IgM Antibodies in Fish Mucus[1] (35443)

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We have reported that serum antibodies of the gar, *Lepisosteus platyrhincus*, a fresh water holostean fish, are restricted to the macroglobulin fraction (1). In studies to be reported we will present evidence that this species forms only one class of immunoglobulin, *i.e.*, IgM (Bradshaw, C. M., Clem, L. W., and Sigel, M. M., to be published). In more recent work we have detected IgM antibody in mucus secretions of the gar. These findings are presented in the present paper.

Materials and Methods. Animals. The collection, maintenance and bleeding of the gar, *Lepisosteus platyrhincus*, were as described previously (1).

Fish were immunized with 1 ml of sheep erythrocytes (ShE) prepared in 0.15 *M* phosphate buffered saline (PBS), pH 7.2, and standardized spectrophotometrically to 1×10^9 cells/ml, administered intramuscularly into two sites on either side of the backbone.

Mucus preparation. Mucus was collected by wiping seven fish with filter paper. 150 ml of Tris–HCl buffer, 0.14 *M* NaCl, pH 7.4, was added and allowed to stand overnight at 4°. The material was then centrifuged at 1500 rpm for 20 min and the supernatant was concentrated by positive pressure dialysis (1 atm of pressure) at 4°.

Antibody assay. Antibodies were detected by agglutination of ShE or other erythrocytes using the microtiter assay system (Cooke Eng. Co., Alexandria, Va.). The standard diluent for all antibody assays was PBS. Erythrocytes were washed three times with PBS and standardized spectrophotometrically to 1×10^8 cells/ml for titration. Serum or mucus samples were serially diluted in a twofold step using 0.25 ml of PBS containing 0.1% gelatin.

Assay of antibody to *S. typhosa* H antigen was performed as described previously (1).

Immunochemical procedures. Sephadex G-200 gel filtration, reduction of antibody with 2-mercaptoethanol (2-ME), preparation of rabbit antisera to gar proteins, Ouchterlony, and immunoelectrophoresis were all conducted as described previously for the lemon shark (2).

Results. Pools of normal and immune gar mucus and corresponding pools of serum were assayed for antibody activity to a variety of antigens at three different temperatures, 37°, 20°, and 4°. The assay was performed at these temperatures because we have previously reported that normal gar serum contains cold agglutinin activity (3). The results in Table I illustrate several interesting points. Normal mucus contains hemagglutinating activity and this activity was augmented by immunization. The activity in the mucus differs from that in the serum in two respects: (i) It was not enhanced by a temperature downshift to 4° although, like in serum, it was increased by the downshift from 35 to 20°. (ii) No agglutinins for *Salmonella typhosa* H antigen were detected in mucus. However, we have not quantified the amount of immunoglobulin present in mucus.

Pools of normal and immune mucus were treated with 2-mercaptoethanol (2-ME) and iodoacetamide to determine whether this antibody activity was sensitive to reduction. The antibody activity in the mucus was totally susceptible to reduction with 2-ME. Furthermore, by the use of rabbit anti-gar IgM (RAGIgM) serum which removed all hemagglutinating activity from mucus, the antibody was found to be associated with a

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TABLE I. Effect of Assay Temperature on Titer of Natural and Immune[a] Agglutinins in Gar Mucus and Serum.[b]

Material	Antigen	Temp of assay (°) 35	20	4
Mucus				
Normal	ShE	2	16	8
	Guinea Pig E	Neg[c]	4	4
	Pigeon E	Neg	Neg	Neg
	S. typhosa H	Neg	Neg	Neg
Immune	ShE	16	64	64
	Guinea Pig E	4	32	32
	Pigeon E	Neg	2	2
	S. typhosa H	Neg	Neg	Neg
Serum				
Normal	ShE	32	16	64
	Guinea Pig E	4	8	32
	Pigeon E	4	8	32
	S. typhosa H	4	8	64
Immune	ShE	32	256	2048
	Guinea Pig E	4	32	128
	Pigeon E	4	8	32
	S. typhosa H	4	8	64

[a] Immunized with 1×10^9 ShE.

[b] Antibody titers expressed as reciprocal of dilution.

[c] Negative at 1:2.

protein possessing gar IgM antigenic determinants.

