hæmorrhage in 1938 and 1948. In 1941 a gastroenterostomy was performed, but symptoms and hæmorrhage have recurred at intervals. The patient was again admitted to hospital in 1958, at which time he was reported to have low red-cell counts, low hæmoglobin levels, and high white-cell and platelet counts. X-ray examination suggested an ulcer crater in the proximal duodenum. The bleeding

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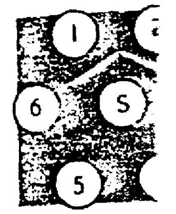

Fig. 1

Fig. 1—The precipiti... plate, between the ... some, but not all, ... persons. Unstained

Fig. 2—The precipitin ... the centre well agai... sera.

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continued for a month and then ceased, and with the help of blood-transfusions the hæmoglobin was raised to 14 g. per 100 ml. Because of the high white-cell count and the presence of numerous megakaryocytes in the bone-marrow and some in the peripheral blood, the possibility that the patient might have leukæmia was raised. No convincing evidence that this is so has been obtained.

Without transfusion the patient's hæmoglobin continued to fall, and in February, 1958, he was admitted to the Clinical Center of the National Institutes of Health. During his time in

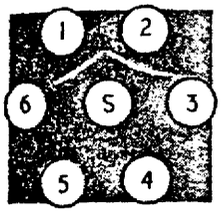

Fig. 1

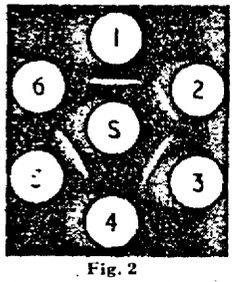

Fig. 2

Fig. 1—The precipitin reaction, seen in an agar-gel Ouchterlony plate, between the serum of the patient in the centre well and some, but not all, members of a panel of sera from normal persons. Unstained plate after 24 hours.

Fig. 2—The precipitin reaction between the serum of the patient in the centre well against some, but not all, members of a panel of sera.

The two degrees of positive reaction are shown: nos. 1; 3, and 5 are 2+ reactors and no. 2 is a weak + reactor.

hospital the patient had persistent anæmia without reticulocytosis. The leucocyte count varied from 10,000 to 15,000 and the platelet count was consistently above 500,000 per c. mm. The differential count showed predominantly neutrophils with a relative increase of immature forms. Bone X-rays showed no abnormalities. Refractory anæmia of unknown cause was diagnosed. The patient was given cells from 4 units of blood, which raised his hæmoglobin to 11 g. per 100 ml., and was discharged.

When the patient first received blood-transfusions in 1958 they were well tolerated. Since that time he has received 47 units of blood. The patient is of blood group AB, Rh+, and there has never been evidence of red-cell incompatibility with donor bloods. In the spring of 1960 the patient began to have transfusion reactions. Within about 1 hour of some, but not all, transfusions there was a fever, rising to 38–39°C (100·4–102·2°F) accompanied by headache and muscle pain, but no other symptoms. The fever and symptoms persisted for about

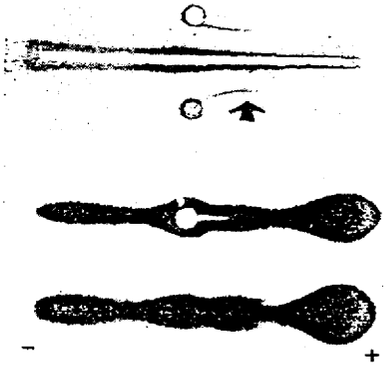

Fig. 3—Agar-gel immunoelectrophoresis to characterise antigen.

The serum of a strongly positive reactor was concentrated three times by ultrafiltration and submitted to electrophoresis. The serum of the patient was placed in the rectangular antibody well. The position of the precipitin line (arrow) suggests that the antigen is an α_2-globulin. Above, unstained precipitin lines; below, protein stain.

5 hours, and then slowly subsided. The patient had been discharged from hospital before the precipitin in his blood was discovered, so that it has not yet been possible to ascertain whether only bloods containing the precipitating antigen give transfusion reactions.

