PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **189**, 196–200 (1988)

A Nonimmunoglobulin Precipitin to Tissue Extracts in Pathological Human Sera

DONG SOO KIM AND FELIX MILGROM

Department of Microbiology, School of Medicine, State University of New York, Buffalo, New York 14214

Abstract. An α-globulin component was noted in pathological human sera, which produced gel precipitation reactions with extracts of human and animal liver. The highest incidence of the precipitin was found in malaria (95%), renal graft rejection (81%), and rheumatoid arthritis (57%). The precipitinogen was thermostable and ethanol soluble; of two precipitation lines formed by this component, one merged into identity reaction with a line produced by commercial lecithin of bovine origin. The possible diagnostic application of the reactions noted was considered.

Reactions between antigens and their corresponding antibodies have frequently been named "specific serological reactions," whereas other interactions of human and animal sera which were mimicking true serological reactions but which were produced by serum proteins other than antibodies were referred to as "nonspecific serological reactions" (1). Such nonspecific reactions have been recognized since the beginning of the 20th century and already at that time, it was noted that some of these reactions were capable of distinguishing pathological from normal human sera. Several tests were described in which pathological sera could be distinguished from normal sera by precipitation of serum proteins by various salts of heavy metals.

Perhaps the most important nonspecific serological reaction was described by Tillett and Francis (2) as precipitation of C-carbohydrate of pneumococci by sera originating from patients with various acute diseases. The term "acute phase reactions" was used by Abernethy and Avery (3) to denote such reactions. The serum protein responsible for precipitation of C-polysaccharide was referred to as C-reactive protein. This β-globulin produced by hepatocytes appears at considerably increased levels in pathological conditions. At present, C-reactive protein is detected in human sera as an antigen by means of rabbit immune sera, and these tests have some diagnostic value. Several other "acute phase" responses have been measured in human sera. This was comprehensively reviewed by Pepys and Baltz (4).

The present study was conducted following our observation that many pathological human sera, including those from patients with malaria and rheumatoid arthritis, frequently gave gel precipitation reactions with extracts of human and animal liver. The precipitin proved to be an α-globulin component of human sera and the precipitinogen an ethanol-soluble component of liver extract identical with or related to lecithin.

Materials and Methods. Pathological human sera originated from the collection of this Department, assembled since the 1940s. These sera were obtained from patients in the local area hospitals, as well as from various other health institutions in this country and abroad. All sera were preserved at −20°C and thawed just prior to use. Sera which appeared grossly turbid were not included into this study. Normal human sera originated from blood donors from the local American Red Cross and from healthy staff members of this Department.

Precipitinogen preparations. Extracts of human or bovine liver were prepared by mincing the tissue in RPMI 1640 medium, by means of a Waring blender. A 20% (w/v) suspension obtained in this way was left for 1 hr at 37°C and 16 hr at 4°C. The preparation was centrifuged for 15 min at 10,000*g* and the supernatant liver extract (LE) was withdrawn. Ethanol-soluble preparation of precipitinogen was obtained by mincing human or bovine liver in 96% ethanol by means of a Waring blender to obtain a 20% (w/v) suspension. This suspension was left for 3 days at 37°C in tightly corked bottles. Thereafter,

0037-9727/88 $1.50

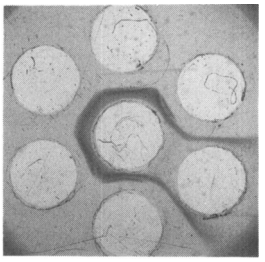

FIG. 1. Double diffusion gel precipitation test. Central well: extract of human liver. Peripheral wells, malaria sera, clockwise from the uppermost well: 3, 4, 8, 10, 12, and 13.

the ethanol extract was separated by centrifugation and evaporated in open dishes on an electric heater at a temperature of 70°C. The dried preparation was homogenized in a carbonate–bicarbonate buffer (a mixture of 0.5 *M* solutions of sodium bicarbonate with sodium carbonate, pH 9.6), at the original volume of the ethanol extract. The resulting opalescent preparation, referred to as ethanol-soluble liver preparation (ESLP), was used for the tests.

