Effects of Aging on Intracellular Transport of Vitamin B₁₂ (B₁₂) in Rat Enterocytes

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(Received June 18, 1982)

Summary To elucidate the effect of aging on the vitamin B₁₂ (B₁₂) transport in enterocytes, young and old (3–4 months and over 1.5 years, respectively) female rats were studied. Two units of rat intrinsic factor (IF), saturated with ⁵⁷Co-labeled cyanocobalamin were orally administered, and the amount of B₁₂ absorbed into each subcellular fraction of enterocytes was assayed. Concentration of endogenous B₁₂ in each subcellular fraction was also studied. Absorption of radioactive B₁₂ in the lysosomal fraction was maximum between 2 and 4 hr in each age group. In the older rats, the amount absorbed was lower than in the young rats. The older rats showed a significantly lower value of endogenous B₁₂ in the mitochondria. It has already been reported by us that there exist two B₁₂ binders in enterocytes: lysosomal and microsomal binders. The concentrations of lysosomal and microsomal binders as well as B₁₂ uptake in the mitochondria were significantly lower in the older rats than in the young rats. These data might help to explain the lower B₁₂ absorption in the lysosomal fraction and lower B₁₂ contents in the mitochondrial fraction.

Key Words aging effect, vitamin B₁₂ (B₁₂), intrinsic factor (IF), lysosomal binder, microsomal binder, intracellular transport

Decreased absorption of vitamin B₁₂ (B₁₂) in the intestine of the aged has been reported (1–3). This decrease was thought to be due to impaired secretion of gastric intrinsic factor (IF) and was corrected by oral administration of IF to the aged (1, 2). On the other hand, there were other reports, in which no decrease in absorption of B₁₂ could be found in the aged (4–6). Hyams reported a sufficient secretion of IF in the gastric juice in the aged (7). The serum level of B₁₂ was found to be low in the aged by others (8–13).

However, nutritional status including B₁₂ intake must be taken in consideration; low serum B₁₂ levels in the aged do not necessarily reflect decreased B₁₂ absorption, because the intake of foods containing B₁₂ is frequently low in the aged.

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In our laboratory, it has previously been reported that B_{12} binders exist in rat enterocytes and that they have an important role in the intestinal absorption of B_{12} (14–16). Based on these results, the effect of aging on B_{12} absorption was studied in subcellular fractions of rat enterocytes.

MATERIALS AND METHODS

Two groups of female Wistar rats were used in this experiment. One group (young) was 3 to 4 months old and the other, one and a half years old. The results obtained between the two groups were compared.

Oral administration of IF- ^{57}Co labeled cyanocobalamin complex (IF- ^{57}Co - B_{12}). Rat IF was prepared from rat gastric mucosa (17). Before the experiments, the rats were fasted for two days. ^{57}Co - B_{12} (specific radioactivity: 150 $\mu\text{Ci}/\mu\text{g}$. Amercham England) was added to rat IF, capable of binding 2 ng B_{12} (2 units of IF). Unbound B_{12} was removed by changing water outside a Visking tube (Visking Company). Two units of rat IF, saturated with ^{57}Co - B_{12} were orally administered into the stomach through a gastric tube. The rats were then sacrificed under slight anesthesia 1, 2, 4, and 6 hr, after the administration of IF- B_{12} complex. The middle two-thirds of the small intestine was extirpated and the intraluminal contents were washed out with a 0.25 M cold sucrose solution. Subsequently, the intestine was gently everted with a glass rod, and the mucosal surface was washed with cold sucrose solution. The mucosa was scraped off with a glass slide, and the amount of B_{12} absorbed in the mucosa was determined by measuring the radioactivity in the mucosal scraping in a well-type γ scintillation counter.

Subcellular fractionation of enterocytes. The scraped mucosa was homogenized in 5 volumes of 0.25 M sucrose Tris-HCl buffer, pH 7.5, with a Teflon homogenizer, and the subcellular fractions (mitochondria, lysosomes, microsomes, and cellular sap) were prepared by differential ultracentrifugation by a modification of the Robinson's method. These procedures were described in detail in our previous paper (14). The purity of microsomal, mitochondrial and lysosomal fractions was assessed by measurement of alkaline phosphatase, cytochrome oxidase and acid phosphatase. The amount of B_{12} absorbed in each fraction was determined by measuring the radioactivity.

