IDENTIFICATION OF SECRETORY COMPONENT AS AN IgA RECEPTOR ON RAT HEPATOCYTES*

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Polymeric IgA is transported from blood to bile by the rat liver (1, 2) in a manner which may be analogous to the transport of polymeric IgA across the mucosal epithelium. Such transport is thought to be mediated by secretory component (SC) an epithelial cell glycoprotein which acts as a receptor for polymeric IgA on these cells (3–8).

Fisher et al. (9) recently reported that Ig transport by the rat liver is selective for polymeric IgA and that secretory component is involved in this process because prior addition of SC to polymeric IgA reduced its transport. Moreover, although IgG is not transported, IgG which could bind SC (i.e., anti-SC antibodies) was transported by the liver (9).

Recently, Birbeck et al. reported that transport of polymeric IgA by the rat liver proceeds through hepatic parenchymal cells (10). Thus, if SC is involved in the IgA receptor which mediates transport, it should be expressed on rat hepatocytes.

The experiments presented in this paper indicate that SC is synthesized by rat hepatocytes and is involved in mediating the binding of polymeric IgA to these cells.

Materials and Methods

Isolation of Hepatocytes. Rat hepatocytes were prepared as previously described (11, 12). Briefly, the portal vein of 190-g Wistar rats was cannulated and the liver perfused with balanced Hanks' solution containing collagenase. The perfused liver was dissected from the abdomen, cut into pieces, and transferred to the same collagenase-containing medium where the cells were dissociated by stirring for 10 min. The resulting cell suspension was filtered through surgical cotton gauze and the hepatocytes were isolated by sedimentation at 1 g as previously described (12). This method permitted the harvesting of a cell population that was 99% viable (as determined by trypan blue exclusion), consisting of >95% hepatocytes as judged by electron microscopy (kindly performed by Dr. M. J. Phillips, University of Toronto).

Culture of Hepatocytes. Rat hepatocytes were maintained in spinner flasks at a concentration of 1–2 × 10⁶ cells/ml of Waymouth's MB 752/1 medium (Grand Island Biological Co., Grand Island, N. Y.), containing 17.5% (vol/vol) heat-inactivated horse serum, insulin (0.5 IU/ml, Connaught Medical Research Laboratory, Willowdale, Ontario), and gentamycin (50 μg/ml, Schering Corp., Kenilworth, N. J.). The pH and oxygen content were continuously monitored and the cells could be kept viable (≥65%) for up to 2 d as previously described (12). Cell viability was determined by taking a sample (0.1 ml) of cell suspension and mixing with 0.05 ml of trypan blue in 0.9% NaCl, and counting the cells in a Neubauer chamber. Four 1-mm² areas were counted.

* Supported by grants from the Medical Research Council of Canada.

Abbreviations used in this paper: BSA, bovine serum albumin; PBS, phosphate-buffered saline; SC, secretory component.
Isolation of Intestinal Epithelial Cells, Peripheral Blood Lymphocytes, Spleen Cells, and Erythrocytes. Suspensions of epithelial cells were obtained from the jejunum-ileum portion of the rat small intestine by the method of Levine and Weintraub (13). When incubated in RPMI-1640 (Grand Island Biological Co.) at a concentration of 10^6 cells/ml, the isolated epithelial cells were up to 85% viable for 3 h.

Rat peripheral blood lymphocytes were isolated from heparin-treated whole blood by centrifugation on a Ficoll-Hypaque density gradient (density = 1.077 g/ml). Cells were incubated at a concentration of 10^6 lymphocytes/ml in RPMI-1640.

Rat spleen cells were obtained by cutting the spleen into small pieces, mincing in McCoy's medium (Grand Island Biological Co.), and filtering through a steel gauze to obtain a single cell suspension. They were centrifuged and the cell pellet was washed twice with phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.3).

Rat erythrocytes were collected into tubes containing heparin (10 U/ml of blood) and washed three times with PBS.