A few experiments were performed in which the precipitinogen was extracted from human sera. To this end, 1 vol of pooled human serum, normal or pathological, was mixed with 4 vol of ethanol and then the procedure of extraction described above for ESLP was followed.

Reagents. α_2-Macroglobulin, α_1-apolipoprotein, α_1-acid glycoprotein, and C-reactive protein were commercial preparations of Sigma, St. Louis, Missouri.

Cholesterol and phosphatidylethanolamine, in powder form, and cardiolipin, in a solution in absolute ethanol, 4.3 mg/ml, were purchased from Sigma. Lecithin preparation was purchased from Sylvana Chemical Co., Orange, New Jersey. This was a solution of lecithin of bovine origin in absolute ethanol at a concentration of 39.5 mg/ml. Ethanol was evaporated in an atmosphere of nitrogen from solutions of cardiolipin and lecithin. Thereafter, these compounds as well as cholesterol and phosphatidylethanolamine were homogenized in carbonate-bicarbonate buffer at concentrations of 5–20 mg/ml.

Double diffusion gel precipitation (DDGP) test. Agarose dissolved in 0.15 *N* NaCl solution, was used to obtain a 5-mm-thick agar layer in plastic petri dishes with tightly fitted lids. The wells were 4 mm in diameter and the diffusion distance between two adjacent wells was 2 mm. The plates with wells charged with reagents were left for 16 hr at room temperature and then the precipitation lines were inspected and pictures were taken.

Immunoelectrophoresis was performed following the procedure of Scheidigger (5) using barbital buffer, pH 8.8.

Results. This study was initiated by an observation that some pathological human sera produced precipitation lines when tested in DDGP against human LE. In studying this phenomenon, we noted that most malaria sera we examined would produce such reactions. This is exemplified by the experiment in Fig. 1. It may be noted that of six sera studied, five precipitated the LE, whereas one serum, 8, failed to do so. Instead, this serum contained apparently the precipitinogen, since distinct precipitation lines were noted in reaction of this serum with two malaria sera in adjacent wells. Furthermore, in some instances we observed two partially superimposed precipitation lines in reactions between positive sera and LE. This may be distinguished in Fig. 1 in reactions produced by sera 4 and 12. When LE was titrated against a precipitin-containing serum 4, definite precipitation lines were discernible up to the extract's dilution of 1:8 (Fig. 2).

Subsequently, we found that results very similar to those with LE may be produced with ESLP; here again, several sera produced

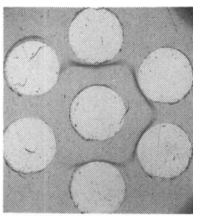

FIG. 2. Double diffusion gel precipitation test. Titration of extract of human liver. Central well: malaria serum 4. Peripheral wells: extract of human liver at dilutions of, clockwise from the upper most well, 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32.

TABLE I. DOUBLE DIFFUSION GEL PRECIPITATION TESTS: REACTIONS OF VARIOUS PATHOLOGICAL AND NORMAL HUMAN SERA WITH ETHANOL-SOLUBLE PREPARATION OF HUMAN LIVER

Diagnosis	Number of sera		Percentage of positive
	Tested	Positive	
Malaria	60	57	95.0
Rheumatoid arthritis	40	23	57.5
AIDS	54	19	35.2
Kawasaki disease	20	11	55.0
Syphilis	60	3	
SLE	20	2	
Lepromatous leprosy	20	2	9.2
Infectious mononucleosis	18	2	
Urinary tract infection	16	3	
Myocardial infarction	24	3	
Gastrointestinal adenocarcinoma	60	5	
Renal graft recipients in rejection crisis	41	25	60.9
Normal human sera	103	3	2.9

two discernible lines. Precipitation lines formed by a malaria serum with these two precipitinogen preparations merged into reactions of identity. Since ESLP gave better reproducibility of results than LE, we employed ESLP for most of the further studies. Using this preparation, the precipitating activity of strongly positive malaria sera was titrated. Precipitation lines up to a serum dilution of 1:16 were observed.

