IMMUNOCYTOCHEMICAL STUDIES IN SCHISTOSOMIASIS

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Immunocytochemistry has recently been applied to the demonstration within liver tissue of both antigen [1,2] and gamma globulin,[3,4] the latter probably representing antibody. The nature of the antigen as well as the antibody target has not been established. It appeared intriguing, therefore, to utilize immunocytochemistry in the study of the distribution of a known antigen and of gamma globulin in the livers of mice with experimental schistosomiasis. This is a disorder in which the granulomatous nature and the presence of lymphocytes and plasma cells in the lesions suggest an immunologic component in the tissue reaction.[5]

Specific antibodies in the serums of patients and animals infected with *Schistosoma mansoni* have been demonstrated serologically.[6-9] They have been applied in different stages of parasitism; namely, in relation to infective cercariae, diecious adults, miracidia and ova. More recently a fluorescent antibody technique has been developed for the serodiagnosis of human schistosomiasis [10]; in this, isolated cercariae have been utilized. Little is known about the localization, fate and pathogenetic implications of the schistosomal antigens in the tissues of the host.

MATERIAL AND METHODS

White mice of both sexes (Webster strain), 18 in number and approximately 25 gm. in weight, were infected with *S. mansoni* (approximately 50 cercariae to each animal). The infected mice were obtained from Dr. H. Schalie, Ann Arbor, Michigan, and from Dr. A. Cheever, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Maryland. Four noninfected mice served as controls. The animals were sacrificed weekly from the second through the 18th week after infection. Small blocks of liver and spleen were rapidly frozen in a dry ice-isopentane mixture at $-70°$ C. and stored at $-30°$ C. Sections were cut at 5μ in a cryostat, dried in a vacuum at 5° C., fixed in dehydrated acetone for 10 minutes, washed 3 times for 5 minutes each with buffered saline solution, pH 7, and subsequently treated with a

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few drops of fluoresceinated serums or antiserums. They were then washed 3 times for 5 minutes, mounted in phosphate buffered glycerol and examined within 12 hours with a Zeiss fluorescence microscope, utilizing an Osram 200 light source, 2 BG-12 exciter filters and one OG 5 barrier filter. Super Anscochrome film, tungsten type, was used for photomicrography; black and white photographs were made from Anscochrome slides.

Anti-mouse gamma globulin was prepared in rabbits using as antigen gamma globulin obtained from mouse serum by precipitation with ammonium sulfate. This fraction was subsequently purified through a DEAE cellulose column.[11] The specificity of the antiserum was checked by the agar double diffusion technique.[13] The gamma globulin fraction of the antiserum was conjugated with fluorescein isothiocyanate (N.B.C.).[12] In addition, slides were treated with antiserum mixed with rhodaminated * bovine albumin [14] to decrease the nonspecific uptake of fluorescein. To avoid nonspecific fluorescence of eosinophils, the antiserum was absorbed with red bone marrow powder and sections were pretreated with normal mouse serum.

To study the localization of antigen in tissues, globulin from serums of 22 patients infected with *Schistosoma mansoni* and of 15 controls was isolated by ammonium sulfate precipitation and conjugated with fluorescein isothiocyanate. Fluoresceinated serums and antiserums were twice absorbed with acetone-extracted pork liver powder before use. In addition, for the indirect technique, cryostat sections of infected mouse liver were treated with serums from either humans or mice infected with *Schistosoma mansoni;* this was followed by fluoresceinated rabbit anti-human or anti-mouse gamma globulin. Anti-human gamma globulin antiserum was prepared in rabbits using as antigen gamma globulin (Cohn Fraction II, N.B.C.) purified through a DEAE cellulose column.[11]

To elute the antibodies from the antigen-antibody complexes,[15] unfixed cryostat sections were treated for 2 hours with pH 3.2 citrate-buffered saline, and for control with pH 7 phosphate buffer. Sections were subsequently treated with fluoresceinated rabbit anti-mouse gamma globulin. In a few instances sections were subsequently washed thoroughly and treated with fluoresceinated gamma globulin from patients with schistosomiasis to demonstrate schistosoma antigen.

Duplicate cryostat sections were also stained with hematoxylin and eosin. In addition, paraffin sections of liver and spleen were stained with hematoxylin and eosin, Mallory's aniline blue, the periodic acid-Schiff (PAS) procedure after digestion with diastase, and silver impregnation according to Gomori.