Proteins. Rat monoclonal proteins were obtained from the serum of rats bearing immunocytomas (14). Rat IgA monoclonal protein (IR22) was isolated from serum by methods previously described by Fisher et al. (9).

Rat SC was isolated by affinity chromatography employing human polymeric immunoglobulins coupled to Sepharose 4B as the affinity immunosorbent, as described by Underdown and Socken (15). The rat SC was able to competitively inhibit the binding of human 125I-SC to either human or rat polymeric IgA in vitro.

Antisera to rat IgA, IgM, IgG, albumin, and whole serum were purchased from Miles Laboratories Inc., (Miles Research Products, Elkhart, Ind.) or prepared in our own laboratory by multiple immunizations of rabbits with purified monoclonal proteins, as previously described (9). Antiserum specific for rat SC was prepared as previously reported (15). The anti-rat SC antiserum gave a pattern of identity when purified rat SC and secretory IgA were tested in adjacent wells by immunodiffusion. No reactions were observed when the anti-SC antiserum was tested against rat serum or rat myeloma proteins. Specific rabbit antibodies to rat SC were isolated and purified by affinity chromatography as previously described (9).

Purified polymeric rat IgA and rat SC were labeled by the iodine monochloride method as previously described (5). The specific activity of 125I-proteins was calculated in counts per minute per micromole by the measurement of radioactivity in a gamma counter and absorbance at 278 nm. The elution volumes of 125I-proteins were identical to the unlabeled proteins when filtered on Sepharose 6B and Sephadex G-200 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) in PBS. Iodinated proteins were stored at 4°C.

Determination of Rat SC Concentration. Rat SC was measured by a double-antibody radioimmunoassay technique according to Gleich et al. (16). Rabbit antiserum to rat SC (100 μl in a 1% solution diluted in PBS) was added to 200 μl of inhibitor (either bovine serum albumin [BSA]/PBS, SC standard solutions in BSA/PBS, or samples of hepatocyte-containing medium). Preliminary experiments were carried out to determine the dilution of anti-rat SC that, in the absence of inhibitor, would bind 60 or 70% of the radiolabeled rat SC subsequently added. The mixture was incubated for 1 h at room temperature after which 50 μl of rat 125I-SC was added (0.1 μg containing 1.0 × 10^4 cpm).

The resulting mixture was incubated for a further hour at room temperature and then 200 μl of sheep antiserum to rabbit IgG was added, which was previously determined to be in antibody excess by quantitative precipitation analysis. Precipitates formed after incubation at 4°C for 18 h, and were washed three times in cold PBS, isolated by centrifugation (1,200 g), and counted in a gamma counter. In the absence of inhibitor, 68% of the rat 125I-SC was precipitated by the addition of anti-rat SC, whereas only 6% was precipitated in the presence of 100 μl of normal rabbit serum.

SC Synthesis by Hepatocytes. The method used was similar to that previously reported for albumin (11, 12). 3-ml aliquots of a suspension of cultured hepatocytes were taken at several times after the initiation of culture and ultrasonicated (model W140D, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). The sample was then used as an inhibitor in the double-antibody assay for SC as described above. Alternatively, in some cases only the incubation supernate was assayed.

Secretory component associated with the surface of various cell types was also analyzed by
the double-antibody radioimmunoassay. Cells ($10^6$-$10^8$ cells in 150 µl of medium) were added to 100 µl of rabbit anti-rat SC and the mixture was incubated at 4°C for 1 h, after which the cells were removed by centrifugation. The supernatant was then added to rat $^{125}$I-SC, followed by sheep anti-rabbit IgG as described in the standard double-antibody assay. The quantity of SC associated with each cell type was estimated by comparing the degree of inhibition obtained with each cell type to that of soluble secretory component.