Employing ESLP, we found the frequency of positive results in human sera as listed in Table I. Malaria sera gave the highest frequency, 95%. This was followed by rheumatoid arthritis (57%) and renal graft rejection sera (61%). Sera of patients with AIDS were positive in 35%. On the other hand, there was a surprisingly low incidence of positivity in lepromatous leprosy and syphilis sera as well as sera of patients with gastrointestinal adenocarcinomas. The precipitation lines formed by sera from patients with different diseases produced identity reactions.

Significantly, only less than 3% of normal human sera were positive. However, several normal human sera would produce "subthreshold" reactions in that a normal serum shortened the precipitation line formed by a positive pathological serum, but failed to produce the reaction line by itself. This is exemplified by an experiment in Fig. 3.

It was of interest to learn more about the nature of the precipitin combining with the liver preparations. Heating of the precipitin-containing serum up to 66°C for 30 min did not change appreciably the reaction. All efforts to obtain complement fixation tests between the precipitin-containing sera and ESLP were unsuccessful. We also failed to produce positive results in enzyme immunoassay with plates coated by ESLP. Since at that time we did not have any information about the nature of precipitin, we employed the "double reagent" technique to detect the binding of the precipitin to the tissue preparation. We used rabbit antiserum to whole human serum as the first reagent and alkaline phosphatase-conjugated goat antiserum to rabbit IgG as the second reagent. Immunoelectrophoresis experiments showed that the precipitation line developed with electrophoretically separated malaria serum by ESLP was an α-globulin (Fig. 4), which argued against the possible antibody nature of the serum factor under investigation. Fur-

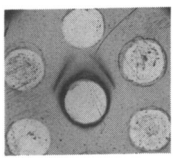

FIG. 3. Double diffusion gel precipitation test. Central well: ethanol-soluble preparation of human liver. Peripheral wells: uppermost, saline; upper left, malaria serum 4; upper right, malaria serum 12; and lower left and right, normal human sera.

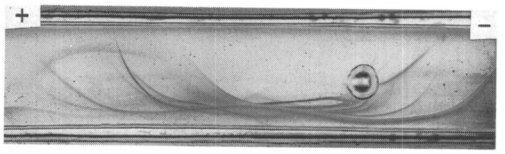

FIG. 4. Immunoelectrophoresis. Malaria serum 12 was separated electrophoretically from the central well. Upper trough was filled with ethanol-soluble preparation of human liver and lower trough was filled with rabbit antiserum to whole human serum. The arc in the lower left part of the field represents albumin and the arc in the lower right part of the field represents IgG.

thermore, we studied purified preparations of α_2-macroglobulin, α_1-apolipoprotein, and α_1-acid glycoprotein, all obtained from pooled normal human serum, as well as C-reactive protein isolated from human pleural effusion. Consistently negative results were obtained in DDGP with these proteins and ESLP.

A rather intriguing finding about the precipitinogen was its presence in some malaria sera, which was shown in Fig. 1. Accordingly, we assumed that the precipitinogen may be present in the sera even if it is not detectable by DDGP test as was the case with serum 8. Following this trend of thought, we prepared an ethanol extract of a pooled malaria serum, and for control, an ethanol extract of a pooled normal human serum, as described under Materials and Methods. Significantly, the former, but not the latter, contained the precipitinogen.

