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EVIDENCE FOR THE SOLUBILIZATION OF THE INTESTINAL INTRINSIC FACTOR RECEPTOR BY SONICATION OF ILEAL BRUSH BORDERS

RICHARD P. SCHNEIDER[a,*], ROBERT M. DONALDSON, Jr[b,**] and BERNARD M. BABIOR[c,***]

[a]*Department of Medicine, Tufts-New England Medical Center, Boston, Mass.*, [b]*Boston University School of Medicine, Boston, Mass. and* [c]*Department of Medicine, Tufts-New England Medical Center, Boston, Mass. (U.S.A.)*

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SUMMARY

The uptake of intrinsic factor–cobalamin complex by guinea pig ileal brush borders is inhibited by particle-free sonicates of distal but not proximal brush border preparations. This finding suggests that sonication releases intrinsic factor receptor from ileal brush borders in a form which is not sedimented by centrifugation for 1 h at 105 000 × *g*.

INTRODUCTION

Intestinal absorption of cobalamin requires intrinsic factor, a glycoprotein secreted by the gastric epithelium which combines with cobalamin to form a macromolecular complex [1]. In the distal small intestine, intrinsic factor–cobalamin complex attaches to a specific receptor on the epithelial brush border [2]. This attachment is thought to be the first step in the complex process by which cobalamin is absorbed from the gastrointestinal tract.

To date, studies of the intrinsic factor receptor have been restricted to whole cell or particulate systems in which the receptor is associated with membrane. We wish to report evidence suggesting that the receptor can be released from the membrane by sonication under mild conditions. A new inhibition assay has been designed to measure the presence of solubilized receptor.

MATERIALS AND METHODS

Cyano-[^{57}Co]cobalamin was obtained from Amersham-Searle, Chicago, Ill. Unlabeled cyanocobalamin and Triton X-100 were purchased from Sigma. Other

* Present address: Department of Medicine, St. Thomas Hospital, Nashville, Tenn., U.S.A.
** Present address: Department of Medicine, West Haven Veterans Administration Hospital, New Haven, Conn., U.S.A.
*** To whom correspondence should be addressed.

reagents were the best grade commercially available, and were used without further purification. Guinea pigs were purchased from Allison Acres, Lunenberg, Mass. U.S.A.

Isolation of brush borders

Brush borders were isolated from guinea pig intestines in a manner similar to that previously described [2]. All steps were carried out in a cold room at 4 °C. Guinea pigs weighing 300–900 g were killed by a blow on the head. The small bowel was immediately removed and divided into thirds, and the middle third discarded. Proximal and distal thirds were washed separately by flushing with 0.9 % saline. The mucosa was expressed from 8–12 washed intestinal segments by pressing on the serosal surface with a glass rod. The expressed mucosa from proximal and distal thirds was pooled separately in 100 ml of 5 mM EDTA solution in 0.15 M sodium phosphate buffer, pH 7.5. This was homogenized a in Waring blender for 25 s at a setting previously determined to be maximally efficient [2]. The homogenate was filtered through No. 25 bolting silk, and the filtrate was centrifuged in an International PR-2 refrigerated centrifuge at 1500 rev./min for 10 min. Thereafter the sediment was washed twice with 50–60 ml of cold 5 mM EDTA and centrifuged again at 1500 rev./min for 10 min. The pellet was then suspended in Krebs–Ringer bicarbonate solution, pH 7.4, centrifuged at 500 rev./min for one min, and the sediment, containing clumped debris and nuclei, was discarded. The supernatant was then spun for 10 min at 2500 rev./min for 10 min. This pellet was resuspended in approximately 4 ml Krebs–Ringer bicarbonate solution and usually contained 4–8 mg/ml protein by biuret determination [3].

Preparation of intrinsic factor–Cyano-[^{57}Co]cobalamin complex

One ml of depepsinized human gastric juice [2], 0.4 ml of cyano-[^{57}Co]cobalamin solution containing 0.23 μg cobamide/ml (spec. act. 13 μCi/mg) and 10 ml of 0.155 M NaCl were incubated together at room temperature for 30 min. Unbound cyano-[^{57}Co]cobalamin was removed from the resulting solution of intrinsic factor–cyano-[^{57}Co]cobalamin complex by dialysis against 0.155 M NaCl as described elsewhere [4]. The solution was diluted with 0.155 M NaCl to a concentration of 2 ng cobalamin per ml and stored at −20 °C. Prior to use, the solution was diluted another 10-fold (final cobalamin concentration 0.2 ng/ml) with 0.155 M NaCl.