Binding of IgA to Hepatocytes. To study the binding of rat $^{125}$I-IgA to hepatocytes, cells were taken from spinner flasks, centrifuged at 150 g, and washed in BSA/PBS three times. The cells were resuspended and filtered through glass wool to remove aggregates. Varying quantities (0.1-10 µg) of radiolabeled rat IgA were added to 3 x $10^5$ hepatocytes in a final vol of 200 µl of 30 mg BSA/ml PBS, in the presence or absence of 1.0 mg of human IgA dimer to detect specific binding. (Our previous studies indicated that human IgA dimer could block the biliary transport of rat IgA [9].) The mixture was incubated for 30 min at 22°C. Cells were centrifuged (150 g) and washed three times in PBS and the quantity of radioactivity bound in the presence or absence of unlabeled protein was then determined. The ability of various proteins to inhibit the binding of rat $^{125}$I-IgA to hepatocytes was also studied. In the inhibition experiments, the quantity of rat $^{125}$I-IgA used was sufficient to saturate 16% or alternatively 100% of the IgA-binding sites.

Immunofluorescence. The IgG fraction of sheep antiserum to rabbit IgG was purified by fractionation on DEAE-cellulose using 0.05 M Tris-HCl, 0.01 M NaCl, pH 8.0. The purified sheep IgG (anti-rabbit IgG) was conjugated with rhodamine (Lissamine rhodamine B, RB 200 sodium salt, lot 27285-A, ICN K & K Laboratories, Inc., Plainview, N. Y.) by the method of Nairn et al. (17). The rhodamine conjugate was gel filtered over a Sephadex G-25 column equilibrated with PBS to remove free dye. An adsorption step using mouse liver powder (N. L. Cappel Laboratories, Inc., Cochrantonville, Pa.) at a final concentration of 50 mg/ml of conjugate (stirred for 30 min at room temperature and then centrifuged at 12,000 g for 5 min) was used to decrease diffuse background staining. The molar F/P ratio was 2.5.

To demonstrate SC on the surface of hepatocytes, cells were incubated with either purified rabbit IgG anti-rat SC antibodies (50 µg/10⁵ cells in 100 µl of 30 mg BSA/PBS) or an equal weight of rabbit IgG for 20 min at 22°C. The cells were washed in BSA/PBS three times and incubated with rhodamine-sheep anti-rabbit IgG for 20 min at 22°C. The cells were washed three times and placed on a microscope slide to air dry. Buffered glycerin (90% glycerin in PBS) was added to the slide and a cover slip placed on the slide and the cells examined with a Zeiss epi-fluorescent microscope (Carl Zeiss, Inc., New York).

Results

Synthesis of Rat SC by Hepatocytes. Radioimmunoassay of sonicated culture fluid containing hepatocytes demonstrated synthesis of SC over a 48-h period. Synthesis was calculated by subtracting the SC contained in the sonicated samples at the initiation of culture from that present at the time points shown (Fig. 1). No significant difference between the sonicated samples of cells plus culture fluid and culture fluid alone was detected, which indicated that the majority of SC measured under these conditions was secreted.

To confirm that the double-antibody assay was specifically determining SC, the culture medium was gel filtered on Sephadex G-200 in PBS and the effluent analyzed for SC activity. It was revealed that the SC activity appeared in two fractions, one corresponding to free rat SC and the other at the void volume consistent with SC bound to polymeric horse immunoglobulins.

When 0.5 µg of Actinomycin D per milliliter of medium (Sigma Chemical Co., St. Louis, Mo.) was added after cells had been incubated for 12 h, synthesis and secretion of SC ceased (Fig. 1). This further suggested that the presence of SC represented de novo synthesis and not release of SC which had been taken up by hepatocytes in vivo.
Fig. 1. Demonstration of the synthesis of SC by hepatocytes over a 48-h period. When a metabolic inhibitor, Actinomycin D, was added after the cells had been incubated for 12 h, the synthesis and secretion of SC ceased.

