Radioautographic localisation of iodinated human intrinsic factor in the guinea pig ileum using electron microscopy

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SUMMARY The uptake of iodinated human intrinsic factor by guinea pig ileum was studied *in vivo* using electron microscopy radioautography. Iodination of intrinsic factor cobalamin complex with N-chlorobenzene-sulphonamide beads did not modify its physicochemical properties in gel filtration, nor in iso-electrofocusing. It didn't affect its biological activity either *in vitro* in presence of solubilised receptor, or *in vivo* in the Schilling test. The ^{125}I -intrinsic factor cobalamin complex was instilled *in vivo* in ileal blind loops in presence of either CaCl_2 , EDTA, bile or intestinal juice. The labelled complex was predominantly located in the intermicrovillous pits of the apical membrane and in the apical cytoplasm. The uptake was about five-fold lower in presence of EDTA. On the apical membrane, the number of grains (per 80 μm length of epithelium) increased from 2.7 (0.2) up to 9.4 (1.7) respectively for 15 minutes and two hours of delay. This suggests a recycling of the intrinsic factor receptor complex. In the apical compartment, the silver grains were often detected over non-coated vesicles and over the infoldings of the lateral membrane. These results show that intrinsic factor is internalised into enterocytes during cobalamin absorption, and that a part of intrinsic factor enters the blood circulation through transcytosis.

The absorption of cobalamin needs its binding to a glycoprotein secreted by the stomach, the intrinsic factor (IF) and the subsequent calcium dependent binding of the Cbl-IF complex to a specific ileal receptor. The number of ileal IF receptors is restricted.¹⁻⁴ It has been shown that Cbl enters the enterocytes in less than one hour⁵ but the mechanism of the transfer has not yet been elucidated. The enterocyte subcellular distribution of the labelled Cbl orally administered to guinea pigs⁶ or dogs⁷ showed the presence of the vitamin in the lysosomes of enterocytes after two hours.

Contrary to that of Cbl, the fate of IF remains debatable.⁸ Several recent findings suggest that IF is internalised into the enterocytes in the guinea pig (i) after incubation of ^{35}S -methionine labelled rabbit IF

with isolated guinea pig ileal enterocytes for periods of up to 30 minutes, only half of the radioactivity was removed by washing the cell surface with acid or EDTA;⁵ (ii) [^{125}I]-iodinated IF was localised in lysosomes, brush border membrane, and cytosol of guinea pig ileum maintained in organ culture.⁷ The internalisation of IF has also been observed in rabbit ileal explants.⁹

Other findings suggested that only Cbl, but not IF, enters the cell. Hines and coworkers observed on everted sac of guinea pig ileum that the permease activity of IF for Cbl was maintained after sequential incubations with labelled Cbl.¹⁰ Finally, a morphological investigation using immunocytochemical localisation of IF found the molecule associated only with the microvillous pits of enterocytes from guinea pig ileum.⁴ In the present work, we have studied the fate of IF after its binding to ileal mucosa in the guinea pig *in vivo*, using high resolution electron microscopy radioautography instead of immunocytochemistry.

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Methods

CHEMICALS

[¹²⁵I]-iodine (1 Ci/67 μmol) was obtained from the radiochemical Centre (Amersham, England). Iodo beads™ were purchased from Pierce (Rockford, USA) and Sephacryl S 300 from Pharmacia Fine Chemicals (Uppsala, Sweden). Other reagents were obtained as described elsewhere.^{11,13}

IODINATION OF INTRINSIC FACTOR

Intrinsic factor was purified from human gastric juice as recently described.¹¹ One nanomole IF was iodinated using the method of Markwell *et al.*¹²

Iodo beads were incubated at room temperature with 100 μ Ci of [125 I]-iodine in 200 μ l 20 mM Tris HCl pH 8.0 during five minutes. Two nanomoles of IF-Cbl complex were then added in a total volume of 400 μ l 20 mM Tris HCl buffer pH 8.0. After 15 minutes of incubation, the beads were discarded and 200 μ l of 20 mM Tris HCl buffer pH 8.0 containing 0.125 NaCl and 2% (wt/vol) of potassium iodide was added. The incubation mixture was poured onto a Sephacryl S 300 column dimensioned 1 $\times$ 40 cm, filtered with 20 mM Tris HCl buffer pH 8.0 containing 0.5 M NaCl, 2% (wt/vol) of potassium iodide, and 0.2% (wt/vol) bovine serum albumin at a flow rate of 0.2 ml/min.