Inhibition assay

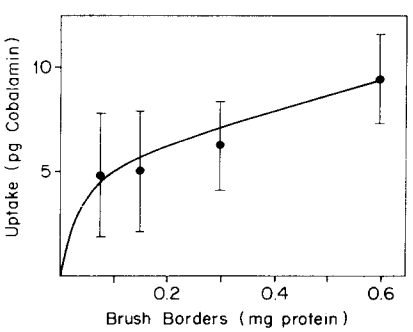
If active ileal intrinsic factor receptor were present in a particle-free preparation, it would be expected that this preparation would inhibit the uptake of intrinsic factor-cobalamin complex by ileal brush borders by competing with the brush border for intrinsic factor-cobalamin complex. To measure inhibition of uptake by particle-free preparations derived from brush border, a modification of the previously described assay [2] was developed which permitted uptake to be measured in mixtures containing roughly equimolar quantities of receptor sites and intrinsic factor-cobalamin complex, instead of the large molar excess of intrinsic factor-cobalamin usually present.

Two incubation mixtures were prepared, each in a 5 ml conical glass centrifuge tube. Each incubation mixture contained 0.1 ml (40 pg) labeled intrinsic factor–

cobalamin complex. To one mixture was added 0.2 ml of a suspension of brush borders in Krebs–Ringer bicarbonate buffer, while the other received 0.2 ml Krebs–Ringer bicarbonate buffer without brush borders. (The quantity of brush borders in the suspension varied as shown in Fig. 1.) After stirring briefly on a Vortex mixer, the reaction mixtures were incubated with agitation in a Dubnoff metabolic shaker for 30 min at 37 °C. The brush borders were then sedimented by centrifugation at 3000 rev./min in a refrigerated International PR-2 centrifuge for 60 min at 4 °C. A 200 μl portion of supernatant was withdrawn from each reaction mixture and counted in a Packard Autogamma detector. Uptake was calculated from the difference between the amount of radioactive intrinsic factor cobalamin complex in the control supernatant and the amount in the supernatant from the brush border-containing reaction mixtures.

The data of Fig. 1, obtained by the foregoing procedure, show that intrinsic factor-cobalamin complex is taken up by brush borders prepared from the distal third of guinea pig small intestine, the amount of uptake rising as the quantity of brush borders is increased. Little uptake is seen when brush borders from the proximal third of the guinea pig intestine are used, or when radioactive cyanocobalamin is substituted for the intrinsic factor-cobalamin complex (Fig. 2). These findings confirm that the process being measured by this assay is the uptake of intrinsic factor-cobalamin complex by the specific receptors of the guinea pig ileum.

The inhibition assay represents a modification of the above procedure. Two incubation mixtures were prepared, each in a 5 ml conical glass centrifuge tube. Each mixture contained 0.2 ml (40 pg) labeled intrinsic factor–cobalamin complex and 0.2 ml distal brush border suspension (ca. 2.0 mg of brush border protein per ml Krebs–Ringer bicarbonate buffer). To one mixture was added 0.2 ml of the solution to be tested for solubilized receptor, while the other mixture received 0.2 ml of Krebs–Ringer bicarbonate buffer. From this point on, the assay was conducted as described above, except that 500 μl portions of supernatant were counted. The extent of inhibi-



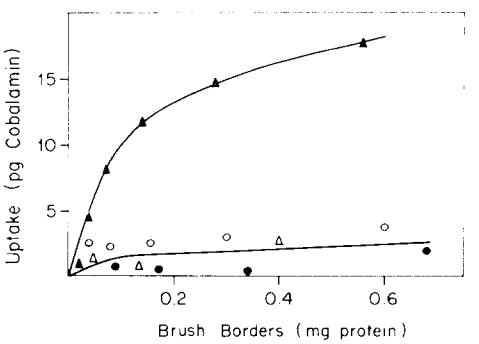


Fig. 1. Uptake of very dilute intrinsic factor–cobalamin complex by ileal brush borders. For details see text. Results shown are the mean ± S.E. for 5 experiments. Separate brush border preparations were used for each experiment.