Hepatocyte-associated SC Determined by Radiolabeled and Rhodamine-labeled Antibodies. Because hepatocytes synthesized SC, we turned our attention to the possibility that SC might be present on the surface of hepatocytes. Preliminary experiments indicated that $^{125}$I-anti-rat SC antibodies were capable of binding to washed hepatocytes. Moreover, the binding of $^{125}$I-anti-SC was specifically inhibited by unlabeled specifically purified rabbit IgG anti-rat SC antibodies but not by normal rabbit IgG (Fig. 2). Although 30 µg of specifically purified rabbit IgG anti-rat SC antibodies caused 50% inhibition, as much as 10 mg of normal rabbit IgG had little effect (Fig. 2).

Immunofluorescent staining was also employed to demonstrate SC on the surface of hepatocytes. The results observed when live hepatocytes were stained are shown in Fig. 3. A variety of patterns were observed including diffuse membrane staining and patching and/or capping (Fig. 3).

In different experiments, between 25 and 50% of cells were stained with anti-SC whereas <5% of cells treated with normal rabbit IgG were positive. The results shown in Fig. 3 were obtained when cells were stained at room temperature. In separate experiments in which cells were stained at 2°C, no caps were observed. A detailed study on the movement of SC within the hepatocyte membrane will be reported elsewhere.

Detection of SC Molecules Associated with Hepatocytes by Radioimmunoassay. SC molecules
on the surface of hepatocytes were detected by using washed cells as inhibitors in the double-antibody radioimmunoassay for SC. The results are shown in Table I. Between 1.4 and 3.4 × 10⁵ molecules of rat SC per hepatocyte were calculated. This number was similar to that found for rat jejunum-ileum epithelial cells, which had between 1.0 and 2.0 × 10⁵ molecules of SC per cell. In contrast, the number of SC molecules was found to be <5.0 × 10⁵ per cell in peripheral blood lymphocytes, unfractionated spleen cells, and rat erythrocytes. Experiments were carried out to exclude the possibility that the calculated number of SC molecules per cell was influenced by secretion of SC during the 1-h incubation period with the anti-SC antiserum. Cells were incubated in BSA/PBS for 1 h and the supernate assayed for rat SC. Under these conditions insufficient SC was secreted to interfere with the determination of membrane-bound SC.

Role of SC in Binding of Rat ¹²⁵I-IgA to Hepatocytes. Our previous studies (9) indicated that the transport of IgA by the rat liver may proceed by a secretory component-mediated mechanism similar to that proposed for the transport of IgA across the mucosal epithelium. Therefore, it was of interest to determine whether rat IgA could bind to isolated rat hepatocytes and if such binding was mediated by hepatocyte-associated SC.

The binding of rat ¹²⁵I-IgA was studied in competitive inhibition experiments in which ¹²⁵-IgA was added to hepatocytes in the presence or absence of various unlabeled proteins (Fig. 4). Such competitive inhibition experiments were carried out at two different doses of rat ¹²⁵I-IgA added, corresponding to ~16 and 100% of the IgA-binding sites saturated, with similar results. A representative experiment is shown in Fig. 4. Unlabeled rat dimeric IgA consistently inhibited the binding of rat ¹²⁵I-IgA, whereas while <20% inhibition was obtained when as much as 10-fold greater quantities of a series of rat and human proteins were tested. Inhibition by rat IgG, IgE, and albumin were considered to be nonspecific because inhibition by these proteins was not significantly different from control tubes containing BSA/PBS. Moreover, inhibition by these latter proteins did not take place in a dose-dependent fashion and was <20% at all doses. In contrast, inhibition by rat IgA was dose-dependent (Fig. 5). Rat IgM, at a 10-fold greater concentration than rat IgA, did not inhibit the binding of rat ¹²⁵I-IgA as well. This was expected because rat IgM-SC
interactions are weak (15). In a series of additional experiments (not shown), human myeloma IgA dimers were able to competitively inhibit the binding of rat $^{125}$I-IgA whereas human secretory IgA dimer or human IgA monomer were unable to compete with rat $^{125}$I-IgA for binding to rat hepatocytes.