Dextran Blue 2000 and tritiated water were used to calibrate the column. The molecular mass of the iodinated IF ($[^{125}\text{I}]\text{-IF}$) was estimated using labelled human serum albumin and IgG as described previously.¹³

A part of the pooled fraction of [125 I]-IF-Cbl was poured onto a 110 ml LKB isoelectrofocusing column packed with carrier ampholytes of pH 2.5–4.0 and of pH 3.5–6.0 in a final concentration of 1% and in a 0–50% (wt/vol) sucrose gradient.

BIOLOGICAL ACTIVITY OF THE IODINATED INTRINSIC FACTOR

[¹²⁵I]-IF was tested *in vitro* in presence of a solubilised IF receptor extract. The receptor extract was prepared from guinea pig ileal mucosa after the method of Kouvonen *et al.*¹⁴ About 0.04 nmol of [¹²⁵I]-IF-Cbl was incubated with 0.8 ml of the receptor extract in presence of either 1 mM CaCl₂ or 1 mM EDTA in a total volume of 1 ml. Each incubation mixture was then poured onto a Sephacryl column dimensioned 2.5×100 cm filtered with 20 mM Tris HCl buffer pH 8.0 containing 0.5 M NaCl and 1 mM CaCl₂ at a flow rate of 30 ml/h. The ability of iodinated IF-Cbl complex to be degraded was also tested by incubating 0.04 nmoles of [¹²⁵I]-IF-Cbl with 20 µl ileal guinea pig intestinal juice in a total volume of 1 ml 20 mM Tris HCl buffer pH 8.0 containing 0.5 NaCl and 1 mM CaCl₂, during one hour at room temperature. The

sample was then filtered as described above. The *in vivo* biological activity of IF iodinated with non-isotopic iodide was also tested by a Schilling test in an Addison Biermer anaemia patient.

IN VIVO GUINEA PIG INCUBATIONS

The experiments were carried out as described by Levine *et al.*⁴ Nine fasted male guinea pigs were anaesthetised with pentobarbital (25 mg/kg ip). After laparotomy, three 1 cm ileal loops were made by three double ligatures of the terminal ileum per animal. The border circulation was carefully pre-

Fig. 1 (a) Elution profile of the iodinated human intrinsic factor cobalamin complex (^{125}I -IF-Cbl) in Sephacryl S 300 gel filtration. The ^{125}I -IF-Cbl complex was eluted in a position corresponding to a molecular mass of about 50 000 daltons. (b) In presence of ileal intrinsic factor receptor extract from guinea pig and of 1 mM CaCl_2 , the ^{125}I -IF-Cbl was eluted in the same position as dextran blue (DB) corresponding to its binding to the receptor (—). This phenomenon was not observed when 1 mM EDTA was added in the incubation mixture instead of CaCl_2 (— · —); in that case, IF was eluted as a monomer and a dimer.

Fig. 2a, b, c.

Table 1 Distribution of iodinated intrinsic factor-cobalamin complex (^{125}I -IF-Cbl) in the enterocytes of guinea pig ileum using radioautography

Instilled sample	Incubation time (min)	Analyses (n)	Grains/80 μm length of epithelium (n)				
			Total	Microvilli	Apical cytoplasm	Basal cytoplasm	Nucleus
^{125}I -IF-Cbl+SPB	15	3	8.3 (3.0)	2.7 (0.2)	4.7 (0.4)	0.4 (0.2)	0.3 (0.2)
^{125}I -IF-Cbl+SPB	120	6	21.4 (12.3)	9.4 (1.7)	8.3 (2.1)	1.3 (0.6)	2.1 (0.6)
^{125}I -IF-Cbl+KB	120	3	20.6 (5.1)	9.5 (0.6)	7.4 (2.5)	1.2 (1.0)	2.5 (1.0)
^{125}I -IF-Cbl+IJ	120	3	20.3 (8.0)	10.3 (2.4)	6.5 (2.0)	1.6 (0.8)	1.8 (0.6)
^{125}I -IF-Cbl+Bile	120	3	13.4 (5.2)	5.0 (0.4)	6.2 (0.7)	1.1 (0.5)	1.2 (0.1)
^{125}I -IF-Cbl+EDTA	120	3	4.9 (4.7)	1.4 (0.7)	1.6 (0.2)	0.8 (0.3)	1.1 (0.4)