Fig. 2. Uptake of very dilute cyanocobalamin or intrinsic factor–cobalamin complex by brush borders from proximal and distal small intestine. For details see text. ●, ○ indicate proximal brush borders and ▲, △ indicate distal brush borders. Closed and open symbols refer to experiments with complexed and free cyanocobalamin respectively.

tion was determined from the difference in intrinsic factor–cobalamin complex uptake in the two incubation mixtures.

RESULTS

Experiments with Triton X-100

The first attempts to solubilize the ileal brush border intrinsic factor receptor were carried out with a non-ionic detergent, Triton X-100 [5]. Ileal brush borders incubated with Triton X-100 were found to lose their ability to take up intrinsic factor–cobalamin complex (Table I). Uptake of intrinsic factor-cobalamin complex by brush borders incubated in 0.4 % Triton X-100 for 2 h at 4 °C was half that of brush borders incubated in Krebs–Ringer bicarbonate buffer alone, while uptake by brush borders incubated in 1.0 % Triton X-100 was almost abolished. However, it was not possible to establish that this finding reflected release of intrinsic factor receptor into the medium, since the detergent itself was found to interfere with uptake of intrinsic factor–cobalamin complex by brush borders. Table II shows substantial impairment of intrinsic factor-cobalamin complex uptake when the Triton X-100 concentration in the assay medium was 0.2 %. Solubilized receptor could therefore only be assayed in medium containing less than 0.2 % Triton X-100.

Because the detergent was to be used at such a low concentration, a sonic disruption step was added to facilitate the solubilization. The medium from an experiment in which ileal brush borders were incubated with 0.1 % Triton X-100 after sonication was found to inhibit intrinsic factor–cobalamin complex uptake by fresh ileal brush borders, an observation suggesting the presence of solubilized intrin-

TABLE I

LOSS OF UPTAKE OF INTRINSIC FACTOR–COBALAMIN COMPLEX BY DISTAL BRUSH BORDERS INCUBATED WITH TRITON X-100

Distal brush borders (0.1 ml of a suspension containing 3.6 mg protein/ml) were incubated for 2 h at 4 °C in a conical centrifuge tube with 1.0 ml of Krebs–Ringer bicarbonate solution containing Triton X-100 at the concentrations indicated (v/v). The brush borders were then reisolated by centrifugation at 15 000 × *g* for 20 min at 4 °C and washed once with Krebs–Ringer bicarbonate solution. They were then suspended with the aid of a Vortex mixer in 0.2 ml of a solution of intrinsic factor–cyano–[^{57}Co]cobalamin complex (0.2 ng cyanocobalamin/ml) and incubated for 30 min at 37 °C in a Dubnoff shaker. The brush borders were then precipitated by centrifugation at 15 000 × *g* for 60 min at 4 °C, and the radioactivity in 0.2 ml of the supernatant was determined in a Packard Autogamma counter. The radioactivity in 0.2 ml of the original solution of complex was also determined, and uptake of the complex was calculated from the difference between these two values.

Triton X-100 (%)	Uptake of complex (pg cobalamin)	
	Expt 1	Expt 2
0	5.2	3.2
0.1	4.1	—
0.2	4.4	—
0.4	2.6	—
0.8	1.5	—
1.0	0.4	0.0

TABLE II

UPTAKE OF INTRINSIC FACTOR-COBALAMIN COMPLEX BY DISTAL BRUSH BORDERS IN THE PRESENCE OF TRITON X-100

Uptake was measured by the inhibition assay (see Materials and Methods), using as the "solution to be tested" 0.2 ml of Krebs–Ringer bicarbonate containing various concentrations of Triton X-100. In the table is given the final concentration of Triton X-100 in the incubation mixture.

Triton X-100 (%)	Uptake of complex (pg cobalamin)
0	5.4
0.05	3.8
0.1	5.4
0.2	1.5

sic factor receptor in the medium. A similar observation was made, however, with medium from an experiment in which brush borders were subjected to sonication alone. Since this experiment suggested that detergent was not required for solubilization of the ileal intrinsic factor receptor, and since certain technical problems could be avoided by omission of detergent, subsequent experiments were carried out using only sonication to separate the intrinsic factor receptor from the ileal brush border.

