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Blocking and Binding Type Antibodies against All Major Vitamin B_{12}-Binders in a Pernicious Anaemia Serum

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SUMMARY. The simultaneous occurrence of blocking and binding antibodies to intrinsic factor (IF), transcobalamin (TC- II, TC I) and other R type vitamin B_{12} proteins in the serum of a patient with treated pernicious anaemia (PA) is reported here for the first time. The dialysed and purified immunoglobulin-G (IgG), but not the immunoglobulin-M (IgM), from a PA patient neutralized the total unsaturated vitamin B_{12} binding capacity ($UB_{12}BC$) of human gastric juice, saliva and serum and also of rabbit serum, suggesting that the PA IgG contained blocking antibodies against, IF, TC II, TC I and other R-binders. In addition, the PA IgG and IgM preparations contained binding antibodies since they could form macromolecular complexes with ^{57}Co-B_{12} bound to IF, TC I or TC II so that each one of the latter was totally excluded from Sephadex G-200. The presence of the binding antibodies was further confirmed by the formation of radioactive precipitation lines on agarose with each one of the vitamin B_{12}-binders bound to ^{58}Co-B_{12}. The PA serum did not exhibit any measurable $UB_{12}BC$ after dialysis against 7·5 M guanidine-HCl followed by renaturation with phosphate buffer (pH 7·4). But it did form TC I and TC II complexes with ^{57}Co-B_{12} when the latter was added during the renaturation step indicating that the serum contained circulating immunoglobulin–TC complexes. The blocking antibodies should be distinguished from the previously described binding antibodies. The blocking of the binding of vitamin B_{12} to TCs resulted in relatively lower serum vitamin B_{12} levels in the present case in contrast to the presence of binding antibodies where high serum vitamin B_{12} levels have been reported. In addition, the binding antibodies form immunocomplexes with TCs which can easily be detected because they can bind radioactive vitamin B_{12} while the corresponding immunocomplexes of blocking antibodies are hidden because they prevent the binding of the vitamin to TCs.

Three immunologically and physicochemically distinct vitamin B_{12}-binding proteins (B_{12}-binders) are known to be involved in the transport of vitamin B_{12} of most animals studied (Gräsbeck, 1975). Intrinsic factor (IF) is a gastric glycoprotein (Hoedemaeker *et al*, 1964) and binds most of the dietary vitamin B_{12}. The complex of IF and vitamin B_{12} is transported distally

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where it attaches to specific IF-vitamin B_{12}-receptors located on the apical membrane of the ileal enterocyte. The vitamin B_{12} subsequently appears in the portal blood bound to transcobalamin I (TC I) and transcobalamin II (TC II) (Hall & Finkler, 1965). The TC II delivers vitamin B_{12} to various tissue cells (Cooper & Parachych, 1961; Retief *et al*, 1967). The biological importance of IF and TC II can readily be demonstrated by the fact that in their absence severe megaloblastic anaemia occurs (Cooper & Castle, 1960; Hakami *et al*, 1971; Hitzig *et al*, 1974; Burman *et al*, 1977). In the most common form of the Addisonian pernicious anaemia (PA), autoantibodies against gastric parietal cells and IF are found in a high percentage of the cases (see Chanarin, 1969; Schwartz, 1958). Antibodies preventing the coupling of vitamin B_{12} to IF are called blocking or type II. The binding or type I antibodies react with antigenic determinants remote from the vitamin B_{12}-binding site (see Glass, 1974) and interfere with the uptake of the IF–vitamin B_{12} complex by ileal mucosa (Schade *et al*, 1966). The physiologic activity of IF is not adversely affected by the presence of anti-IF antibodies in the serum (Kaplan *et al*, 1963). Probably the antibodies produced locally in the stomach are of greater importance (Fisher *et al*, 1966; Rose & Chanarin, 1969). These have been suggested to cause the final failure in the secretion of IF (Rose *et al*, 1970) or to inhibit the vitamin B_{12} absorption from the alimentary tract (Schade *et al*, 1966). Antibodies against each one of the serum TCs were identified in PA patients overloaded with hydroxocobalamin (Skouby *et al*, 1971; Carmel & Shurafa, 1977). These antibodies did not result in any clear-cut pathological state although their presence in PA patients has been associated with increased cobalamin levels (Carmel & Shurafa, 1977).

Blocking antibodies against TCs have not been reported to occur in human sera. The present report describes the identification in a PA serum of blocking and binding antibodies against all known B_{12}-binders. A preliminary report of this work has been presented (Marcoullis *et al*, 1978b).

SUBJECTS, MATERIAL AND METHODS

Subjects and collection of material. 20–30 ml of venous blood was collected from healthy adult volunteers and subjects with proven PA, allowed to clot at room temperature, and the serum was separated by centrifugation at 3000 rpm for 30 min.