Precipitation Reactions

The serum of the patient gave well-defined precipitation reactions in agar with some, but not all, panel sera (fig. 1). The reaction was clearly visible at 18 hours and was intense after 2 days. The precipitation line was convex toward the centre (precipitin) well, indicating that the antigen is of relatively high molecular weight (Korngold et al. 1959). Two intensities of reaction were definable in most groups of sera studied (fig. 2). Some sera lacked detectable antigen, the precipitation lines extending into the corresponding wells without deviation (fig. 1). The precipitation lines were clear-cut and quite unlike the diffuse reaction which slowly develops between rheumatoid factor and γ-globulin in some subjects (Franklin 1960a). Rheumatoid-factor reaction was excluded because neither the patient nor most of the positive subjects had rheumatoid factor, as determined by bentonite-flocculation and latex-fixation tests. Some panel subjects with rheumatoid factor gave reactions with the antiserum, but others did not. A second sample of serum from the patient, drawn 3 weeks after the first, at a

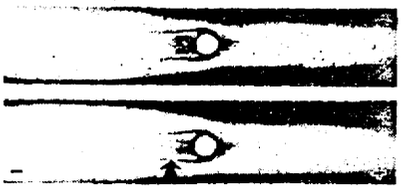

Fig. 4—Agar-gel immunoelectrophoresis to characterise the antibody.

The serum of the patient was placed in the circular wells and submitted to electrophoresis, and a strongly positive serum concentrated three times was placed in the rectangular wells. A broad precipitin line (arrow) is seen in the position of the γ-globulin.

time when the patient had not received any medication since discharge, gave identical reactions, as did a third sample drawn 1 month later, and a fourth 3 months later.

Support for the interpretation that the precipitin in the serum of this patient is an antibody came from experiments showing that the active protein is a 7S-γ-globulin. We propose the designation Ag(a+) for sera containing the α-globulin antigen and Ag(a−) for sera that do not contain the antigen. The precipitin in the serum of the patient thus has the specificity anti-Ag(a+).

Characterisation of Antigen

A panel serum giving a strongly positive precipitation line in the gel-diffusion test was concentrated three times by ultrafiltration and used for immunoelectrophoresis. The antibody well was filled with serum from the patient. A well-defined precipitation line developed between the α_2 region and the antibody (fig. 3). This result, along with that in the Ouchterlony plates, suggested that the antigen is a high-molecular-weight α_2-component. The only α_2-macroglobulin so far defined is the 19S-α_2 component described by Brown et al. (1954) and Wallenius et al. (1957), which appears to be equivalent to the slow-α_2 component found on starch-gel electrophoresis (Poulik and Smithies 1958). Purified α_2-macroglobulin (Behringwerke, Marburg) in a wide range of concentrations failed to give precipitation with the patient's serum.

Furthermore, when an Ag(a+) serum was submitted to starch-gel electrophoresis, the slow-α_2 fraction recovered by the technique of Gordon (1960) failed to show precipitation with the anti-Ag(a+) serum. The further identification of the antigen present in the α-globulin is currently being undertaken.

Characterisation of Antibody

The patient's serum was submitted to immunoelectrophoresis, and a serum giving a strong reaction in the Ouchterlony tests was placed in the side well. Within 18 hours a well-defined precipitation line appeared in the γ-globulin region (fig. 4). This result, together with the shape of the precipitation curve in the Ouchterlony plate, suggested that the antibody was a relatively low-molecular-weight γ-globulin. This interpretation was confirmed by an experiment in which the 7S- and 19S-γ-globulin components in the patient's serum were separated by density-gradient centrifugation. Precipitation lines in Ouchterlony plates were obtained with low-molecular-weight fractions giving no reaction with immune-rabbit serum against 19S human γ-globulin as described by Franklin (1960b). It can, therefore, be concluded that the antibody is a 7S-γ-globulin. The serum of the patient's daughter showed no reaction when tested against panel sera nor against her father's serum.

Stability of the Antigen

The antigen is unaffected by heating at 57°C for 40 minutes, by hæmolysis with its own or foreign human hæmoglobin, or by storage at room temperature for up to 7 days. The antigen was not lost by dialysis or ultrafiltration. Sera of either type (reacting or non-reacting) stored for as long as 4 years at −20°C were consistently found to give the same reaction as fresh sera from the same subjects. The reaction in the fresh sera, however, was in some cases stronger and better defined than in the stored sera. Repeated freezing and thawing of the sera up to at least fifteen times did not affect the reaction. Blood-samples were withdrawn twice weekly for 4 weeks from two normal volunteers whose sera reacted strongly with that of the patient. The reaction remained unchanged during this period. Sera withdrawn approximately twice yearly over a 2-year period from six subjects, two of whom reacted and four of whom did not, consistently gave the same reaction. Of 2 sera withdrawn in 1955, 1 was a reactor and the other not; the reactions in sera withdrawn from the same subjects in 1959 were unchanged.