Results

The liver of mice exhibited granulomas with or without schistosoma ova. The latter showed various stages of degeneration and a shell with bright brown-yellow autofluorescence. The material in the schistosoma ova gave a dense PAS reaction. In later stages of infection a crescent-shaped necrotic area which also gave a bright PAS reaction (Fig. 1) appeared within the granuloma. In sections treated with fluoresceinated gamma globulin from patients infected with *Schistosoma mansoni* (Table I), structures within the schistosoma ova (Fig. 2) (mature or immature, preserved or disintegrating miracidia) gave a strong apple-green fluorescence. This was also evident in adult worms (Fig. 3) within intrahepatic portal vein branches and in necrotic areas around the ova.

* Lissamine rhodamine B was donated by A. Hoffman and Co., Providence, R.I.

Sometimes a multilobulated mass in an ovum, suggesting penetration glands of the miracidium, was the only structure having strong fluorescence. Specific fluorescence also appeared in the cytoplasm of many macrophages (Fig. 4) and occasionally was noted extracellularly in the granulomas. Specific fluorescence was not observed in the spleen of infected mice treated with fluoresceinated serums from patients with schistosomiasis. Infected livers treated with fluoresceinated gamma

TABLE I

IMMUNOCYTOCHEMICAL OBSERVATIONS IN MICE INFECTED WITH *Schistosoma mansoni*

	Weeks after infection			
	0	2–4–6	8–10–12	14–16–18
	Number of animals			
	4	6	6	6
Binding with rabbit anti-mouse gamma globulin				
Liver				
Necrotic zone	−	−	±	+
Basophilic cells in granuloma	−	±	+	−
Basophilic cells in sinusoids	−	±	+	+
Spleen				
Basophilic cells in pulp	±	+	+	+
Binding with gamma globulin of patients with schistosomiasis				
Schistosomal granuloma				
Worm	−	+	+	+
Ovum	−	+	+	+
Macrophages	−	±	+	+
Extracellular	−	±	+	+

globulin from serums of hospital patients without schistosomiasis showed no binding (Fig. 5). This was also the case when the indirect technique was utilized.

Cells containing gamma globulin were encountered in 3 locations in infected mice: (a) in hepatic schistosomal granulomas; (b) in littoral cells of hepatic sinusoids; and (c) in the red pulp of the spleen. In earlier stages of infection, cells with specific fluorescence were noted at the periphery of occasional granulomas (Fig. 6). After 8 weeks few fluorescent cells lay within the granulomas. Later, when the granulomas became partially or wholly fibrotic, fluorescent cells were absent but the connective tissue showed diffuse fluorescence, much less intense in control sections. In the hepatic sinusoids (Fig. 7) and in the red pulp of the spleen (Fig. 8) cells containing gamma globulin increased in number progressively with duration of the infection. This coincided with a progressive increase in weight and consistency of the spleen and with reticuloendothelial hyperplasia, plasmocytosis, chronic passive conges-

tion, lymphoid atrophy and increase in collagen and reticulin fibers. Yellow autofluorescence in the spleen, caused by lipofuscin pigment, was increased in later stages.

In conventional sections the gamma globulin-containing cells lining the hepatic sinusoids in the position of Kupffer cells and in the red pulp of the spleen had round or oval configuration, abundant basophilic cytoplasm and round, occasionally eccentric nuclei with fine chromatin and nucleoli. In addition, the spleen contained many mature plasma cells. However, not all cells with the appearance described in conventional sections contained gamma globulin.

The necrotic portions of granulomas exhibited a specific fluorescence when treated with fluorescein-labeled anti-mouse gamma globulin. This was almost abolished when the sections were exposed to a citrate buffer at pH 3.2. Specific fluorescence could be restored when the sections were re-treated with fluoresceinated gamma globulin from patients with schistosomiasis (Fig. 9).

Discussion

Serums and gamma globulin from patients with schistosomiasis showed binding to structures within the adult schistosoma and its ova as well as to material in granulomas outside the ova. This observation indicates that recent serodiagnostic findings based on the use of isolated cercariae[10] also apply if liver tissue is used. The reaction with liver tissue may be an aid in the serodiagnosis of schistosomiasis if unknown serum is tested with tissue infected with *Schistosoma mansoni*. The relation of the state of the disease to the serum reactivity requires further investigation. The same reaction can be applied in the histologic diagnosis of schistosomiasis since with known serums, antigenic products of schistosoma may be demonstrated, either extracellularly or within macrophages, in the absence of recognizable ova in the granulomas. The method may be useful in determining the etiology of nonspecific granulomas in liver biopsy specimens from patients suspected of infection with *Schistosoma mansoni*.