The patient, whose serum was studied here (T.W. serum), was a female aged 82 in 1974 when a diagnosis of PA was made. At the time of diagnosis the patient had a haemoglobin of 6·8 g/dl, a red blood cell count of $1{\cdot}7 \times 10^{12}$/l, a white blood cell count of $3{\cdot}4 \times 10^{9}$/l and fulfilled the usual criteria of macrocytosis and megaloblastic bone marrow changes. The serum vitamin B_{12} was 10 ng/l. Vitamin B_{12} absorption was measured by the Schilling test (Schilling, 1953) with use of two sequential 24 h urine collections, each preceded by an injection of 1 mg cyanocobalamin (Lowenstein *et al*, 1960). Doses of 1·0 μg of ^{57}Co-B_{12} were given free or bound to gastric juice. After administration of the mixture of gastric juice and ^{57}Co-B_{12}, the fraction of the oral dose excreted during 2 d rose to 12·4% from a basal value of 0·6% when the dose was given without gastric juice. Anti-IF and anti-parietal cell antibodies were also present in her serum. Collected and assayed as described below, the patient's IF could bind only 9 ng vitamin B_{12} per ml gastric juice.

Gastric juice, after augmented histamine stimulation (Kay, 1953), was collected through a

nasogastric tube from human healthy volunteers as well as from the PA patients. The juice was depepsinized first by raising the pH to 10·0 with dropwise addition of 1 M NaOH, followed 20 min later by a reduction of the pH to 7·0 with 1 M HCl. The pH was stabilized at 7·4 by adding 0·5 volume 0·1 M phosphate buffer, pH 7·4, containing 0·15 M NaCl. The preparations were centrifuged at 27 000 ***g*** for 30 min. The results of these gastric juices were expressed per vol of original gastric juice. 20 ml saliva were collected from a normal subject, ultrafiltered to 20 ml volume, and centrifuged as described above. All sera, gastric juices and the saliva were stored at −20°C until use.

Gel exclusion chromatography. Sephadex G-200 (Pharmacia, Sweden) columns were packed in the form of beds 2·5 cm wide and 100 cm long. When serum TCs were studied, the buffer used was 0·05 M phosphate (pH 7·4) containing 0·75 M in respect to NaCl and 0·02% (w/v) NaN_3. In all other runs the buffer was the same except that the concentration in respect to NaCl was lowered to 0·154 M. The runs were carried out at +4°C with a flow of 12–13 ml/h. 2 ml fractions were collected.

Purification of vitamin B_{12}-binding proteins. The antisera prepared and reported previously (Marcoullis *et al,* 1977) were used for purification of vitamin B_{12}-binding proteins. The IF and R binder from gastric juice and the TC I and TC II from sera were purified (Marcoullis *et al,* 1977) by using the above-mentioned specific immune sera and gel filtration; the B_{12}-binder not required in an experiment was removed as its immune-complex with a specific antibody in the excluded volume of Sephadex G-200, while the other required binder was recovered as a well-defined peak in the included volume. Due to lack of specific anti-human TC II serum, serum TC I was purified solely by gel filtration (Marcoullis *et al,* 1978a). Thus, each of the B_{12}-binders in human gastric juice and serum was purified in the sense that the preparation contained only one type of B_{12}-binder.

Double immunodiffusion in gels. Double immunodiffusion in gels followed by autoradiography was carried out according to Samloff & Barnett (1965). Briefly, 2·5 μl incubation mixtures containing the serum under study and B_{12}-binder preparations saturated with ^{58}Co-B_{12} (peripheral wells) were allowed to react with an equivalent volume of horse anti-human serum (Hoechst, Germany) (central well) on 1·5% (w/v) agarose gels prepared with 0·075 M phosphate buffer (pH 7·4) containing 0·15 M NaCl. After precipitation lines were formed, the ^{58}Co-radioactive lines were visualized by autoradiography using X-ray dental film (Kodak) in contact with the dried gel for 7–28 d. Precipitation lines were stained after autoradiography using Amido black (Ouchterlony & Nilsson, 1973).

Radioassays. The unsaturated vitamin B_{12}-binding power ($UB_{12}BC$) of biological fluids and tissue extracts was measured using haemoglobin-coated charcoal and ^{57}Co-B_{12} (1 Ci/g) (Gottlieb *et al,* 1965). To quantitate the IF content, the preparations were incubated with excess blocking type antibody from PA sera. The residual $UB_{12}BC$ was taken to represent the concentration of the non-intrinsic factor (non-IF) B_{12}-binders (Gottlieb *et al,* 1965).

Preparation of marker—and other—proteins. Immunoglobulin (IgG) was purified, using the caprylic acid method of Steinbuh & Audran (1969). Immunoglobulin M (IgM) was prepared by precipitations at low ionic strength and pH 6·0 (Wager *et al,* 1971). To ensure that both proteins were stable under the present experimental conditions and to further purify them, the IgG and IgM preparations were filtered through columns equilibrated with the buffers described above. Sephadex G-200 and Sepharose 6B were used for fractionating the IgG and

IgM preparations, respectively. The protein peaks eluting with K_d values characteristic for each one of the immunoglobulins (Marcoullis & Gräsbeck, 1977) were concentrated by ultrafiltration to required volumes and stored at +4°C until use.