Variation in the position of the slow-α_2-globulin after starch-gel electrophoresis has been noted. By means of vertical electrophoresis, using borate buffer at pH 8·6 (Smithies 1959), a slow-moving, a fast-moving, and a double band can be distinguished. It has been suggested that this variation may be due to storage (Smithies 1955); but this may not be the only explanation: Sera from different persons stored for the same length of time under identical conditions may show the variation, and not all stored sera show it (Blumberg 1961). In any event, this does not appear to affect the reaction with the patient's serum. Persons of each of the three slow-α_2-types were both positive and negative reactors.

It appears, therefore, that the antigen is relatively stable and persistent in any one person.

Distribution of the Antigen in Populations

Because the supply of antibody serum is limited, extensive population surveys have not been possible. However, positive and negative sera were found in all the populations studied. On the basis of preliminary examinations, the frequency seems to vary in different populations. The frequencies of positive reactors in some populations are shown in table I. More extensive studies are planned when micromethods are developed.

TABLE I—FREQUENCY OF POSITIVE REACTORS

Population	No. studied	No. positive
Parents of white U.S. families	56	32 (57%)
U.S. Negroes	21	10 (48%)
Micronesians (Rongelap, Marshall Islands) ..	51	50 (98%)

Inheritance of the Antigen

126 sera from 29 American families (25 white, 4 Negro) were studied. The results are shown in table II. In addition, 38 sera from 8 Micronesian families were tested. Since the frequency of positive reactors was high in the Micronesians, all of these were Ag(a+) × Ag(a+) matings, and nearly all the offspring were Ag(a+). These are also shown in table II, but are not included in the statistical evaluation, since they are not decisive in testing the genetic hypothesis. In the families from the United States, among the offspring of positive × positive and positive × negative matings, positive and negative offspring were found, whereas among the offspring of negative × negative matings only negative offspring appeared. This result suggests that the inheritance of the antigen follows mendelian segregation, with negative subjects homozygous for a recessive gene *Ag*, and positive subjects homozygous or heterozygous for the allelic gene Ag^A. It is suggested that the Ag^A gene controls the synthesis of the *Ag*(a+) antigen. If a product of the allelic gene is found, the gene could be termed Ag^B and the antigen *Ag*(b+).

TABLE II—INHERITANCE OF THE ANTIGEN

Mating type	No. of families	Offspring		Total
		Ag(a+)	Ag(a−)	
United States:				
Ag(a+) × Ag(a+) ..	11	21	5	26
Ag(a+) × Ag(a−) ..	10	9	13	22
Ag(a−) × Ag(a−) ..	8	0	20	20
Micronesian:				
Ag(a+) × Ag(a+) ..	8	21	1	22

The family data were analysed by the methods summarised by Smith (1956). In this, four types of comparison are made, in each of which a correction is included for family size. The comparisons of observed and expected figures involve:

1. The number of recessive Ag(−) children resulting from Ag(+) × Ag(a−) matings, given the number of families in which there is at least one recessive child.
2. The number of recessives resulting from Ag(a+) × Ag(a+) matings in which there is at least one recessive offspring, given the total number of families with at least one recessive offspring.
3. In Ag(a+) × Ag(a−) matings, the total number of families with at least one recessive offspring, given the total number of Ag(a+) × Ag(a−) matings and the gene frequencies in the population from which the families were selected (q = frequency *Ag* = 0·66).
4. In Ag(a+) × Ag(a+) matings, the total number of families with at least one recessive offspring, given the total number of Ag(a+) × Ag(a+) matings and the gene frequency.

The first two computations are independent of the gene frequencies in the population study. For the third and fourth computations, however, an estimate is needed of the gene frequency in the population from which the families were drawn. We were unable to obtain a large random sample from the population from which the families were selected, and the gene frequency was estimated from the frequency of the phenotypes in the

TABLE III—OBSERVED AND EXPECTED NUMBERS OF Ag(a−) CHILDREN FROM VARIOUS MATINGS

Test	Mating	χ^2
Comparison of observed and expected number of Ag(a−) children in families where there is at least one Ag(a−) child	Ag(a+)×Ag(a−)	0·99
Comparison of observed and expected number of Ag(a−) children in families where there is at least one Ag(a−) child	Ag(a+)×Ag(a+)	1·37
Comparison of observed and expected number of families with at least one Ag(a−) child, assuming an Ag-gene frequency of 0·66	Ag(a+)×Ag(a−)	0·49
Comparison of observed and expected number of families with at least one Ag(a−) child, assuming an Ag-gene frequency of 0·66	Ag(a+)×Ag(a+)	0·03
Comparison of observed and expected number of recessives in Ag(a−)×Ag(a−) matings	Ag(a−)×Ag(a−)	0

$\Sigma\chi^2 = 2{\cdot}88$ with 5 degrees of freedom; $0{\cdot}8 > P > 0{\cdot}7$.

parental members of the families (table I). Of 56 parents, 57% were found to be Ag(a+).