The extracellular substance in the necrotic portions of granulomas seemed to be bound to antibodies, since upon treatment with acid pH its reactivity was abolished, probably by splitting the antigen-antibody complexes. A strong fluorescence reappeared when the sections were treated with fluoresceinated anti-schistosoma antibodies, indicating that the PAS-positive diastase-resistant antigen remained in place. These observations and the prevention of such necrosis by ACTH treatment[16] provide support for the immunologic nature of the tissue reaction in schistosomiasis. The antigenic character of the PAS-positive substance around schistosoma ova has been suggested previously,[5] and in other

liver disorders PAS-positive antigenic material has been demonstrated in bile.[1] The necrosis about the ova is apparently a response to antigen-antibody complexes rather than to free antigen; the solubility experiment suggests that an antibody is bound to an antigen in this location. Others have demonstrated the tissue damaging effects of antigen-antibody complexes.[17]

Granulomas frequently represent immune responses in tissue, and hepatic granulomas occur in a variety of conditions conventionally considered to be hyperergic in nature.[18] The demonstration of antigen in the reactive site supports this concept. Moreover, the detection in the cells of schistosoma granulomas of gamma globulin similar to that seen about granulomas induced in other conditions[19] also points to an immunologic process. The presence of gamma globulin similar to that in granulomas in basophilic Kupffer cells may also indicate hypersensitivity. These cells seem to show transformation to plasma cells despite their littoral position. This has also been observed in examples of human cirrhosis.[3] The basophilic cytoplasm in cells containing gamma globulin in the liver and spleen suggest local formation rather than phagocytosis of protein as indicated previously.[3]

Splenic enlargement appears in mice with schistosomiasis very early in the course of the infection. This seems to be not only the result of portal hypertension but also of reticuloendothelial hyperplasia, presumably on an immunologic basis in view of the many gamma globulin-containing cells in the red pulp. Occasionally, in an otherwise typical "pipestem fibrosis" of hepatic schistosomiasis,[20] fine but long connective tissue septums are noted; these resemble the pattern seen in inactive postnecrotic cirrhosis.[21] Recently studied cases of human schistosomiasis have shown accumulations of plasma cells and lymphocytes, presently designated as "immunological competent cells."[22] These are similar to the collections seen in postnecrotic cirrhosis or chronic active hepatitis[23] and might conceivably indicate an immunologic basis for the self-perpetuating hepatic process in schistosomiasis. Self perpetuation in typical postnecrotic cirrhosis, particularly that of unknown etiology, has been related hypothetically to an immunologic process.[24–26] Demonstration of an established antigen and of gamma globulin formation in schistosomiasis might represent a useful model for studies of other types of cirrhosis in which the mechanism of self perpetuation has not yet been established.

Summary

The gamma globulin in mesenchymal cells in the liver and spleen and hepatic granulomas of mice infected with *Schistosoma mansoni* may be seen by immunocytochemical methods to increase in proportion to the

development of the granulomas. Serums from patients with schistosomiasis were observed to bind antigenic substances in adult worms and ova as well as material derived from them.

The usefulness of a serodiagnostic method based on this principle and its value in the histologic distinction of granulomas is apparent. The antigen in necrotic portions of the granulomas appeared to be bound to antibodies. The visualization of gamma globulin and antigen throws light on the pathogenesis of the hepatic lesions in schistosomiasis, exemplifies the role of antigen-antibody complexes in granuloma formation and provides a model for immunocytochemical studies of other hepatic disorders.