Other procedures and materials. ^{125}I-labelling was performed using chloramine-T according to the method of Greenwood *et al* (1963) and human serum albumin (Miles Laboratories, U.S.A.) and IgG prepared from patients as well as from normal subjects were iodinated. Protein determinations were carried out (Lowry *et al*, 1951) using human serum albumin as standard. Vitamin B_{12} was measured by microbiological assay using *Lactobacillus leichmannii* (Spray, 1955; Biggs *et al*, 1964). Ultrapure guanidine-HCl was purchased from Schwarz/Mann (U.S.A.) and ^{57}Co-B_{12} (250 Ci/g), [^{125}I]sodium iodide (100 mCi/ml) and [^{3}H]water ($^{3}H_2O$, 100 mCi/g) were purchased from Amersham (England).

RESULTS

Preliminary Observations Made with the Pernicious Anaemia Serum

Following diagnosis, the PA patient (T.W.) received a transfusion of packed red blood cells and several intramuscular injections of hydroxocabalamin. The patient required 10 consecutive injections given weekly (totally 12 mg vitamin) before a haematologic remission was observed. At this stage her haematological status was now found to be markedly improved; the haemoglobin was 14 g/dl, RBC $4{\cdot}23 \times 10^{12}$/l and WBC $4{\cdot}6 \times 10^{9}$/l and examination of a bone-marrow aspirate showed normal proliferation and maturation of all the cellular elements. The serum vitamin B_{12} was 1600 ng/l. The patient refused to receive any further treatment but her serum, prepared and re-examined 6 months later by the haemoglobin-coated charcoal procedure, was found to be capable of neutralizing the total $UB_{12}BC$ of several normal gastric juices (a volume of pooled gastric juices having a total $UB_{12}BC$ of 80 000 ng vitamin B_{12} could be neutralized by 1 litre T.W. serum). the T.W. serum itself did not exhibit any measurable $UB_{12}BC$ and this might have indicated that the serum contained excess endogenous vitamin B_{12} or perhaps vitamin B_{12} from previous injections of hydroxocobalamin. The serum vitamin B_{12}, however, measured at the same time was found to be as low as 350 ng vitamin B_{12}/l. In addition, the serum behaved similarly after exhaustive dialysis against 250 volumes of 0·15 M NaCl with changes at 12, 24 and 48 h, or after removing free vitamin B_{12} with haemoglobin-coated charcoal (Gottlieb *et al*, 1965).

Evidence for the Presence of Blocking Antibodies against Gastric Vitamin B_{12}-Binders

Since the presence of excess endogenous vitamin B_{12} could not be mistaken as the reason for neutralizing the $UB_{12}BC$ of gastric juice, the most plausible cause for this would be the presence of blocking antibodies against both the IF and the R-binders. Indeed, the T.W. serum was tested similarly and found to neutralize also the $UB_{12}BC$ of concentrated human saliva (a volume of saliva having a total $UB_{12}BC$ of 16 400 ng vitamin B_{12} could be neutralized by 1 litre T.W. serum).

Evidence for the Presence of Blocking Antibodies against Transcobalamins

2-ml aliquots of pooled normal human sera, having a $UB_{12}BC$ of 700 ng vitamin B_{12}/l, were mixed with excess ^{57}Co-B_{12} (2·5 ng vitamin B_{12}, 0·1 μCi) and filtered separately through

Sephadex G-200. Fig 1(a) shows the mean radiochromatogram of ^{57}Co-B_{12} complexed to serum vitamin B_{12}-binding proteins, i.e. TC I and TC II. 0·5-ml aliquots of T.W. serum were subsequently incubated (1 h at room temp.) with the normal human sera (0·5–2·0 ml) described above. Following addition of excess ^{57}Co-B_{12}, the amount and form of bound vitamin B_{12} in the incubation mixtures were assayed using haemoglobin-coated charcoal and gel filtration. The precipitation of vitamin B_{12} with haemoglobin-coated charcoal indicated that the ^{57}Co-B_{12} previously added into the mixtures remained completely free (unbound). The Sephadex G-200 radiochromatogram (Fig 1b) confirmed that TC I and TC II, previously shown to carry ^{57}Co-B_{12} (see Fig 1a), had now been neutralized by the T.W. serum (67·1 litres of normal human serum having a total $UB_{12}BC$ of 47 300 ng vitamin B_{12} could be neutralized by 1 litre of T.W. serum). These observations indicated that the T.W. serum contained blocking antibodies against both serum vitamin B_{12}-binders, i.e. the TC II and TC I. To provide further evidence for this, ordinary rabbit sera known to contain only, or mainly, TC II (Finkler *et al,* 1969) were tested similarly as described above after incubating with the T.W. serum. Again, neutralization of the $UB_{12}BC$ of the rabbit serum occurred (0·8 litre rabbit serum having a total $UB_{12}BC$ of 11 500 ng vitamin B_{12} could be neutralized by 1 litre T.W. serum).