Measurements of endogenous B_{12} in subcellular fractions. After fasting for 2 days, ten rats of each age group were sacrificed, and the subcellular fractions of their enterocytes were separated by differential ultracentrifugation. Each fraction was previously solubilized by addition of Triton solution to obtain a final concentration of 0.2% (v/v). The amount of endogenous B_{12} contained in each fraction was measured by the radioactive competition method using Phadebas kit (Pharmacia Diagnostics, Sweden).

Separation of large and small binders in microsomal and lysosomal fractions. Lysosomal and microsomal fractions were pre-treated with Triton solution to obtain a final concentration of 0.2% (v/v), and 300 pg of radio- B_{12} was added to

each fraction followed by incubation overnight.

The treated subfractions were applied to a 2 × 30 cm Sephadex G-200 column. B₁₂ binders were eluted with 0.5 M NaCl at a flow rate of 20 ml/hr. Unsaturated B₁₂ binding capacities were determined by measuring radioactivity at each elution peak. Half of the remaining fractions were applied to the Sephadex column without radio-B₁₂. Gel fractions corresponding to these radio-peaks were collected for the assay of endogenous B₁₂.

B₁₂ uptake to mitochondria enhanced by small binder. The second radioactive peak of lysosomal and microsomal fractions in the young rats was collected and concentrated by carbowax No. 20000 (14). After addition of radio-B₁₂, the collected peak was completely dialyzed against 0.01 M sodium phosphate buffer, pH 7.5, and applied to a 1 × 19 cm DEAE-cellulose column, and eluted by stepwise elution using 0.01 M sodium phosphate buffer, pH 7.5, followed by 0.06 M sodium phosphate, pH 6.3, and finally by 1 M sodium chloride. The radioactive peak eluted by the second buffer described above was collected as the purified small binder, which promotes B₁₂ uptake to the mitochondria. Fresh mitochondria were prepared from rat enterocytes (14). The incubation mixture consisted of aliquots of small binder obtained from lysosomal or microsomal fraction and fresh mitochondrial suspensions. In one group, CaCl₂ containing solution (10 meq) was added to the mixture, while, in the other group, it was not. In control experiments, free radio-B₁₂ unbound to the binder was used instead of the binder-B₁₂ complex, the mixtures were incubated at room temperature for 2 hr and were centrifuged at 15,000 × *g* for 20 min. Mitochondrial pellets obtained after centrifugation were washed three times with a 0.25 M sucrose solution to exclude radio-B₁₂ unbound to the pellets, the radioactivity found in mitochondrial pellets was measured.

Table 1. Total amount of B₁₂ absorbed in enterocytes and amounts of B₁₂ absorbed in subcellular fraction of enterocytes (pg B₁₂).

		Time after oral administration			
		1 hr	2 hr	4 hr	6 hr
Young	Total	62.3 ± 32.5	123.6 ± 48.2	28.8 ± 14.5	24.0 ± 11.0
	Lysosome	22.6 ± 12.1	67.9 ± 32.9	7.5 ± 4.4	8.1 ± 4.8
	Microsome	7.7 ± 3.8	33.7 ± 13.7	7.6 ± 4.1	5.3 ± 2.4
	Mitochondria	2.5 ± 0.8	5.5 ± 2.5	1.1 ± 0.6	0.8 ± 0.4
	Sap	37.3 ± 16.2	21.6 ± 7.4	14.3 ± 3.6	9.6 ± 3.9
Old	Total	82.7 ± 21.8	84.2 ± 6.9	33.0 ± 18.4	26.0 ± 15.6
	Lysosome	33.2 ± 4.2	36.5 ± 3.1	13.3 ± 8.6	13.8 ± 9.3
	Microsome	9.8 ± 1.1	18.7 ± 7.5	9.8 ± 5.5	6.1 ± 3.3
	Mitochondria	3.0 ± 2.0	3.6 ± 2.3	0.7 ± 0.4	0.6 ± 0.3
	Sap	36.7 ± 17.3	18.9 ± 2.7	9.2 ± 4.2	7.0 ± 4.1

Each value represents mean ± SD of four rats.

