Immunoglobulin M-Immunoglobulin G Mixed Cryoglobulinemia in Schistosoma japonicum-Infected Rabbits

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Cryoprecipitation was observed in defibrinated serum from Schistosoma japonicum-infected rabbits. Ouchterlony immunodiffusion, sodium dodecyl sulfate-polyacrylamide gels, and anti-cryoprecipitate antiserum demonstrated the presence of immunoglobulin G (IgG), IgM, C3, fibrinogen, and an alphamacroglobulin. Parasite antigen was not detected. IgG and IgM from pooled cryoprecipitates failed to react with each other in Ouchterlony immunodiffusion gels after separation by column chromatography. The data suggest that the IgG and IgM are not specifically complexed with each other but may simply be aggregates of altered proteins.

Schistosomiasis is a chronic helminthic disease of humans and animals which in the absence of reinfection may persist for 10 to 12 years or perhaps for the lifetime of the host. The host inflammatory response to eggs produced by intravascular female worms is the basis for the pathophysiological changes which result in hepatosplenic schistosomal disease.

In view of the extensive endeavors to isolate a putative immune complex from human as well as experimental schistosomiasis (1, 5, 9, 14), the present study was initiated to isolate and characterize an abnormal serum protein(s) which spontaneously precipitated from sera of Schistosoma japonicum-infected rabbits after exposure to lower temperatures. The abnormal precipitation of serum proteins under a defined condition such as temperature is an indication of the presence of immune complexes or protein aggregates. Cryoglobulins are immunoglobulins which precipitate or gel spontaneously at lower temperatures and return to a soluble form upon elevation of the temperature. The cryoprecipitate may be composed of complexes of an immunoglobulin and its specific antibody, a complex of foreign protein or autologous tissue antigen and specific antibody, an immune complex and an antibody to the complex, or simply altered or aggregated proteins (2). The combinations of mixed cryoglobulins that have been reported are, in decreasing order of occurrence, immunoglobulin M (IgM)-IgG, IgM-IgG-IgA, IgA-IgG, and IgG-IgG. In all of these cryoglobulins, IgG is the antigen, and both monoclonal and polyclonal antiglobulin components have been detected. These mixed cryoglobulins are believed to have clinical significance as opposed to a cryoprecipitate containing a monoclonal immunoglobulin (2) and may become deposited in tissue and mediate injury at that site. Although none has been definitive, a number of investigations have correlated the presence of a cryoglobulin with the existence of glomerulonephritis (2, 6–8, 10–12). The cryoprecipitate in S. japonicum-infected rabbits offered a potentially valuable opportunity to obtain and characterize an abnormal serum protein which might mediate the glomerulonephritis which is a frequent sequela of chronic schistosomiasis japonica in rabbits (13).

Immunoochemical and physical chemical studies were performed on pooled cryoprecipitates which were collected from rabbits with 8-week-old infections initiated with 1,000 S. japonicum cercariae of the Philippine-Leyte strain. The animals exhibited average worm burdens of 200 adult worm pairs. A second group of 18 rabbits infected with 250 or 500 S. japonicum cercariae were bled from the ear artery to obtain sera for cryoprecipitate processing. These animals were used in a previously published study of immune-complex glomerulonephritis and amyloidosis in S. japonicum-infected rabbits (13). After the initial preinfection bleed, blood was collected at 2, 4, 6, 8, 11, 14, 17, 20, 23, 26, 29, and 32 weeks postinfection. To avoid spontaneous gel formation, blood was immediately defibrinated (4) at 37°C. The defibrinated serum was placed at 4°C for an interval of 1 week to obtain a flocculent precipitate which was dissolved in 0.01 M phosphate-buffered saline (PBS) (pH 7.8) by incubating for 1 h at 37°C. After centrifugation to remove any insoluble material, the supernatant

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was placed at 4°C for 1 week to permit reprecipitation of the cryoprecipitate. The purification cycle of solubilization at 37°C followed by precipitation at 4°C was repeated two more times.

Although trace amounts of cryoprecipitate were present in normal rabbits, there was qualitatively at least 20 times more precipitate in animals at 7 weeks after infection with 250 to 1,000 S. japonicum cercariae. Each infected animal exhibited some degree of cryoprecipitation, and these precipitates were observed throughout the 8-month infection. Although the initial formation of cryoprecipitate at 7 weeks postinfection correlated with rising serum levels of IgG, IgM, and C3, cryoprecipitates persisted at 8 months in spite of IgG and IgM returning to near normal levels (A. Robinson and R. M. Lewert, submitted for publication).