Identification and Classification of the Blocking Antibodies

Purified IgG and IgM fractions prepared from the T.W. serum were added separately to aliquots of normal human sera, gastric juices and saliva and incubated as described above followed by addition of excess ^{57}Co-B_{12}. Precipitation of free vitamin B_{12} with haemoglobin-coated charcoal indicated that the IgG, but not the IgM, preparation had the capacity to completely neutralize the $UB_{12}BC$ of normal gastric juice, saliva and sera. This further supported the suggestion that the neutralization could not be due to the presence of free vitamin B_{12} as the latter would not be present in the purified IgG preparation.

In addition, special methodology was designed in order to demonstrate and identify the presence of the blocking antibodies. First, ^{57}Co-B_{12} was added to the crude T.W. serum, and, after a 1 h incubation, filtered through Sephadex G-200. All the vitamin eluted in the total volume of the column (Fig 1b), indicating that the serum did not contain a free vitamin B_{12}-binder capable of carrying some of the vitamin. This confirmed the previous observation in which no measurable $UB_{12}BC$ could be detected in the T.W. serum when the haemoglobin-coated charcoal procedure was used. The same observation (absence of any protein bound to ^{57}Co-B_{12}) was made when 1 mg of the purified IgG fraction from the T.W. patient was incubated with 0·5 ml normal human serum, having a $UB_{12}BC$ of 700 ng vitamin B_{12}/l, followed by saturation with ^{57}Co-B_{12} and finally filtration through the same columns (Fig 1b). When, however, the same experiments were repeated using the T.W. IgM instead of the IgG fractions, two proteins corresponding to TC I and TC II were found to carry the bulk of the added ^{57}Co-B_{12} as in Fig 1(a). These results show that the serum contained an excess of blocking antibodies against the serum TC I and TC II, and that these antibodies are present in the IgG class. The same observations were made when normal gastric juice was used instead of normal serum.

Demonstration of in Vivo Formed Immune-complexes with TC I and TC II

The patient's serum was mixed with 1·5 vol and dialysed against 25 vol of 0·1 M phosphate