To investigate whether the antibody molecules in mucus were of the same molecular size as the purified macroglobulin from serum, normal and immune mucus pools were applied to Sephadex G-200. The fractionation of normal and immune mucus gave identical results. The first peak contained hemagglutinating antibody to ShE, and represented 70% of the recovered optical density (Fig. 1, normal gar mucus). Two additional small peaks were detected but did not contain detectable hemagglutinating activity. The second peak corresponds in position to the region where 7S immunoglobulin elutes. The three peaks were separately concentrated by pressure dialysis and analyzed by the Ouchterlony reaction. Figure 2 shows normal and immune gar mucus in wells 1 and 2, respectively, the void volume fraction in 3 and 6, the "7S" fraction in 4, and rabbit

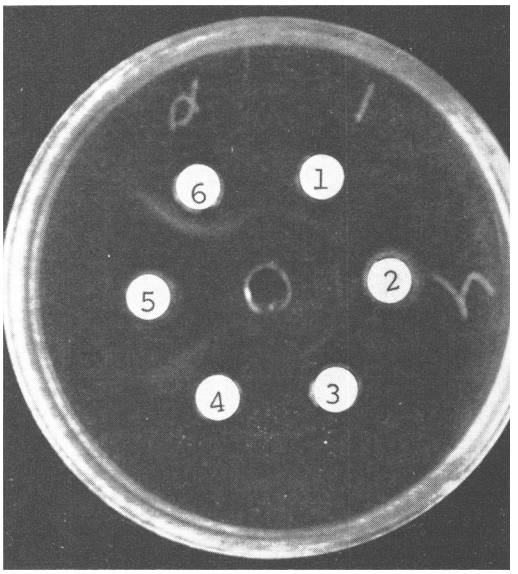

FIG. 1. Sephadex G-200 gel filtration of normal gar mucus. Fractions were assayed for hemagglutination activity of ShE.

antigar whole serum (RAGWS) in 5. The center well contains RAGIgM. The void volume fraction formed a single line of identity with normal and immune gar mucus. RAGIgM did not precipitate the protein in

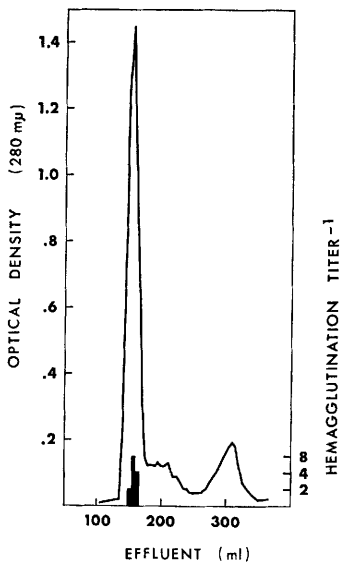

FIG. 2. Ouchterlony analysis of normal gar mucus fractionated by Sephadex G-200: (1) normal gar mucus; (2) immune gar mucus; (3 and 6) void volume fraction; (4) the "7S" fraction; and (5) RAGWS. The center well contains RAGIgM.

the 7S region, although this fraction contained two proteins when reacted with RAGWS. The third peak also was devoid of any reactivity with RAGIgM. These findings were confirmed by Ouchterlony analysis with rabbit antigar IgM heavy and light chain antisera. Thus the large size protein of mucus had the antigenic specificity of IgM.

Discussion and Summary. As early as 1935, Nigrelli (4) observed that the mucus of certain fishes was toxic to parasites and suggested that this activity might be due to antibody or that the mucus itself might be toxic to parasites.

Our studies have indicated that normal gar mucus contains detectable antibody to several erythrocytes. Other work in this laboratory (Bradshaw, C. M., and Sigel, M. M., unpublished work) with mucus of normal snapper, *Lutjaneus griseus,* and normal bowfin, *Amia calva,* also demonstrated detectable antibody activity in surface mucus. After primary immunization of gar with SHE their surface mucus antibody levels increases considerably. Lower titers of hemagglutinin were recently reported by Fletcher and Grant in the mucus of plaice (5).

We have previously reported that normal gar serum contains cold agglutinins with maximum reactivity at 4°. Interestingly, the normal and immune mucus antibody titers were increased by downshifting the temperature from 35 to 20° but no further enhancement was noted at 4°.

The hemagglutinin in gar mucus has several properties of macroglobulin antibody. It is located in the excluded fraction from Sephadex G-200, its activity is removed by absorption with RAGIgM and it is totally susceptible to reduction. The absence of 7S immunoglobulin is consistent with our findings (Bradshaw, C. M. and Sigel, M. M., to be published) on the absence of 7S IgM in gar serum.

Fletcher and Grant (5) found that the antibody present in plaice serum and mucus was not retained by Sephadex G-200, and had similar carbohydrate and amino acid compositions. Although they did not determine the S value of the mucus antibody, the serum antibody had an $S_{20,w}$ of 12.4. The provisional conclusion based on our investigation and on the findings of Fletcher and Grant (5) is that IgM of fish occurs as a macroglobulin secretory protein. Its presence in mucus has an obvious survival value for lower vertebrates.

Beyond their phylogenetic implications these findings provide a new approach for the study of local synthesis and excretion of IgM immunoglobulin relevant to several problems of mammalian immune mechanisms. It has already been demonstrated that IgM is present in human (6) and porcine intestinal secretions (7, 8). Brandztaeg *et al.* (9) reported that parotid secretions from patients deficient in IgA contained IgM. Moreover, Eidelman and Davis (10) demonstrated IgM in intestinal mucosal plasma cells of children with ataxia telangiectasia.

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