Solubilization of the ileal intrinsic factor receptor

The supernatant from a preparation of distal brush borders subjected to sonication followed by centrifugation at 105 000 × *g* for 1 h was found to inhibit uptake of intrinsic factor–cobalamin complex by untreated brush borders (Table III). The supernatant from a similarly treated preparation of proximal brush borders had no effect on uptake of complex. In a single experiment, supernatants from proximal and distal brush border preparations centrifuged without prior sonication were also

TABLE III

EFFECT OF PARTICLE-FREE BRUSH BORDER PREPARATIONS ON UPTAKE OF INTRINSIC FACTOR–COBALAMIN COMPLEX BY PARTICULATE ILEAL RECEPTOR

Brush border suspensions (proximal and distal; protein concentration 3 mg/ml) were divided into two portions. One portion (2 ml) of each suspension was sonicated for 45 s at maximum power with a Branson Sonic Oscillator (Model W185), using a microtip attachment and cooling in melting ice. Both portions of each brush border preparation were then centrifuged at 105 000 × *g* for 60 min at 4 °C in a Spinco Model L2-65B ultracentrifuge, using a Model SW-65K swinging bucket rotor. Inhibition assays were then carried out as described in Materials and Methods, using the 105 000 × *g* supernatant from each of the brush border preparations as the solution to be tested for solubilized receptor.

Addition	Uptake of complex (pg cobalamin)
Krebs–Ringer bicarbonate	5.7
Sonicated proximal brush border supernatant	6.2
Sonicated distal brush border superanatant	3.0
Unsonicated proximal brush border supernatant	5.3
Unsonicated distal brush border supernatant	5.1

TABLE IV

COMPARISON OF THE EFFECT OF PARTICLE-FREE PROXIMAL AND DISTAL BRUSH BORDER SONICATES ON UPTAKE OF INTRINSIC FACTOR–COBALAMIN COMPLEX BY PARTICULATE ILEAL RECEPTOR

2-ml Portions of proximal and distal brush border suspensions at the concentrations shown were sonicated for the times indicated at maximum power with a Branson Sonic Oscillator (Model W185), using a microtip attachment and cooling in melting ice. The sonicated suspensions were then centrifuged at 105 000 × *g* for 1 h at 4 °C in a Spinco Model L2-65B ultracentrifuge, using a Model SW-65K swinging bucket rotor. Inhibition assays were then carried out as described in Materials and Methods, using the 105 000 × *g* supernatant from each of the brush border preparations as the solution to be tested for solubilized receptor.

In some cases, uptake of complex by the brush borders before and after sonication was also determined. For these experiments, the brush border pellet from the 105 000 × *g* centrifugation was resuspended in 2 ml Krebs–Ringer bicarbonate. Intrinsic factor–cyano-[^{57}Co]cobalamin complex (0.6 ml, 120 pg) was added to the resuspended sonicated brush border and to 2 ml of the original brush border suspension. The brush border–complex mixtures were incubated for 30 min at 37 °C. Brush borders were then reisolated by centrifugation at 15 000 × *g* for 1 h at 4 °C, washed twice with Krebs–Ringer bicarbonate, and finally suspended in 2 ml of Krebs–Ringer bicarbonate and counted in a Packard Autogamma counter.

Each experiment represents the results obtained with proximal and distal brush borders from the same group of animals. The difference in inhibition between particle-free sonicates of proximal and distal brush borders is significant at the level of $p < .01$ (sign test [6]).

Experiment	Sonication conditions		Uptake of complex by brush borders (pg cobalamin)		Uptake of complex in inhibition assay (pg cobalamin)		
	Duration (sec)	Concentration (mg protein/ml)	Before sonication	After sonication	Control*	Supernatant from proximal brush borders	Supernatant from distal brush borders
1	15	3	–	–	9.8	9.1	4.9
2	30	3	23.1	6.1	5.9	6.2	2.9
3†	15	7	46.8	20.4	16.5	15.7	13.6
4†	45	7	46.8	19.3	16.5	11.0	7.4
5	40	11	–	–	11.9	9.9	9.0
6	30	3			11.9	10.1	6.8
7	45	6	–	–	6.1	5.0	4.0
8	45	7	–	–	3.5	2.1	1.4

* Krebs–Ringer bicarbonate used instead of supernatant.