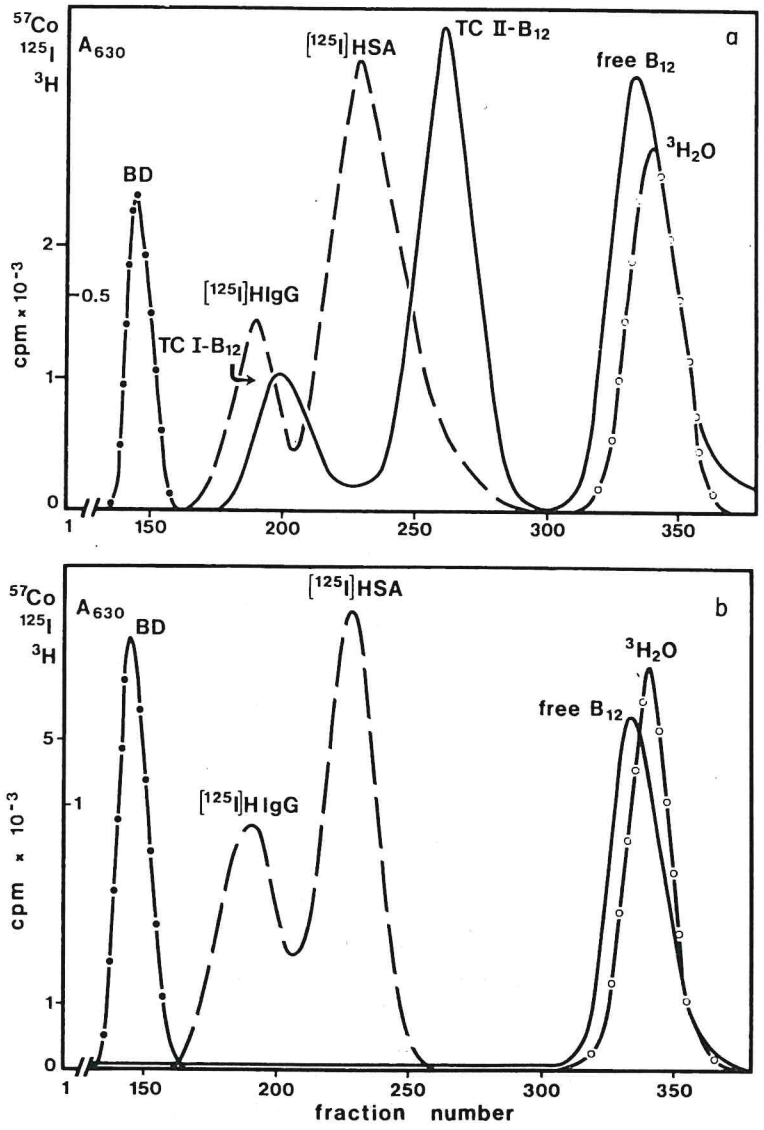
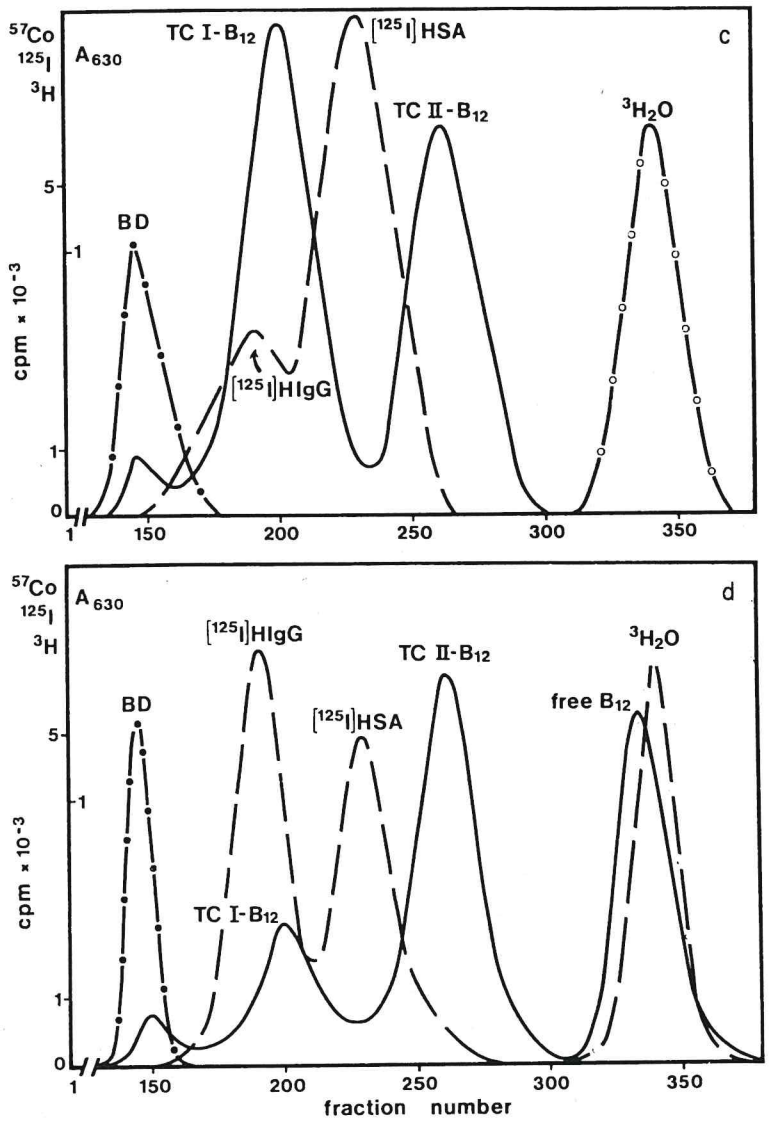


FIG 1. Sephadex G-200 radiochromatograms. ^{125}I-labelled marker proteins (----) were mixed and filtered simultaneously with the samples. Normal human serum (NH serum) saturated with [^{57}Co]vitamin B_{12} resolved (a) into three ^{57}Co-radioactive complexes (———). The first peak elutes after ^{125}I-labelled human immunoglobulin-G ([^{125}I]HIgG) and corresponds to the serum transcobalamin I–vitamin B_{12} complex (TC I-B_{12}). The second peak elutes after the ^{125}I-labelled human serum albumin ([^{125}I]HSA) and it corresponds to the transcobalamin II–vitamin B_{12} complex (TC II-B_{12}). The last peak eluting near the total volume of the column (see $^{3}H_2O$; [^{3}H]water, line o–o) corresponds to the free [^{57}Co]vitamin B_{12} (free B_{12}). When the same normal serum was mixed and incubated with the the pernicious anaemia serum under study (T.W. serum) prior to adding ^{57}Co-B_{12}, all the vitamin eluted (b) as free B_{12} near the total volume of the column. A similar pattern (see b) was observed when the experiment was repeated using IgG purified from the T.W. serum instead of the crude T.W. serum indicating that the 'neutralizing' factor in the T.W. serum is an IgG