Cryoprecipitates from seven animals with 8-week infections were pooled and dissolved in 0.01 M PBS (pH 7.8). The cryoprecipitate pool was concentrated to 4.0 mg/ml in B15 Amicon concentrators (Amicon Corp., Lexington, Mass.) and reacted in Ouchterlony double-diffusion gels with undiluted goat anti-normal rabbit serum, goat anti-rabbit IgG, goat anti-rabbit IgM (Cappel Laboratories, Cochrane, Pa.), or sera from humans and rabbits with chronic S. japonicum infections for 48 h at room temperature in 1.0% agarose (Sigma Chemical Co., St. Louis, Mo.) gels buffered with 0.05 M veronal buffer (pH 8.2). Reaction of the precipitate with goat anti-normal rabbit serum demonstrated the presence of only two bands, which were identified as IgG and IgM by specific antisera. No parasite antigens could be detected.

Physical chemical analyses of individual and pooled cryoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out in a discontinuous system as described by Spear et al. (15). The main gel contained 9% acrylamide, and the stacking gel consisted of 6% acrylamide. Before electrophoresis, cryoprecipitates solubilized in 0.01 M PBS at 37°C were denatured by boiling for 2 min in a solution containing 0.15 M Tris-hydrochloride (pH 7.0), 6% sodium dodecyl sulfate, 15% β-mercaptoethanol, 0.003% bromphenol blue, and 30% sucrose. The solubilization mixture for the protein standards, rabbit IgG and IgM, contained 0.05 M Tris-hydrochloride (pH 7.0), 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.001% bromphenol blue, and 10% sucrose. Multiple 50-μg samples of pooled cryoprecipitates were separated by electrophoresis. IgG (Cappel Laboratories) (10 μg) and IgM (15 μg) were used as protein standards. The IgM was prepared by ammonium sulfate precipitation of normal rabbit sera followed by fractionation on DEAE-cellulose (Reeve Angel, Clifton, N.J.), Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.), and Sepharose 6B (Pharmacia) columns. Protein bands, identified as μ-heavy-chain, γ heavy-chain, and light-chain immunoglobulin molecules, were shown to be major components of the cryoprecipitates (Fig. 1). Subsequent electrophoresis of cryoprecipitates from individual rabbits showed banding patterns identical to that found with the pooled precipitates.

Three guinea pigs were immunized with an antigen emulsion containing equal parts of pooled cryoprecipitates (1 mg/ml) and complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). A total of 1 ml of the emulsion was injected intramuscularly and subcutaneously into each of the guinea pigs. The inoculation protocol was repeated 2 weeks later with incomplete Freund adjuvant (Difco). Normal rabbit serum or plasma was electrophoresed in 1% agarose (Sigma) with 0.05 M veronal buffer (pH 8.2) and reacted with either guinea pig anti-rabbit cryoprecipitate, goat anti-rabbit IgG, goat anti-rabbit IgM, goat anti-rabbit C3, or goat anti-rabbit fibrinogen (Cappel Laboratories) for 48 h at room temperature. Similarly, the cryoprecipitate was reacted with the antisera, whole S. japonicum worm antigens, and egg antigens in 1% agarose double-diffusion gels. The S. japonicum worm and egg extracts were obtained by homogenizing lyophilized worms or eggs in 0.85% saline containing merthiolate (1:10,000) and centrifuging at 1,000 × g. In addition to the previously identified IgG and IgM, the guinea pig cryoprecipitate contained two bands of IgG, which were designated as IgG1 and IgG2, and two bands of IgM, which were designated as IgM1 and IgM2. The bands of IgG1 and IgG2 were more intense than the bands of IgM1 and IgM2. The IgG1 and IgM1 bands were detected in the cryoprecipitate from all animals, while the IgG2 and IgM2 bands were detected in the cryoprecipitate from only two animals.
pigs mounted an antibody response to rabbit fibrinogen, alpha-macroglobulin, and C3, as determined by immunoelectrophoresis (Fig. 2). The fibrinogen and alpha-macroglobulin were only minor constituents of the cryoprecipitates as they were not detectable upon direct examination.

