PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 212-214 (1976)

Studies on Vitamin B_{12} Binding Proteins in Human Saliva (39176)

SUDHIR KUMAR, MANOHAR RATHI, AND LEO M. MEYER

Perinatal Laboratory, Christ Hospital, Oak Lawn, Illinois and Hematology Division, Veterans Administration Hospital, Brooklyn, New York

While many workers in recent years have demonstrated the presence of at least three vitamin B_{12} binding proteins in normal serum, human saliva has been described to consist of only one binding protein, namely, transcobalamin I, or the higher molecular weight protein. TC-II and TC-III have not been reported in saliva.

In our attempts to standardize a technique of isolating these vitamin B_{12} binders on a small DEAE-cellulose column (1), we also used saliva from patients to determine the effect of different buffers on the pattern of elution of salivary binding proteins. Data, so accumulated, indicate that saliva not only contains both TC-II and TC-III in small amounts, but that the TC-II of saliva is also capable of mediating B_{12} uptake by human lymphocytes transformed with phytohemagglutinin (PHA) *in vitro*.

Materials and methods. Saliva from normal human volunteers, both male and female, were obtained, centrifuged to remove any particulate matter and the clear saliva stored at −20°. Once thawed, samples were not re-used. Radioactive cyanocobalamin ([^{57}Co]B_{12}, sp act 182 mCi/mg) was obtained from Amersham/Searle.

DEAE-cellulose column chromatography. DEAE-cellulose was prepared according to Gizis and Meyer (7). To 0.5 ml of saliva, 250 pg of [^{57}Co]B_{12} were added and the mixture was incubated at 37° for 20 min. After counting, the mixture was dialyzed against a liter of 0.0175 *M* sodium phosphate buffer, pH 6.3, overnight, and subjected to chromatography on DEAE-cellulose columns as described by Kumar *et al.* (1). The isolated fractions were collected, dialyzed against large volumes of distilled water in the cold for 24 hr and concentrated on a minicon B 15 micro-concentrator (AMICON).

Gel-filtration on Sephadex G-200 column. Two milliliters of saliva were incubated with 1000 pg of [^{57}Co]B_{12} at 37° for 20 min and the mixture dialyzed against 4 liters of 0.005 *M* sodium phosphate buffer, pH 7.4, containing 1 *M* NaCl, overnight at 4°. Dialyzed saliva was placed on a Sephadex G-200 column (2.6 × 60 cm; void vol, 118 ml; prepared according to the manufacturer Pharmacia), pre-equilibrated with the same buffer used for dialysis. Elution was also performed with the same phosphate buffer. Two-milliliter samples were collected at a flow rate of 10 ml per hr, and counted for 2 min each in an automatic well-type scintillation counter (Nuclear Chicago, Model 1185).

Uptake of saliva B_{12}-binding proteins by transformed human lymphocytes. Human lymphocytes were prepared and transformed with PHA, as described by Meyer *et al.* (9). The transformed lymphocytes were suspended in 0.02 *M* tris-saline, pH 7.4, to give a suspension of about 3 × 10^6 cells/ml. Frozen transcobalamins were thawed and suspended in 0.02 *M* tris-saline buffer, pH 7.4. Transcobalamins (each containing about 5000 cpm) and suspensions (1 ml) of transformed lymphocytes were incubated at 37° for 2 hr, centrifuged, pellets washed with normal saline three times, and counted in an automatic scintillation counter. All uptake studies were done in duplicate.

Results and discussion. Chromatography on DEAE-cellulose column. Results of chromatography on DEAE-cellulose columns are presented in Table I. Three distinct peaks of radioactivity were obtained. TC-II with 0.06 *M*, pH 5.85 buffer; TC-III with 0.1 *M*, pH 5.8 buffer; and TC-I with 0.25 *M*, pH 5.4 buffer. Percentage distribution of [^{57}Co]B_{12} binders in saliva of normal adults, both male and female, was TC-II, 10 ± 3; TC-III, 15 ± 3; and TC-I, 75 ± 5. While the total binding of radioactive B_{12} by saliva of normal adults and that from females on oral contraceptives was equal, the distribution

of B_{12} binders in saliva of 15 females on oral contraceptives showed an entirely different pattern with a much higher level of TC-II (63 ± 7) and a lower TC-I (25 ± 5). Level of TC-III was not markedly affected (12 ± 4).

Gel-filtration of saliva on Sephadex G-200 column. Only one peak of radioactivity was obtained when saliva from either male or female donors, including females on oral contraceptives, was chromatographed on a Sephadex G-200 column. More than 95% of the applied radioactivity was recovered in a peak corresponding in elution to TC "L" when normal human serum is chromatographed on Sephadex G-200 column (4).

Uptake of salivary B_{12}-binders by transformed human lymphocytes. Results of a representative experiment on delivery of radioactive B_{12} by binding proteins of saliva, introduced to transformed human lymphocytes, are presented in Table II. While there was no appreciable uptake of [^{57}Co]B_{12} and its complex with TC-III, 8% of total radioactivity of TC-II and 5% of TC-I was taken up by lymphoblasts. A large increase in the uptake of radioactivity of both TC-II and TC-I occurred when the incubation medium also contained 1 m*M* $CaCl_2$, indicating thereby that Ca^{2+} enhances the uptake of radioactivity by these transformed human lymphoblasts.