antibody. The crude T.W. serum mixed with ^{57}Co-B_{12} and run as such also behaved similarly (as in Fig 1b). All endogenous vitamin B_{12}, free or protein bound, was removed from the T.W. serum by dialysis, first against 7·5 M guanidine-HCl, and then against the chromatographic buffer to remove guanidine-HCl and renature the proteins. ^{57}Co-B_{12} added to this preparation all eluted as free vitamin B_{12} (as in Fig 1b). When the ^{57}Co-B_{12} was added during the renaturation step, TC I–^{57}Co-B_{12} and TC II–^{57}Co-B_{12} complexes (see c, line ——) were observed. A portion of the ^{57}Co-radioactivity eluted in the total excluded volume of the column (see Fig 1c, BD; Blue Dextran, line o–o). When normal human serum (e.g. serum used in Fig 1a) was saturated with ^{57}Co-B_{12} and then incubated with the T.W. serum, again totally excluded ^{57}Co-radioactive complexes (d) were observed indicating that binding type antibodies were present in the T.W. serum. Similar totally excluded ^{57}Co-radioactive complexes were also formed when the T.W. serum was incubated with IF–^{57}Co-B_{12}, TC I–^{57}Co-B_{12}, TC II–^{57}Co-B_{12} or with R binder–^{57}Co-B_{12} from human gastric juice.

buffer, pH 7·4, containing 7·5 M ultrapure guanidine-HCl with changes at 12, 24, 48 and 72 h, to denature binding protein and liberate bound vitamin B_{12}. Aliquots of this dialysate were renatured by dialysis against 0·05 M phosphate buffer, pH 7·4, containing 0·75 M NaCl and 0·02% (w/v) NaN_3 in the absence and in the presence of 3 ng ^{57}Co-B_{12} per ml serum. The serum renatured in the absence of vitamin was assayed by the haemoglobin-coated charcoal method, but it did not exhibit any measurable $UB_{12}BC$. A 2 ml aliquot of the same preparation to which 0·1 ng ^{57}Co-B_{12} was added was filtered through Sephadex G-200. No ^{57}Co-radioactive complexes of TC I or TC II were observed (Fig 1b). The radioactivity of the aliquot of serum renatured in the presence of radiovitamin was measured. The undialysed vitamin was calculated to be 989 ng vitamin B_{12}/l of serum and this indicated that the suspension of the T.W. serum in guanidine-HCl containing solution had made free vitamin B_{12}-binding sites available. When this serum was filtered through a Sephadex G-200 column, the bulk of the radiovitamin eluted as TC I (22%) and TC II (75%) bound (Fig 1c). A small fraction (approximately 3%) of the radiovitamin was totally excluded. These results further support the suggestion that the absence of $UB_{12}BC$ in the T.W. serum could not be due to complete saturation of the serum TCs by endogenous vitamin B_{12}. The latter, if present, would have been dissociated in the presence of guanidine-HCl, removed during the dialysis, and would have thus enabled the detection of $UB_{12}BC$ in the T.W. serum renatured in the absence of vitamin B_{12}. The results obtained using the T.W. serum which was renatured in the presence of radioactive vitamin B_{12}, however, indicated the presence of TC I and TC II (see Fig 1c). As the lack of $UB_{12}BC$ in the T.W. serum renatured in the absence of the vitamin could have been due to degradation of TCs because of the treatment with guanidine-HCl, a control experiment using normal human serum instead of the T.W. serum was carried out in the same way. This preparation did not decrease its $UB_{12}BC$ after dialysis against the guanidine-HCl solution but rather increased by 52% its $UB_{12}BC$, apparently because endogenous vitamin B_{12} had been removed during dialysis with guanidine-HCl. Gel filtration demonstrated a more than 100% recovery of both the serum TC I and TC II (Fig 1a).

Demonstration and Characterization of Binding Antibodies

Evidence for the presence of binding antibodies was obtained during the studies on the blocking antibodies (see above) where 3% of the ^{57}Co-B_{12} coupled to the T.W. serum during dialysis to remove guanidine was found to be totally excluded from Sephadex G-200. To verify this, the normal human serum (used in Fig 1a) or normal gastric juice was saturated with ^{57}Co-B_{12} and excess vitamin removed either by dialysis or haemoglobin-coated charcoal. The preparation was then incubated with either crude T.W. serum or with the T.W. IgM preparation or with the T.W. IgG preparation, and then filtered through Sephadex G-200. In all cases, 6–13% of the protein-bound radiovitamin was eluted near or in the excluded volume of the column (Fig 1d) indicating that the T.W. serum did contain binding antibodies against IF, TC I and TC II. In addition, when the ^{125}I-labelled T.W. IgG preparation was used, approximately 5–7% of the ^{125}I-radioactivity was totally excluded from the gel. Similar experiments were repeated using separately purified TC I, TC II and IF, all saturated with radiovitamin. In all cases, ^{57}Co-radioactive complexes were eluted in the excluded volume. The results indicate the presence of IgG and IgM binding antibodies against all these B_{12}-binders. The fact that only a small fraction of the ^{125}I-labelled T.W. IgG preparation was

totally excluded shows that only a small part of this immunoglobulin represents specific binding antibody. The presence of these antibodies in the serum was further confirmed using a double immunodiffusion technique. In these experiments, both the IgG and IgM fractions were tested and found to contain precipitating antibodies against each one of the known B_{12}-binders.