Thus, the competitive-inhibition experiments (Fig. 4) were consistent with a hy-
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TABLE I
Detection of Rat SC Molecules on a Series of Different Cell Types

| Cell type                          | * Number of SC molecules per cell |
|------------------------------------|-----------------------------------|
| Hepatocytes                        | 1.4 x 10^5                        |
|                                    | 2.2 x 10^5                        |
|                                    | 3.4 x 10^5                        |
| Intestinal epithelial cells (jejunum-ileum) | 1.0 x 10^5                        |
|                                    | 1.3 x 10^5                        |
|                                    | 2.0 x 10^5                        |
| Unfractionated spleen cells        | <5.0 x 10^2                       |
| Rat erythrocytes                   | <5.0 x 10^2                       |
| Peripheral blood lymphocytes       | <5.0 x 10^2                       |

* Each value represents the results of an individual experiment.

FIG. 4. Inhibition of binding of rat ^125^I-IgA to isolated rat hepatocytes. The amount of ^125^I-IgA corresponded to 100% saturation in the absence of inhibitor. (IgA)_2, unlabeled rat IgA dimer; IgM, IgG, and IgE were rat proteins; BG, correspondent to the experimental variation in the control points in which only BSA was added. The error bars are standard deviations of the mean of six individual determinations.

FIG. 5. Dose-dependent inhibition of rat ^125^I-IgA by unlabeled rat IgA. The error bars represent standard deviations of the means of six individual determinations.
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Flo. 6. Inhibition of binding of rat 125I-IgA to hepatocytes. (a) Cells were treated with IgG anti-SC antibodies or normal IgG, before addition of 125I-IgA; (b) 125I-IgA was incubated either with rat SC or BSA before incubation with hepatocytes.

Figure 6

Discussion

The results presented in this paper indicate that in the rat, secretory component is

hypothesis that rat 125I-polymeric IgA was binding to SC on hepatocytes because SC in free solution does not bind IgG, IgE, monomeric IgA, or albumin (5, and Socken and Underdown. Unpublished results.) and the majority of secretory IgA already contains SC.

To further investigate the possibility that hepatocyte-associated SC might be responsible for binding of rat 125I-IgA, experiments were carried out in which the binding of rat 125I-IgA to hepatocytes was determined in the presence of purified anti-rat SC antibodies or normal rabbit IgG as a control. The results indicate that the binding of rat 125I-IgA was markedly reduced in the presence of rabbit anti-rat SC but not the presence of an equal or (as shown) 10-fold excess of normal rabbit IgG (Fig. 6a).

A second approach was employed in which rat 125I-IgA was first incubated with rat SC or BSA, after which, the individual mixtures were separately incubated with hepatocytes. Prior incubation with rat SC reduced the ability of rat 125I-IgA to bind to hepatocytes, whereas BSA showed no effect (Fig. 6b).

When hepatocytes were treated with trypsin (0.1-0.25%, Sigma Chemical Co.) for 30 min at 37°C and washed three times in cold 30 mg/ml BSA/PBS, rat 125I-IgA did not bind to these cells. If these cells were washed and incubated for 1 h at 37°C in Waymouth’s medium, cells were capable of binding rat 125I-IgA, suggesting that the IgA-receptor had been newly synthesized.

The results presented in this paper indicate that in the rat, secretory component is
synthesized by hepatocytes. The method of isolation and short-term culture of hepatocytes provided easy access to a highly purified population of hepatic parenchymal cells (11-12). The quantity of SC synthesized by the hepatocyte cultures was significant (Fig. 1) but ~1,000 times less than previously reported for albumin and 100 times less than transferrin (12).

Evidence was obtained that SC is expressed on the surface of hepatocytes and is also secreted by these cells in culture. It should be noted that mucosal epithelial cells behave in similar fashion, i.e., SC has been found on the surface of epithelial cells (6-8), as well as being secreted by these cells (18). The dual distribution of SC (membrane and secretion) is consistent with the transport function of SC as well as its appearance in both biliary (19) and mucosal secretions (20). It is not clear what mechanism (if any) is required to allow SC to act as a receptor for transport as well as to be secreted.