0.02 nmol ^{125}I -IF-Cbl complex was instilled *in vivo* in ileum in each experiment. The scoring of the grains of each cell compartment was calculated from the determination of the distribution of grains in each compartment and the average of total grains detected over 80 μm length of epithelium. SPB: sodium phosphate buffer; KB: Krebs buffer; IF: intestinal juice; EDTA: ethylenediaminetetraacetic acid.

served from the ligatures. One hundred microlitres 0.1 M sodium phosphate buffer pH 7.4 containing 0.15 NaCl, 1.5 mM CaCl_2 and 0.02 nmol of the ^{125}I -IF-Cbl complex (0.18 μCi) was instilled in each loop by means of a needle. In two other series of experiments, the ^{125}I -IF-Cbl complex was instilled with 100 μl bile or of human duodenal juice instead of sodium phosphate buffer. After 15 minutes or two hours of incubation the loops were excised, washed with the sodium phosphate buffer described above, cut in 2 mm wide rings and immersed in a fixative solution containing 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at 4°C for two hours. The postfixation was achieved for two hours in 0.1 M cacodylate buffer pH 7.2 containing 1% OsO_4 (vol/vol).

In a control series, the guinea pig ileal loops were washed with 1 mM EDTA in 0.1 M phosphate buffer pH 7.4 containing 0.15 NaCl and 1 mM EDTA was added to the ^{125}I -IF-Cbl complex solution which was instilled. The incubation time of these control experiments was two hours. In another control series, the guinea pig ileal loops were instilled consecutively with 1 nmol unlabelled saturated IF and 10.02 nmol of iodinated IF-Cbl complex. The incubation time was 15 minutes.

RADIOAUTOGRAPHIC METHOD

After embedding in Epon, thin sections were taken and processed for radioautographic treatment as

previously described.^{15,16} The sections were coated with an emulsion (Ilford L4 nuclear emulsion diluted 1:4 with distilled water) and exposed from four to 10 weeks at 4°C and developed in Microdol X (Kodak) for four minutes at 17°C or in phenidon developer for two minutes at 20°C. The sections were examined on a TEM Siemens 102.

Quantitative analysis

Three kinds of analyses were done by counting a total of 11 000 grains:

(1) Frequency of the grains overlying the epithelium

The scoring of the silver grains was carried out directly on the TEM screen at a magnification of 6000/unit of membrane length, determined by the photographic 'square'. The length of each unit was estimated to 13.3 μm using latex calibrated beads. The frequency was calculated from grain counting over $3 \times 1000 \mu\text{m}$ of epithelium length for each ileal fragment. The results were expressed as the number of grains/80 μm length of epithelium.

(2) Distribution of silver grains over the cellular compartments

The silver grain distribution through the epithelial cells compartments was analysed directly on the TEM screen at a magnification of 6000 using the 'simple grain density procedure' of Ross and Benditt.¹⁷ Four compartments were determined in

Fig. 2 Silver grain distribution of the iodinated intrinsic factor cobalamin complex over the ileal epithelium after instillation of the tracer *in vivo* for 15 minutes (0.35 μCi). (a) The radioautographs showed a restricted number of binding sites of the tracer on the apical microvillous membrane (MV). The iodinated intrinsic factor was also present in the apical cytoplasm (Ap Cyt). No radioactivity was observed over the migrating cells (MC). (b) At a higher magnification, the tracer detected over the brush border was mainly located in the intermicrovillous pits. (c) High magnification showed the association of the silver grains with small apical vesicles (V). The apical vesicles were rare, (a) $\times 6000$, bar = 1 μm ; (b) $\times 30\,000$, bar = 0.2 μm ; (c) $\times 55\,000$, bar = 0.2 μm . (Original magnifications)