Persistence of the Blocking and Binding Antibodies

Although the serum of this patient was prepared in the year 1974, these studies were carried out 3 years later on her serum. Large quantities of serum were then prepared from the same patient in the year 1977 and a similar series of experiments was repeated. The results were the same, although the titres of the blocking antibodies of the T.W. serum were reduced by 25%. Between 1974 and 1977 the patient refused treatment and therefore she did not receive any vitamin B_{12} injections.

DISCUSSION

The intriguing neutralization of the total $UB_{12}BC$ of normal human gastric juice observed following its incubation with the T.W. serum prompted the present studies. Thus, the absence of any measurable $UB_{12}BC$ in a pool of gastric juices preincubated with the T.W. serum indicated that the latter might contain both anti-IF and anti-R binder antibodies. As saliva contains only one B_{12}-binder immunologically related to R binders from other sources (Simons, 1964), the neutralization of the $UB_{12}BC$ of saliva induced by the T.W. serum confirmed the assumption that the serum contained anti-R binder antibodies. Since all the R binders are immunologically similar (Simons, 1964), it is probable that the anti-R binder antibody would react with the serum TC I of the normal sera. Indeed, the reduction of the $UB_{12}BC$ of normal human sera observed after incubation with the T.W. serum supported this view. In addition, the complete neutralization of the $UB_{12}BC$ of normal sera induced by the T.W. serum indicated that this also contained blocking antibodies against the other serum vitamin B_{12}-binder, TC II. The fact that the T.W. serum exhibited similar properties after the removal of free or bound endogenous vitamin B_{12} by haemoglobin-coated charcoal or by dialysis against buffers, and against solutions containing high concentrations of guanidine-HCl, respectively, showed that the 'neutralizing factor' was not dialysable and ruled out the possibility that these phenomena might be due to the presence of excess vitamin B_{12}. Finally, the finding that the 'neutralizing' properties of the T.W. serum reside with its IgG fraction demonstrated that all the above properties were derived from several blocking antibodies present in the IgG class. The fact that these antibodies are not present in the IgM class is consistent with similar observations (Samloff *et al,* 1968) about the blocking anti-IF antibodies from PA sera. Also, the finding that some binding antibodies are present in the IgM class is in line with previous observations (Samloff *et al,* 1968) that these antibodies (as regards IF) are present both in the IgG and IgM class (see also Glass, 1974).

Guanidine-HCl is known to potentially dissociate the bonds of the vitamin from its transport proteins (Allen & Majerus, 1972) as well as of most antigen–antibody complexes (Ruoslahti, 1976). Consequently, the fact that crude T.W. serum did not bind any added ^{57}Co-B_{12} while it bound the vitamin in the presence of guanidine showed that immune-

complexes of TC I and TC II existed in T.W. serum and, as a result of this, the serum appeared to have no measurable $UB_{12}BC$. The same results showed that vitamin B_{12} can compete with these antibodies in the presence of guanidine for binding to TCs. It is well known (Gräsbeck, 1969; Hippe & Olesen, 1971), however, that most of the B_{12}-binders are more resistant to degradation in the presence of vitamin B_{12}. It could be postulated that the absence of $UB_{12}BC$ from the T.W. serum after treatment with guanidine in the absence of the vitamin was due to degradation of the TCs. This possibility was subsequently ruled out by treating normal human serum with guanidine in the absence of the vitamin. The $UB_{12}BC$ of these sera was not reduced but increased.

The identification of the *in vivo* formed immune-complexes containing 'neutralized' TC II may indicate that such blocking antibodies may exist in low concentrations in a number of patients with treated pernicious anaemia but, apparently, were not detected in the past, being masked by native TC II (in the present case the detection of these antibodies was possible because they were present in excess to the total vitamin B_{12} binding capacity of the T.W. serum).

The presence of blocking, and sometimes binding, antibodies against IF in a high percentage of cases of PA patients has been well documented and these findings serve as valuable diagnostic aids (see Chanarin, 1969; Glass, 1974). Several PA patients have been reported having binding antibodies against serum TCs following treatment with long-acting preparations of hydroxocobalamin. The anti-TC II antibodies and especially the blocking anti-TC II antibodies may be of particular importance since this protein represents the important vitamin B_{12} transport protein in the blood and other biological fluids. It is possible, but not established, that as in cases of congenital absence of TC II neutralization by specific antibodies directed towards the vitamin B_{12}-binding site of TC II may have contributed to the vitamin B_{12} deficiency state of patient T.W. as well as of other PA patients.