In addition to demonstrating SC on the surface of hepatocytes, specific binding of radiolabeled rat IgA to hepatocytes was also demonstrated and the data presented are consistent with SC being involved as the IgA receptor on hepatocytes. Thus, prior incubation of polymeric IgA with rat SC prevented the IgA from binding to hepatocytes. Moreover, treatment of the hepatocytes with anti-SC antibodies prevented binding of polymeric rat IgA to the hepatocytes.

Rat IgM did not competitively inhibit the binding of rat 125I-IgA (Fig. 4) to hepatocytes as well as rat IgA. This was not unexpected as we had previously observed that in the rat, SC-IgM interaction was much less prominent than in man (15, and Socken and Underdown. Unpublished results.).

Our study and that of Hopf et al. (21) clearly indicate that binding to hepatocytes is specific for polymeric IgA. However, not all the polymeric IgA proteins studied by Hopf et al. were observed to bind to hepatocytes, which led these authors to conclude that binding of polymeric IgA to hepatocytes was not mediated by SC but by a glycoprotein receptor. However, the data presented by Hopf indicated that secretory IgA bound SC less well (as expected because 85% of secretory IgA contains SC) and showed a corresponding decrease in binding to hepatocytes.

The experiments of Fisher et al. indicate that biliary transport of polymeric IgA is mediated by liver-associated SC. More recently, Birbeck et al. demonstrated that biliary transport of IgA in the rat, proceeds through hepatocyte parenchymal cells. The experiments reported here indicate that SC mediates the binding of polymeric IgA to hepatocytes and that SC is part of the IgA receptor-complex on hepatocytes.

It seems likely that for SC to function as a receptor on the hepatocyte plasma membrane it must either be associated with an integral membrane protein or differ in structure from the freely water-soluble-free SC.

The hepatocyte appears to possess a number of different receptors on its plasma membrane which mediate the transport of a number of different proteins (22-25). The IgA transport system is relatively unique in that a part of the receptor complex is a protein which has been isolated and characterized (i.e., SC). Biliary transport of polymeric IgA may proceed by a vesicular mechanism as has been proposed for other proteins transported by hepatocytes (26) and for IgA transported by intestinal epithelial cells (6). Because the majority of IgA transported to bile is intact polymers (9), a mechanism may exist to prevent the IgA from being degraded by lysozomal vesicles within the cell. It may be that IgA-containing vesicles are protected by a clathrin coat (27) during transport through the hepatocyte. It will be of interest to
determine whether membrane-associated SC is physically related to clathrin or other proteins on the hepatocyte surface.

Summary

Secretory component (SC) was found to be synthesized by isolated rat hepatocytes. SC was detected by radioimmunoassay and cultured hepatocytes were found to synthesize 0.078 μg SC/10⁶ hepatocytes in a 48-h period. SC was also present on the surface of hepatocytes as detected by the specific binding of radiolabeled anti-SC antibodies as well as by the detection of specific membrane staining in indirect immunofluorescence tests using specifically purified anti-SC antibodies. Rat SC was detected on hepatocytes and intestinal epithelial cells but not on peripheral blood lymphocytes, unfractionated spleen cells, or erythrocytes. Specific binding of radiolabeled rat dimeric IgA to rat hepatocytes was also observed and evidence was obtained to indicate that such binding was mediated by SC. Thus, prior incubation of hepatocytes with anti-SC prevented binding of radiolabeled IgA. Moreover, prior incubation of radiolabeled IgA with rat SC prevented binding of the IgA to isolated hepatocytes. Cells treated with 0.25% trypsin lost their ability to bind to radiolabeled dimeric IgA.

We wish to thank Mr. Joseph Ho for his excellent assistance in isolating rat hepatocytes, Ms. Sandra Kuzniak for help in isolating specific antibodies to SC, and Ms. Patricia Rapacchietta for typing the manuscript. We also are indebted to J. M. Schiff for valuable discussions.

Received for publication 25 July 1979.

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