Fig. 3 Silver grains association with the infoldings of the lateral plasma membrane of ileal enterocytes after instillation of [125 I]-IF-Cbl complex (0.35 μ Ci) *in vivo* in guinea pig ileum for 15 min. (a) Clusters of grains overlying the lateral membrane. The cytoplasmic organelles did not exhibit any labelling (G=Golgi apparatus, M=mitochondria, R=endoplasmic reticulum, V=vesicles, D=desmosomes). (b) At a higher magnification, the grains were located at less than 1 HD of the lateral plasma membrane (N=Noyau). (a) $\times 12\,000$, bar=0.5 μ m; (b) $\times 28\,100$, bar=0.5 μ m. (Original magnifications)

the epithelium and their respective area ratio were estimated on 20 cells micrographs using a digitising table (Hewlett Packard) connected to a computer Tektronik 4.

The relative area of the plasma membrane (including microvilli and intermicrovillous pits), the apical cytoplasm (between the plasma membrane and the nucleus midline), the basal cytoplasm (between the nucleus midline and the basal membrane) and the nucleus were estimated to 0.11, 0.44, 0.35, and 0.10 respectively.

Each developed grain lying in a compartment was tabulated until a total of 200 grains/section three counts corresponding to 600 grains were performed per analysed ileal fragment. The results were presented as average number of grains per unit area of the compartment.

(3) Relation between silver grains and apical specific organelles

At higher magnifications, we often observed a proximity of silver grains with the mitochondria, the lateral membranes and the vesicles. This led us to

determine whether or not these structures were associated to the silver grains using a half distance analysis.

Micrographs were taken at an initial magnification of 15 000, 20 000, 30 000. The negatives were then viewed in a microfilm reader projector with an enlargement of $\times 4$. Establishing a half distance (HD) of 90 nmol,¹⁸ each silver grain was centred in a resolution boundary circle of a radius equivalent to one HD up to 5 HD. The organelles located in this area have been considered to be associated to the silver grain centring this circle. Each analysis was done over 3000 μ m length of epithelium.

Results

The human IF-Cbl complex was iodinated with a yield of about 18% and a specific radioactivity of 0.18 μ Ci/ μ g. The [125 I]-IF-Cbl complex was eluted with an apparent molecular mass of 50 000 daltons in Sephacryl S 300 gel filtration (Fig. 1). It focused in four peaks isoelectric at pH 4.6, 4.7, 5.0, and 5.1 in isoelectrofocusing. The iodinated molecule was bio-

Fig. 4 (a) After two hours of instillation of the iodinated intrinsic factor cobalamin complex in guinea pig ileum (0.35 μ Ci), the grains are located very often near the numerous mitochondria (M) of the apical cytoplasm. (b) Some grains were detected close to vesicular structures (V) below the terminal web (TW). The golgi apparatus (G) did not exhibit any labelling. (a) $\times 15\,000$, bar = 0.5 μ m; (b) $\times 12\,000$, bar = 0.5 μ m. (Original magnifications)

logically active since (i) it crossreacted *in vitro* with the guinea pig solubilised ileal receptor as shown by elution of the iodinated IF-receptor complex in the same position as dextran blue 2000 in Sephacryl gel filtration (Fig. 1). (ii) The binding of IF to the receptor did not occur *in vitro* in presence of 1 mM EDTA (Fig. 1). (iii) The absorption of CN [57 Co] Cbl tested by a Schilling test was significantly enhanced from 0.2% up to 10% when the labelled Cbl was ingested by an Addison-Biermer anaemia patient in presence of 10 μ g non-radioactive iodinated human IF. The iodination of either unsaturated human IF or saturated hog IF by a same procedure resulted in a significant loss of their biological activity. The iodinated IF was not degraded when incubated with intestinal juice of guinea pig as no change of its elution position in gel filtration nor liberation of iodine were observed.

In electron microscopy radioautography, the grains were observed in enterocytes but not in mucus secreting cells nor migrating cells (Fig. 2a). The grains overlying the microvilli compartment were mainly located over intermicrovillous pits (Fig. 2b).