RESULTS

Total amount of B₁₂ absorbed in the intestinal mucosa

The total amount of B₁₂ absorbed in the mucosa reached a maximum value at 2 hr after oral administration, and decreased sharply between 2 and 4 hr regardless

Fig. 1. Amounts of B₁₂ absorbed in subcellular fraction of enterocytes. Radioactive B₁₂ in the lysosomal, microsomal and mitochondrial fractions, are given by white columns, shaded columns, and stippled columns, respectively. Each value represents mean $\pm$ SD of four rats.

Table 2. Concentrations of endogenous B₁₂ in each subcellular fraction in rat enterocytes (pg B₁₂/mg protein).

	Lysosome	Microsome	Mitochondria	Sap
Young	298 $\pm$ 108	232 $\pm$ 119	553 $\pm$ 173	155 $\pm$ 103
Old	266 $\pm$ 149	224 $\pm$ 87	310 $\pm$ 133	304 $\pm$ 71

Each value represents mean $\pm$ SD of ten rats.

of the group. In the older rats, absorption of B₁₂ in the mucosa was lower than that in the young rats. Especially, the difference between two groups was the largest at 2 hr (Table 1).

Amounts of B₁₂ absorbed in subcellular fraction

The amounts of B₁₂ absorbed in the lysosomal fraction, microsomal fraction and mitochondrial fraction are shown in Table 1 and Fig. 1. In each fraction,

Fig. 2. Gel filtration of lysosomal and microsomal fractions on Sephadex G-200 column. Unsaturated B₁₂ binding capacity and endogenous B₁₂ concentration of each eluate are shown in the figure. Solid line shows radioactivity bound to binding protein on the left-sided vertical axis. Shaded columns show endogenous B₁₂ shown on the vertical axis to the right. The first elution peak, large binder appeared at the elution site of transcobalamin I in the serum. The second elution peak, small binder, appeared at the elution peak of transcobalamin II. The small binder has the function to transport B₁₂.

maximum absorption was seen at 2 hr, and decreased sharply between 2 and 4 hr after oral administration of the IF-B₁₂ complex. The amounts of B₁₂ absorbed in the lysosomal fraction were lower in the older rats than in the young rats at 2 hr ($p < 0.05$). Little radioactive B₁₂ was absorbed into the mitochondrial fraction.

Concentration of endogenous B₁₂ in subcellular fraction

The contents of endogenous B₁₂ in subcellular fraction are shown in Table 2. The concentrations of endogenous B₁₂ in the mitochondria in the young and older rats were 553 ± 173 (SD) pg B₁₂/mg protein and 310 ± 133 (SD) pg B₁₂/mg protein, respectively. The concentrations in the older rats were less than those in the young ($p < 0.005$). The amount of endogenous B₁₂ in the cellular sap in the older was 304 ± 71 (SD) pg B₁₂/mg protein which was higher than that in the young ($p < 0.005$).

Concentrations of small and large binders in lysosomal and microsomal fraction

The results of gel filtration of the lysosomal and microsomal fractions are shown in Fig. 2. Three main peaks appeared in the elution. The first peak appeared at the elution site of transcobalamin I (TC I) in the serum (molecular size, about 120,000) and the second peak appeared at the elution site of transcobalamin II (TC II) (molecular size, about 40,000). The third peak shows the unbound free B₁₂. The property of each fraction was described in detail elsewhere (14, 15).

Table 3. Concentrations of large B₁₂ binders in enterocytes (pg B₁₂/g mucosa). Unsaturated B₁₂ binding capacity and endogenous B₁₂ contents of large binders.

		Lysosome	Microsome
Young	Unsaturation	17 ± 5	26 ± 7
	Endogenous	171 ± 46	154 ± 114
Old	Unsaturation	12 ± 4	22 ± 11
	Endogenous	197 ± 90	147 ± 37

Each value represents mean $\pm$ SD of seven rats.

Table 4. Concentrations of small B₁₂ binders in enterocytes (pg B₁₂/g mucosa). Unsaturated B₁₂ binding capacity and endogenous B₁₂ contents of small binders.

		Lysosome	Microsome
Young	Unsaturation	107 ± 52	174 ± 31
	Endogenous	160 ± 42	281 ± 61
Old	Unsaturation	66 ± 28	91 ± 38
	Endogenous	94 ± 8	267 ± 58

Each value represents mean $\pm$ SD of seven rats.