In view of the characteristics of other IgM-IgG mixed cryoglobulins, it seemed highly probable that the IgM would exhibit antoglobulin activity toward the IgG component (2). To achieve separation of the cryoprecipitate components, 10 mg of cryoprecipitate from seven infected rabbits was dissolved in 0.4 ml of 0.01 M PBS-1 M NaCl at pH 7.2 and eluted at room temperature on a Sepharose 6B (Pharmacia) column (0.9 by 95 cm). Six peaks were observed (Fig. 3). The first peak corresponds to the void volume and contained nondissociated cryoprecipitate with a molecular weight of greater than $4 \times 10^6$. Peaks I, II, III, IV, and V were each vacuum concentrated and dialyzed against 0.1 M PBS (pH 7.2) to a volume of 0.2 ml. Each peak was reacted with goat anti-rabbit IgG and goat anti-rabbit IgM (Cappel Laboratories) in Ouchterlony double-diffusion gels to determine which peaks contained IgM and IgG and whether those two components were successfully resolved from one another. Samples were applied to micro Ouchterlony templates placed on 0.7% agarose in 0.01 M PBS (pH 7.8), and the slides were incubated for 24 h at room temperature and then for 24 h at 4°C. Duplicate slides were incubated for 48 h at 4°C. Similarly, the separated cryoprecipitate IgM and IgG were reacted with each other and with other column fractions. Peak III contained molecules with a molecular weight of approximately $10^6$, and, therefore, IgM was eluted in this peak and was not contaminated with IgG as demonstrated by immunodiffusion gels. The IgG was located in peak V in the absence of any detectable IgM. None of these separated fractions cryoprecipitated upon incu-

![FIG. 2. Immunoelectrophoretic demonstration of IgG, IgM, C3, alpha-macroglobulin, and fibrinogen in the cryoprecipitate with specific guinea pig antiserum. (A) Normal rabbit serum (NRS) was electrophoresed before the addition of goat anti-rabbit IgG (GAR IgG) to the bottom trough and guinea pig anticyroprecipitate (GP-ANTI-CRYO) to the top trough. (B) NRS was electrophoresed before the addition of GAR IgM to the bottom trough and GP-ANTI-CRYO to the top trough. (C) NRS was electrophoresed before the addition of GAR C3 to the bottom trough and GP-ANTI-CRYO to the top trough. (D) Normal rabbit plasma (NRP) was electrophoresed before the addition of goat anti-rabbit fibrinogen (GAR FIB) to the bottom trough and GP-ANTI-CRYO to the bottom trough.]

![FIG. 3. Sepharose 6B chromatographic profile of pooled cryoprecipitates. O.D., Optical density.]

Vol. 39, 1983
NOTES 1479

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bation at 4°C. Ouchterlony immunodiffusion analysis failed to demonstrate any reactivity between the IgG and IgM cryoprecipitate fractions at either 4°C or room temperature. The IgM and IgG fractions also failed to interact in Ouchterlony immunodiffusions with any of the other Sepharose 6B column fractions. It is possible that the constituents of the cryoglobulin were in some manner altered during the course of separation and, therefore, would not reassociate. It has been reported that the antigenic specificities of the antigen components of mixed cryoglobulins may be more limited than is the case with other rheumatoid factors, since they tend to react preferentially with human IgG determinants exposed by antigen-antibody interaction or IgG aggregation (3). As a result of this limited specificity, antigen-antibody activity may not be demonstrable if the antigen-antibody interaction or IgG aggregation is altered. Additionally, the negative data might be due to concentration effects. However, the precipitates may simply be composed of altered or aggregated proteins, since no parasite antigen was detectable in the cryoprecipitate and the third component of complement was a minor constituent as demonstrated by the studies with guinea pig anti-cryoprecipitate antiserum. Further studies utilizing more sensitive antigen detection techniques need to be instituted to determine whether this is indeed the case, since this would be the first instance in which an IgM-IgG mixed cryoglobulin did not exhibit antigen antibody activity.

The majority of the evidence, albeit circumstantial, indicates that the cryoprecipitate may mediate the renal damage observed in rabbits infected with *S. japonicum* (13). First, the proteins found in the subendothelial deposits are identical to those found in the cryoprecipitate: IgM, IgG, and a low frequency of C3, with a notable absence of parasite antigen. Second, the proliferation of mesangial and endothelial cells in the glomerulus rather than an infiltration of polymorphonuclear leukocytes is consistent with the absence of complement fixation. At present, it is unknown whether the IgM and IgG components of the cryoglobulins from rabbits with schistosomiasis japonica are complexed or mere aggregates of altered immunoglobulins. If they are complexed, then the complex is such that it is usually incapable of fixing complement. On the other hand, if the components are simple aggregates of altered immunoglobulins, then this is the first report of a new type of complex capable of mediating a glomerular lesion which ultrastructurally resembles that seen in systemic lupus erythematosus.

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**LITERATURE CITED**