The results of these computations are summarised in table III, which includes also the results of Ag(a−) × Ag(a−) matings. Although the numbers are small, this preliminary family analysis is consistent with the genetic hypothesis. On the basis of segregation in this group of families, no evidence of close linkage was found with the following blood-group systems and haptoglobins types: ABO, Rh, MN, Duffy, Kidd, and P. Analyses of 30 twin pairs to be reported elsewhere were also consistent with the genetic hypothesis.

Independence of the Ag and Other Known Inherited Systems of Serum-proteins

The reactions in the panel sera showed that the Ag reactions were independent of rheumatoid factor and of haptoglobin, transferrin, and γ-globulin types. The segregation of the Ag^A and Ag genes in families was also independent of these systems. Since the Ag(a+) factor is an α-globulin it is also independent of another recently described inherited γ-globulin system designated In_γ by Ropartz (1960). In collaboration with Dr. J. Hirschfeld, of Stockholm, Ag tests were carried out on 113 sera of known Gc types, which are distinguishable by immunoelectrophoresis (Hirschfeld and Beckman 1960). Positive and negative Ag reactions were found in subjects of Gc types 1-1, 2-1, and 2-2, the percentage of reaction being of the same order in all these groups. Hence the Ag system is independent of all other known inherited serum-protein differences. The Ag(a+) antibody is also distinct from the factor in normal human serum giving a precipitate in agar with red-cell hæmolysates (Peetom et al. 1960); the antigen in the latter case is not present in serum and does not react as an α-macroglobulin. The Ag factor is present in too many normal sera to be C-reactive protein; which in any case migrates in the γ-region in immunoelectrophoresis (Zach and Zimmermann 1959).

Discussion

The precipitin in the patient's serum defines the presence of a factor in some sera from all human populations so far tested. The antigen is an α_2-macroglobulin and the precipitin a 7S-γ-globulin. The presence of the antigen appears to be genetically determined, so that a new polymorphic system in human serum-proteins has been revealed.

Besides being of genetical and anthropological interest, the finding of a specific precipitin in the patient's serum suggests that isoimmunisation by human serum-proteins may occur. Though there is no direct evidence that the precipitin was induced by the many transfusions which the patient received, this would seem to be the most probable explanation of the findings. In all, 61 sera of subjects who had received multiple transfusions have been tested, and 3 of these have given definite precipitation lines with some of the panel sera. The reaction which is the subject of this paper was the strongest, and the specificity of the others, which are different from the Ag system, will be reported later. Sera of forty persons who had not received transfusions failed to give precipitation lines with the panel sera.

Though our patient initially tolerated transfusions well, during the past 2 years he has had pyrexical reactions after most, but not all, transfusions. Whether these reactions take place only when donor serum is Ag(a+), and represent an antigen/antibody reaction, is now being investigated. Other properties of the precipitin will be described elsewhere. The unusual history of this case may be irrelevant, since the other subjects showing precipitins had typical thalassæmia.

Summary

A serum from an anæmic patient who had received approximately 50 transfusions was found to give strong precipitation with sera of some, but not all, normal subjects.

It appears that an antigen/antibody reaction is involved. The antigen in some normal subjects is an α_2-globulin, and the antibody in the patient is a 7S-γ-globulin. The antigen appears to be inherited according to simple mendelian rules and its frequency varies in different racial groups.

It is possible, though not proven, that antigen/antibody reactions were responsible for severe post-transfusion reactions in the patient, and similar processes may be involved in other unexplained post-transfusion reactions.

We are indebted to Dr. Fred Stohlman and Dr. Archie McKinney for supplying the serum from the patient; to Dr. Sheldon Dray and Dr. Kurt J. Bloch for valuable help and advice; to Dr. J. Hirschfeld, Stockholm, for collaboration with the Gc tests; to Dr. George A. Silver, Montefiore Hospital, New York, for allowing access to families under his care; to Dr. Arthur G. Steinberg for supplying blood from American Negro families and carrying out the γ-globulin typing; and to Dr. Lyman Crittenden for assistance with the linkage studies. Most of this investigation was undertaken while one of us (A.C.A.) was a visiting scientist at the National Institute of Arthritis and Metabolic Diseases.

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