Fig. 3. B₁₂ uptake to mitochondria obtained from enterocytes. Amounts of B₁₂ absorbed to mitochondria prompted by small binder is shown in this figure. The result is expressed by pg B₁₂/mg protein/mg binder. White columns are the results of the young rats, and shaded columns are those of the older rats.

Table 3 shows concentrations of the large binders in lysosomal and microsomal fractions. Little difference in the concentration of large binders was found between the young and the older rats.

Table 4 shows concentrations of the small binders found in the lysosomal and microsomal fraction. Unlike the result of large binder, endogenous contents of lysosomal fraction and unsaturated capacity of the binder in the microsomal fraction were significantly lower in the older rats than in the young rats. (Endogenous B₁₂ contents in lysosomal fraction: $p < 0.05$. Unsaturated B₁₂ contents of microsomal fraction: $p < 0.05$).

B₁₂ uptake to mitochondria

The values of radio-B₁₂, shown in Fig. 3, are expressed by pg B₁₂/mg protein of mitochondria/mg binder and have been already subtracted from those of the control. Small binder was prepared from the young rats. B₁₂ uptake to the mitochondria was significantly lower in the older rats than in the young rats ($p < 0.01$), regardless of the presence of Ca²⁺ ion.

DISCUSSION

Mechanisms of intracellular transport of B₁₂ by lysosomes similar to one proposed in this study have been reported by Newmark in rat kidney (18), and by Pletsch and Coffey in rat liver (19). In their reports, it was suggested that the delivery of B₁₂ from serum TC II occurs by pinocytotic mechanisms which are

accompanied by lysosomal fusion. The same conclusion was also reached by Turner *et al.* (20), who used ^{125}I -labeled TC II and chloroquine, an inhibitor of lysosomal proteolysis; they observed that, in human fibroblasts, chloroquine prevented the degradation of TC II. Our results indicate impaired function of lysosomes in this pinocytotic process in older rats.

Concerning the contents of endogenous B_{12} , the most characteristic feature was lower concentrations of B_{12} in the mitochondria in older rats compared to young rats, while greater amounts of B_{12} remained in the sap. To explain this mechanism, the following experiment was performed.

The amount of B_{12} binders in lysosomal and microsomal fraction was assayed and compared between the young and the older age groups. The amounts of small binders in lysosomes and microsomes in the older rats were lower than in the young rats. However, little difference was seen in the concentration of large binders between the two groups.

As previously reported (15), the small binder promotes B_{12} uptake to the brush border and mitochondria in rat enterocytes and rat reticulocytes. The decrease of these small binders in the older rats reflects the decreased uptake of B_{12} to lysosomal fraction, and the decreased amount of endogenous B_{12} in the mitochondrial fraction.

The existence of intracellular B_{12} binder has been demonstrated in several studies. Pletsch and Coffey reported that in rat liver cells, B_{12} binders were in the lysosomal fraction as well as in the microsomal fraction, which were found to be eluted at the site of rat TC II on a column of Sephadex G-100 and CM-cellulose (21). Ryel *et al.* observed TC II-like B_{12} binders in the sap in mouse L 1210 leukemic lymphoblasts (22). Green *et al.* also observed a B_{12} binding protein, the molecular size of which was similar to TC II, in sonicated cell lysate of mouse fibroblasts (23).

The existence of an intracellular large binder, similar in molecular size as R-binder or cobalophilin (24), was reported in many studies. Recently, these large binders were found to be B_{12} dependent enzymes: methyl-malonyl CoA mutase or methionine synthetase (25, 26).

Gams *et al.* reported that B_{12} uptake by isolated rat liver mitochondria was enhanced by rat plasma TC II (27). In our laboratory, it was reported that these small B_{12} binders in the lysosomal and microsomal fractions promoted B_{12} uptake to mitochondria in rat enterocytes (28).

The endogenous B_{12} in the mitochondrial fraction was found to be lower in the older rats. To explain this result, B_{12} uptake to mitochondria obtained from the young and the older rats in the presence of small lysosomal and microsomal binder was studied. B_{12} uptake to mitochondria was significantly impaired in the older rats. Small binders in the older rats were as effective in enhancing B_{12} uptake to mitochondria as those in the young rats. In the older rats, impaired uptake of B_{12} to mitochondria might explain the lower content of endogenous B_{12} in mitochondria. The mechanism for this phenomenon has not been studied yet. Further in-

vestigation is needed for its clarification.

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