† Same brush border preparations.

found not to affect complex uptake. These results suggest that sonic disruption of distal brush border preparations may have released the ileal intrinsic factor receptor in a form which was not sedimentable by ultracentrifugation.

Further evidence for the release of ileal–intrinsic factor receptor from brush borders is provided by the results of Table IV. In this table are shown several experiments in which brush borders from the proximal and distal small intestine of the same group of animals were sonicated and centrifuged as described above. In every case, inhibition of uptake by the supernatant from the distal brush border preparation exceeded that of proximal brush border supernatant. Moreover, in the three experiments in which it was measured, uptake of intrinsic factor–cobalamin complex by the distal brush borders themselves was greater before than after sonic disruption, indicating that sonication depleted the intrinsic factor binding capacity of distal brush borders. This result accords with the view that the intrinsic factor receptor was released from the distal brush border by sonication.

DISCUSSION

It is generally accepted that a specific intrinsic factor receptor is present on the brush border of distal small bowel epithelium of a variety of species [7]. The characteristics of this receptor have been the subject of many investigations, but as yet it has not been possible to separate the receptor from the particle in which it is embedded. In 1967 Donaldson et al. demonstrated a particle-bound receptor which was released by homogenization of hamster microvillous membrane. This receptor was not sedimented at 28 500 × *g* but was precipitated at 54 500 × *g* or more [2]. In 1968 Rothenberg [8] attempted to isolate the receptor of guinea pig mucosa by a salting-out technique with sodium sulfate, but since his technique involved only centrifugation for 30 min at 30 000 × *g*, it is difficult to determine whether the receptor was freed from particles or not.

In the present experiments, sonication under certain conditions released from distal but not from proximal intestinal brush borders a substance not sedimented by centrifugation at 105 000 × *g* for 1 h which inhibited intrinsic factor–cobalamin complex uptake by distal brush borders. This material did not appear in the 105 000 × *g* supernatant of brush border preparations which were not sonicated. Although sonication under sufficiently rigorous conditions was able to release inhibitory activity from proximal brush borders (see Table IV, Expt 4), mild sonication of dilute brush border preparations led to the appearance of substantial inhibitory activity only in the supernatants from distal brush border preparations. The finding that inhibitory activity was released from distal but not proximal brush borders is consistent with the view that the inhibition was attributable to solubilized intrinsic factor receptor, a component restricted to distal brush borders, and did not represent an effect of nonspecific inhibitory material, which would be expected to be released from both proximal and distal brush borders. The release by mild sonication alone would indicate that if the observed inhibition is in fact due to solubilized receptor, the receptor is rather loosely attached to the brush border.

Our experimental conditions were such that under the most favorable conditions, the amount of receptor released into 0.2 ml of 105 000 × *g* supernatant would be approximately equivalent to the amount of receptor present on the brush borders

used in the competition assay. The detection of inhibition by solubilized receptor present at this very low concentration was possible because of the extremely high affinity of intrinsic factor-cobalamin complex for the distal brush border receptor, a property which allowed the assay to be carried out under conditions where concentrations of complex and distal brush border receptor were roughly equal. Under these circumstances, the reduction in the concentration of free intrinsic factor–cobalamin complex resulting from its binding to solubilized receptor was sufficient to cause a detectable diminution in the amount of complex taken up by the brush borders.

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REFERENCES

1 Gräsbeck, R. (1969) in Progr. Hematology, (Brown, E. B. and Moore, C. V., eds), Vol. 6, pp. 233–260, Grune and Stratton, New York
2 Donaldson, Jr, R. M., Mackenzie, I. L. and Trier, J. S. (1967) J. Clin. Invest. 46, 1215–1228
3 Layne, E. (1957) Meth. Enzymol. 3, 450–451
4 Donaldson, Jr, R. M., and Katz, J. H. (1963) J. Clin. Invest. 42, 534–545
5 Razin, S. (1972) Biochim. Biophys. Acta 265, 241–296
6 Goldstein, A. (1964) Biostatistics: An Introductory Text, p. 61, Macmillan, New York
7 Mackenzie, I. L. and Donaldson, Jr, R. M. (1969) Fed. Proc. 28, 41–45
8 Rothenberg, S. P. (1968) J. Clin. Invest. 47, 913–923