Our results indicate that the observation of earlier workers, that human saliva has only a higher molecular weight binder, was incorrect, and that it does have the other binding proteins, namely, TC-II and TC-III, as well. Also, an increase in the level of TC-II in saliva of women on oral contraceptives indicates that hormones play an important role in the metabolism of B_{12} in humans. The actual role played by these hormones is not presently understood.

The recovery of three radioactive peaks on DEAE-cellulose corresponding in elution to TC-II, TC-III, and TC-I when normal serum is chromatographed on DEAE-cellulose, compared to recovery of only one peak of radioactivity on gel-filtration on Sephadex G-200 indicates the possibility of isozymic forms of TC "L" in saliva, having different charges, but similar molecular weight. This is in agreement with our earlier studies, where we observed that the two peaks of radioactivity obtained by gel-filtration of B_{12}-labeled human serum on Sephadex G-200, namely TC "L" and TC

TABLE I. DISTRIBUTION OF [^{57}Co]B_{12} BINDING PROTEINS IN HUMAN SALIVA.

	Number of specimens	Total binding[a] (%)	Distribution of radioactivity % of total bound		
			TC-II	TC-III	TC-I
Normal saliva	25	85 ± 4	10 ± 3	15 ± 3	75 ± 5
Saliva from women on oral contraceptives	15	87 ± 3	63 ± 7	25 ± 5	12 ± 4

[a] The assay system contained 500 pg of [^{57}Co]B_{12}/ml of saliva. The figures represent the percentage of total [^{57}Co]B_{12} bound/ml of saliva.

TABLE II. UPTAKE OF B_{12} BOUND TO TRANSCOBALAMINS BY TRANSFORMED HUMAN LYMPHOCYTES IN VITRO.

Sample	Assay without added Ca^{2+}			Assay with added Ca^{2+}		
	Total initial counts in assay (cpm)	Uptake		Total initial counts in assay (cpm)	Uptake	
		Counts in pellet (cpm)	% total		Counts in pellet (cpm)	% total
Control [^{57}Co] B_{12} only	4970	32	0.6	4929	35	0.6
Saliva + B_{12}	5215	287	5.5	5217	432	8.2
Fractions:						
TC-I B_{12}	5004	275	5.4	5100	425	8.3
TC-II B_{12}	5046	417	8.3	4976	995	20.0
TC-III B_{12}	4976	35	0.7	5123	35	0.7

"S", each resulted in three B_{12} binding proteins when subjected to further chromatography on DEAE-cellulose (11).

In earlier studies, it has been shown that only TC-II B_{12} of serum is capable of mediating uptake of B_{12} by L-1210 leukemic lymphoblasts, whereas saliva tagged with B_{12} is not; however, in the present studies using human lymphoblasts for uptake studies, we have found that saliva is also capable of mediating B_{12} uptake. It is quite possible that the two cell systems have completely different properties, and, hence, we observe these differences. Further studies at elucidating the role of hormones on the metabolism of B_{12} in humans are in progress.

1. Kumar, S., Meyer, L. M. and Gams, R. A., Proc. Soc. Exp. Biol. Med. **147,** 377 (1974).
2. Hall, C. A., and Finkler, A. E., Blood **27,** 611 (1966).
3. Retief, F. P., Gottlieb, C. W., Kochwa, S., Pratt, P. W., and Herbert, V., Blood J. Hematol. **29,** 501 (1967).
4. Lawrence, C., Blood J. Hematol. **33,** 899 (1969).
5. Gizis, E. J., Dietrich, M. F., Choi, G., and Meyer, L. M., J. Lab. Clin. Med. **75,** 673 (1970).
6. Bloomfield, F. J., and Scott, J. M., Brit. J. Hematol. **22,** 33 (1972).
7. Gizis, E. J., and Meyer, L. M., *in* "Methods in Enzymology" (S. P. Colowick, N. O. Kaplan, D. B. McCormick, and L. D. Wright, Eds.), Vol. 18, Part C, p. 127. Academic Press, New York (1971).
8. Gizis, E. J., and Meyer, L. M., Proc. Soc. Exp. Biol. Med. **140,** 326 (1972).
9. Meyer, L. M., Miller, I. F., Gizis, E. J., Tripp, E., and Hoffbrand, A. V., Proc. Soc. Exp. Biol. Med. **146,** 747 (1974).
10. Meyer, L. M., Gams, R. A., Ryel, E. M., Miller, I. F., and Kumar, S., Proc. Soc. Exp. Biol. Med. **147,** 679 (1974).
11. Kumar, S., Meyer, L. M., and Gams, R. A., Fed. Proc. **34,** 905 (1975).

Received July 24, 1975. P. S. E. B. M. 1976, Vol. 151.