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[*Illustrations follow*]

Legends for Figures

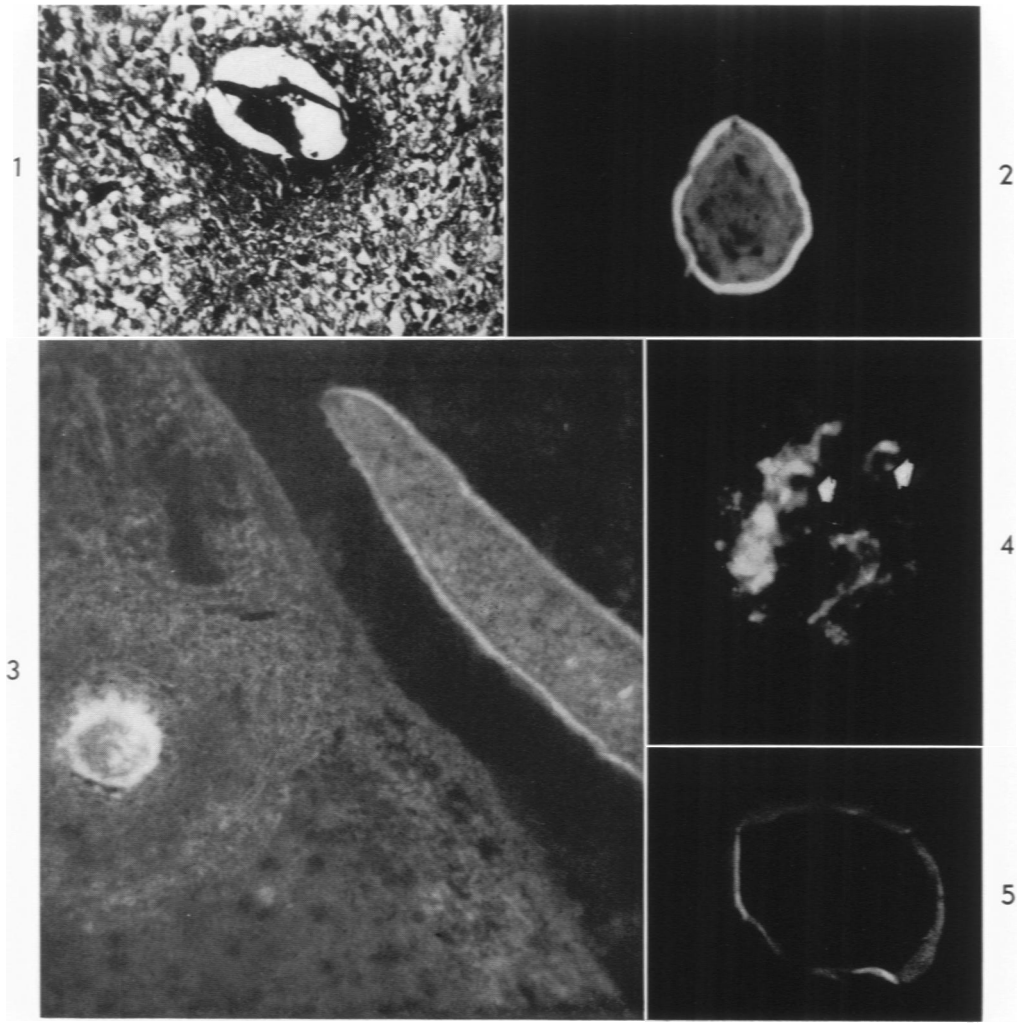
FIG. 1. Hepatic schistosomal granuloma. Note a crescent-shaped PAS-positive necrotic zone about an ovum. Periodic acid-Schiff stain. × 300.

FIG. 2. An ovum in the liver of a mouse infected with *Schistosoma mansoni*. Specific fluorescence is manifest when the section is treated with fluorescein-labeled globulin from a patient with schistosomiasis. × 400.

FIG. 3. Liver of a mouse infected with *S. mansoni*. The section has been treated with fluorescein-labeled globulin from a patient with schistosomiasis. The adult worm, the ovum and a necrotic zone about the ovum show specific fluorescence. × 120.

FIG. 4. Liver of a mouse infected with *S. mansoni*. The section has been treated with fluorescein-labeled globulin from a patient with schistosomiasis. The ovum is not present in the granuloma. Specific fluorescence is seen extracellularly and in macrophages. × 400.