This labelling was enhanced from 2.7 (0.2) grains/80 μ m length of epithelium up to 9.4 (1.7) grains after respectively 15 minutes and two hours of delay (Table 1). After 15 minutes, some grains were

Table 2 Half distance (HD) analysis of iodinated intrinsic factor-cobalamin detected near or over mitochondria, vesicles, and lateral membrane of the apical compartment of enterocytes in radioautographs of guinea pig ileum

Score of grains*						
15 min				120 min		
HD (n)	Mito-chondria	Vesicles	Lateral membrane	Mito-chondria	Vesicles	Lateral membrane
1	0	2	47	18	6	61
2	2	2	18	8	8	33
3	12	12	12	16	26	32
4	14	16	16	28	32	17
5	16	17	11	41	34	25

*The score of grains corresponded to the number of silver grains which could be assigned to the cellular structure for a distance of 1–5 HD at a $\times 15\,000$ – $\times 30\,000$ magnification according to Salpeter *et al.*¹⁸

Fig. 5 A striking decrease of the labelling of the ileal epithelium was observed after instillation of the iodinated intrinsic factor cobalamin complex ($0.35 \mu\text{Ci}$) for two hours in presence of EDTA. The ultrastructure of the microvilli (MV) was not modified, the internalisation of the tracer (arrow) in the apical cytoplasm (Ap Cyt) was about five-fold lower than that observed without EDTA $\times 6000$, bar = $1 \mu\text{m}$. (Original magnification)

observed over the infolds of the lateral membrane of the supra nuclear compartment of enterocytes (Figs 3a, b). The tracer entered the cells and was predominantly located in the apical cytoplasm compartment after two hours as well as after 15 minutes (Table 1). The grains were often found close to the numerous mitochondria located in this compartment and to tubulovesicular structures (about 100 nm diameter) under the terminal web (Figs 2c, 4a, 4b). The detailed study using HD analysis showed that the

radioactive source was located within the infolds of the lateral plasma membrane and near the mitochondria (Table 2). The basal compartment was not significantly labelled, even after two hours. Moreover, no grains were detected over the Golgi apparatus (Fig. 4b). When the labelled IF-Cbl complex was instilled in the ileum in presence of 1 mM EDTA, the number of grains counted/ $80 \mu\text{m}$ length of epithelium was significantly reduced but not abolished (Table 1). EDTA did not induce any disruption of the microvillous membrane (Fig. 5). When the iodinated IF-Cbl complex was instilled in presence of 50 fold its amount of unlabelled saturated IF, a 80% decrease of the frequency of grains overlying the epithelium was seen. The frequency of the intracellular labelling obtained when the iodinated IF-Cbl complex was instilled with phosphate buffer was similar to that observed using either Krebs buffer, intestinal juice, or bile (Table 1).

Discussion

The iodination procedure of IF did not induce any significant denaturation of the molecule as the physicochemical properties and the *in vitro* and *in vivo* biological activity were not modified. The iodination of the molecule was stable as (i) no liberation of $[^{125}\text{I}]$ -iodine was observed in gel filtration, (ii) the radioactivity was not detected in the mucus secreting cells nor in migrating cells, (iii) the radioactivity which entered the enterocyte was much lower than that which remained in the lumen. Moreover, the intracellular distribution of grains was not uniform. Few grains were found in the nucleus and in the basal cytoplasm. This suggested that most of the grains detected in the cells corresponded to protein bound and not to free $[^{125}\text{I}]$ -iodine.