More information was sought about the nature of the precipitinogen. It was found to be remarkably thermostable in that ESLP could be autoclaved at 121°C without losing activity. There was no evidence for any species specificity of the precipitinogen, since we obtained indistinguishable reactions between precipitin-containing sera and ESLP of human and animal origin. It appeared that precipitinogen is a ubiquitous ethanol-soluble tissue component. Accordingly, we tested some ethanol-soluble normal tissue components against precipitin-containing sera. We obtained negative results with cholesterol, cardiolipin, and phosphatidylethylamine, but the results were consistently positive with lecithin. As seen in Fig. 5, the lecithin preparation produced a definite reaction line with the precipitin-containing serum and this line merged into identity reaction with one of the two lines formed by ESLP.

Discussion. The reactions described in this communication were noted very seldom with normal human sera but quite frequently with pathological human sera. Most impressive was the positivity of 95% observed in sera of malaria patients. The precise evaluation of the occurrence of this reaction in other diseases will have to be ascertained on larger material. At present it is rather surprising that the reactions were quite frequent in rheumatoid arthritis, renal graft rejection, and AIDS, whereas they were relatively infrequent in lepromatous leprosy and gastrointestinal adenocarcinomas. The appearance of precipitin was not related to the long-term preservation of the sera in the freezer. We tested several freshly obtained rheumatoid arthritis sera and they showed positivity similar to that of the preserved sera. Furthermore, we tested many normal sera that were preserved for 10–30 years and none of them was positive.

The precipitin was apparently present at subthreshold levels in many normal human sera. It was shown to be a serum protein with the electrophoretic mobility of an α-globulin. It appeared to be distinctly different from acute phase reactants: C-reactive protein, α_1-acid glycoprotein, serum amyloid A protein, and haptoglobin.

Precipitinogen was found regularly in plain extracts of human and bovine liver. It was an ethanol-soluble, remarkably thermostable component that withstood autoclaving at 121°C. Interestingly, in some malaria sera which did not have precipitin, the precipitinogen could be demonstrated by means of plain precipitation reaction with

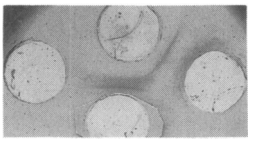

FIG. 5. Double diffusion gel precipitation test. Lower well: ethanol-soluble liver preparation. Upper well: malaria serum, 12. Left well: saline. Right well: lecithin at a concentration of 20 mg/ml.

whole serum. From other sera, it could be extracted by ethanol.

In following the lead obtained from the observation on ethanol solubility of precipitinogen, we tried to produce the reactions between the precipitin-containing sera and a few purified ethanol-soluble normal tissue components, and positive reactions were obtained with lecithin. The results observed seemed to indicate that lecithin represents only one of the two components detectable in the precipitinogen of the tissue extracts.

We are presenting these data since we believe that the significance of "nonspecific" serological reactions in medicine has been underestimated. Further exploration of the biological significance of these reactions should be of interest for pathologists. The diagnostic application of such reactions should not be neglected in view of their outstanding simplicity which may be of great significance in performing mass examinations. In the particular case of the findings discussed in this communication, the diagnostic application of the described test in malaria, rheumatoid arthritis, AIDS, and renal graft rejection warrants further exploration.

This work was supported by NIH Research Grant DK17317 from the National Institute of Diabetes, Digestive and Kidney Diseases and by a grant from The Richard W. and Mae Stone Goode Estate.

1. Landsteiner K. The Specificity of Serological Reactions. Cambridge, Harvard Univ. Press, 1946.
2. Tillett WS, Francis T Jr. Serologic reactions in pneumonia with a non-protein somatic fraction of pneumococcus. J Exp Med **52**:561–571, 1930.
3. Abernethy TJ, Avery OT. The occurrence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patients' sera and the effect of calcium on the flocculation reaction with C polysaccharide of pneumococcus. J Exp Med **73**:173–182, 1941.
4. Pepys MB, Baltz ML. Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. Adv Immunol **34**:141–212, 1983.
5. Scheidigger JJ. Une micro-methode de l'immuno-electrophorese. Int Arch Allergy Appl Immunol **7**:103–110, 1955.

Received April 4, 1988. P.S.E.B.M. 1988, Vol. 189.
Accepted July 7, 1988.