FIG. 5. Ovum in the liver of a mouse with schistosomiasis. There is no specific fluorescence when the section is treated with fluoresceinated globulin from a patient without schistosomiasis. The shell of the ovum shows brown-yellow autofluorescence. × 400.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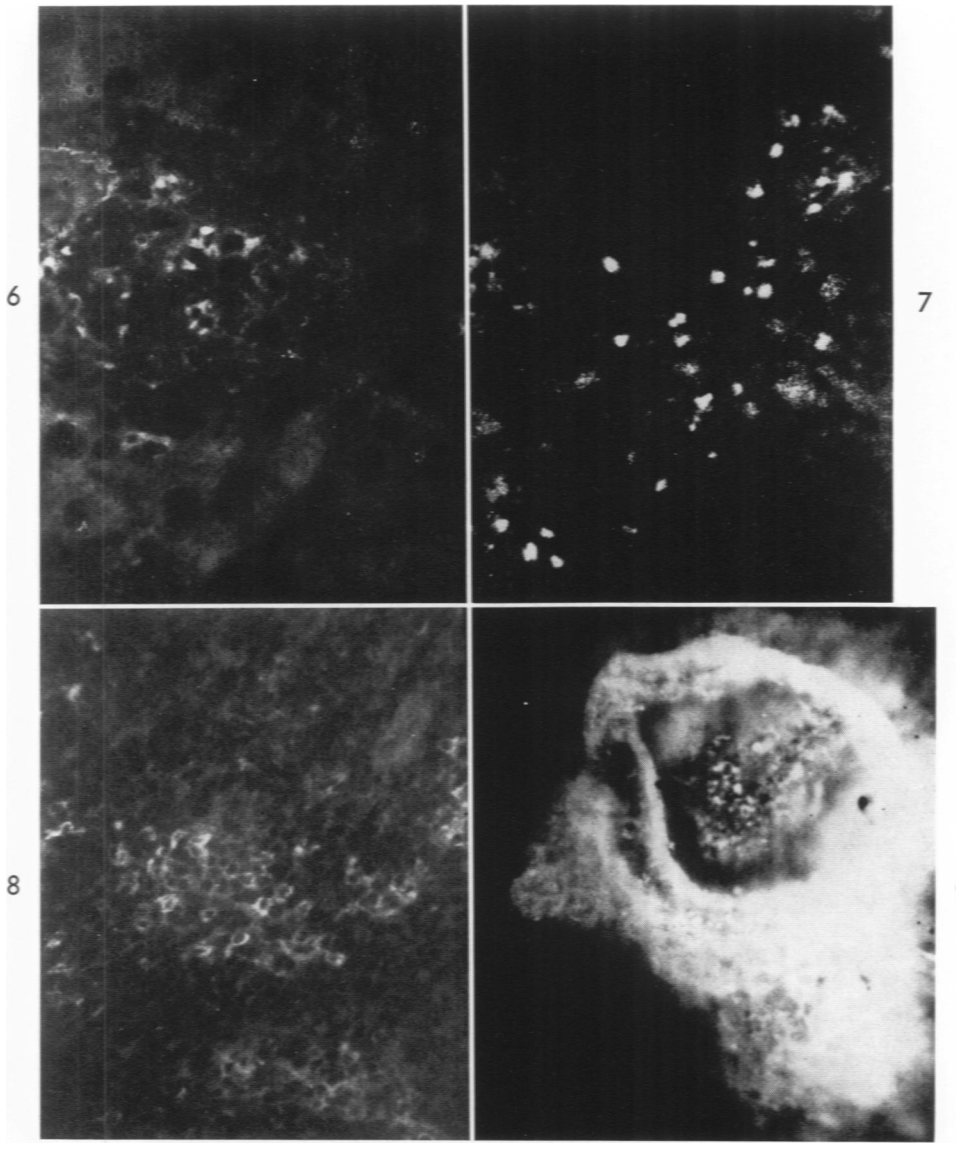

FIG. 6. A schistosomal granuloma in a mouse with infection of 8 weeks' standing. The section has been treated with fluorescein-labeled anti-mouse gamma globulin. A few gamma globulin-containing cells appear within the granuloma and at its periphery. × 400.

FIG. 7. A section from the liver shown in Figure 1. Many littoral cells contain gamma globulin in proximity to a schistosomal granuloma. × 250.

FIG. 8. Spleen of a mouse 8 weeks after infection with *S. mansoni*. Section has been treated with fluoresceinated anti-mouse gamma globulin. Many gamma globulin-containing cells appear in the pulp. × 250.

FIG. 9. Liver of a mouse 16 weeks after infection with *S. mansoni*. The section has been treated with citrate buffer (pH 3.2) followed first by fluoresceinated anti-mouse gamma globulin and then by fluoresceinated serum gamma globulin from patients with schistosomiasis. Only the latter stained the necrotic area around the ovum. × 400.