The uptake of the $[^{125}\text{I}]$ -IF-Cbl complex was specific because it was significantly reduced either in presence of 1 mM EDTA or in presence of unlabelled saturated IF and because it was observed only in enterocytes but never in mucus secreting cells. Contrary to the observation of Levine *et al*⁴ we did not observe any damage of the ileal epithelium in presence of 1 mM EDTA (Fig. 5). EDTA inhibited the uptake of IF-Cbl rather by chelating the divalent cations than by disruption of the apical membrane. The discrepancy observed between our results and those of Levine *et al* was difficult to explain. In fact, the main difference between both *in vivo* experiments was the tissue fixation procedure.⁴ The medium in which the $[^{125}\text{I}]$ -IF-Cbl complex was instilled (buffers, intestinal juice, or bile) seemed to not modify the internalisation of the complex. In the apical membrane, the IF-Cbl complex was very often seen in the intermicrovillous pits. The same result

was described by Levine *et al.*,⁴ who suggested that the IF receptor was located there. About 40–60% of the grains were detected in the apical cytoplasm compartment. It may, therefore, be assumed that IF enters the cell during Cbl absorption because the iodinated IF which was instilled was saturated with Cbl. Our results are in accordance with those of Kapadia *et al.* who found biosynthetically labelled IF to the cytosol of enterocytes after incubating it with isolated ileal enterocytes.⁵ Internalisation of iodinated IF has also been shown using cell fractionation of guinea pig ileal mucosa.¹⁹ Levine *et al.*, however, did not observe any intracellular IF using immunocytochemical localisation of IF in the guinea pig.⁴ In fact, these authors carefully noticed that the absence of internalisation of IF could be the consequence of either an inadequate penetration of the anti-IF antisera or a masking or a denaturation of the antigenic site of IF during the internalisation of the molecule.

The labelling of the cells was about 2.5 fold higher after two hours of delay than after 15 minutes. Moreover, the proportion of labelled IF localised on the microvilli increased from 33% to about 44%. This result may correspond to a recycling of the iodinated IF in the microvillous membrane and could explain that everted sacs of guinea pig ileum would take up free [⁵⁷Co] Cbl after being previously incubated with IF-[⁵⁷Co]-Cbl.¹⁰ Robertson and Gallagher had speculated that the IF receptor complex could be recycled because the uptake of cobalamin in the mouse was not modified after treatment of animals by cycloheximide, an inhibitor of protein synthesis, and because the animals recovered their capacity to take up Cbl 30 minutes after ingestion of a saturation dose of the vitamin.^{20,21}

Although our results provide evidence for internalisation of IF, it still remains to determine if this phenomenon corresponds to receptor mediated endocytosis. The concentration of labelled IF on the intermicrovilli pits of the apical membrane is compatible with such a mechanism. Some of the internalised labelled IF was found near or over very small vesicles at a high magnification. These vesicles cannot be definitely considered as endosomes because of the distance between them and the apical membrane. Moreover at the magnification used, we have not observed silver grains on coated pits nor on coated vesicles. This may correspond to a non-coated endocytosis but until now, the receptor bound protein has been shown to enter cells through coated pits and coated vesicles.²² In most of cellular protein internalisation, the endocytotic phase occurs very quickly, in less than 15 min.²² The longer delays used in our experiment cannot completely explain the absence of the tracer in endocytotic vesicles since the labelling of

enterocytes increased in function of the duration of the experiment, showing that the labelled IF-Cbl complex was continuously internalised by the cells. The iodinated IF-Cbl complex detected in the apical cytoplasm was often localised near the mitochondria. The half distance analysis showed that some of the grains could be assigned to mitochondria for the 120 minutes experiments (Table 2). Such a localisation of the tracer could correspond to an interaction between IF-Cbl complex and the mitochondria as previously speculated.^{23,24} We have not seen any lysosomal localisation of the tracer.⁷ In fact, our experiment needs to be completed not only using short times of delay from 30 seconds up to 10 minutes but also using toxic agents as chloroquine.

Another remaining question was the significance of the presence of the tracer within the infolds of the lateral plasma membrane and within the small vesicles which were seen along this part of the lateral membrane. It seemed that IF was transported *via* small carrier vesicles to the lateral membrane. This may correspond rather to a transcytosis of the molecule from the lumen to the subepithelial tissue spaces and to the blood vessels than to a recycling of IF on the apical membrane. Such a mechanism has been already described for IgG in intestinal epithelial cells of newborn rats²⁵ and for cationic ferritin in epithelial cells of the vas deferens of adult rats.²⁶ In these examples, as well as in our experience, the tracer did not apparently originate directly from the apical membrane and the source of the small transcytotic vesicles remains to be determined.

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