A finding which distinguishes the anti-TC blocking antibodies here from previously identified anti-TC binding antibodies is the relatively lower serum vitamin B_{12} level measured in the present case in contrast to the presence of binding antibodies where high serum vitamin B_{12} levels have been reported (Carmel & Schurafa, 1977). In addition, the immune-complexes of TCs with binding antibodies can bind radioactive vitamin B_{12} which in turn enables their identification using numerous techniques, e.g. gel filtration or electrophoretic techniques. The analogous immune-complexes, however, with blocking antibodies cannot be readily detected because they do not bind the vitamin.

Perhaps the formation of all these antibodies, but not of the pre-existing anti-IF antibodies, had been induced by treatment of the patient with hydroxocobalamin. In addition, the simultaneous occurrence of antibodies against each one of the B_{12}-binders in this patient (T.W.) who had been treated with hydroxocobalamin supports the suggestion that a genetically determined defect in immunological tolerance of antigens (Whittingham *et al*, 1969) may exist in PA.

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REFERENCES

ALLEN, R.H. & MAJERUS, P.W. (1972) Isolation of vitamin B_{12}-binding proteins using affinity chromatography. I. Preparation and properties of vitamin B_{12}-Sepharose. *Journal of Biological Chemistry*, **247**, 7695–7701.

BIGGS, J.C., MASON, S.L.A. & SPRAY, G.H. (1964) Vitamin-B_{12} activity in red cells. *British Journal of Haematology*, **10**, 36–42.

BURMAN, D.L., MOLLIN, R.A., SLADDEN, N.S., SOURIAL, N. & GREANY, M. (1977) Inherited deficiency of transcobalamin II causing megaloblastic anaemia. (Abstract). *British Journal of Haematology*, **35**, 676–677.

CARMEL, R. & SCHURAFA, M. (1977) Circulating immunoglobulin–transcobalamin I complex in patients with elevated serum vitamin B_{12} levels. (Abstract). *Federation Proceedings*, **36**, 1121.

CHANARIN, I. (1969) *The Megaloblastic Anaemias*. Blackwell Scientific Publications, Oxford.

COOPER, B.A. & CASTLE, W.B. (1960) Sequential mechanisms in the enhanced absorption of vitamin B_{12} by intrinsic factor in the rat. *Journal of Clinical Investigation*, **39**, 199–214.

COOPER, B.A. & PARANCHYCH, W. (1961) Selective uptake of specifically bound cobalt-58 vitamin B_{12} by human and mouse tumour cells. *Nature*, **191**, 393–395.

FINKLER, A.E., HALL, C.A., GREEN, P.D. & YOUNG, C.C. (1969) Cross reactivity of mammalian B_{12} transport proteins. (Abstract). *Federation Proceedings*, **28**, 700.

FISHER, J.M., REES, C. & TAYLOR, K.B. (1966) Intrinsic factor antibodies in gastric juice of pernicious-anaemia patients. *Lancet*, **ii**, 88–89.

GLASS, G.B.J. (1974) *Gastric Intrinsic Factor and Other Vitamin B_{12} Binders*. Thieme, Stuttgart.

GOTTLIEB, C., LAU, K.-S., WASSERMAN, L.R. & HERBERT, V. (1965) Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity. *Blood*, **25**, 875–884.

GRÄSBECK, R. (1969) Intrinsic factor and the other vitamin B_{12} transport proteins. *Progress in Hematology*, **6**, 233–260.

GRÄSBECK, R. (1975) Absorption and transport of vitamin B_{12}. *British Journal of Haematology*, **31** (Suppl.), 103–110.

GREENWOOD, F.C., HUNTER, W.M. & GLOVER, J.S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochemical Journal*, **89**, 114–123.

HAKAMI, N., NEIMAN, P.E., CANELLOS, G.P. & LAZERSON, J. (1971) Neonatal megaloblastic anemia due to inherited transcobalamin II deficiency in two siblings. *New England Journal of Medicine*, **285**, 1163–1170.

HALL, C.A. & FINKLER, A.E. (1965) The dynamics of transcobalamin II. A vitamin B_{12} binding substance in plasma. *Journal of Laboratory and Clinical Medicine*, **65**, 459–468.

HIPPE, E. & OLESEN, H. (1971) Nature of vitamin B_{12} binding. III. Thermodynamics of binding to human intrinsic factor and transcobalamins. *Biochimica et Biophysica Acta*, **243**, 83–89.

HITZIG, W.H., DOHMANN, U., PLUSS, H.J. & VISCHER, D. (1974) Hereditary transcobalamin II deficiency: clinical findings in a new family. *Journal of Pediatrics*, **85**, 622–628.

HOEDEMAEKER, P.J., ABELS, J., WATCHERS, J.J., ARENDS, A. & NIEWEC, H.O. (1964) Investigation about the site of production of Castle's gastric intrinsic factor. *Laboratory Investigation*, **13**, 1394–1399.

KAPLAN, M.E., ZALUSKY, R., REMINGTON, J. & HERBERT, V. (1963) Immunologic studies with intrinsic factor in man. *Journal of Clinical Investigation*, **42**, 368–382.

KAY, A.W. (1953) Effect of large doses of histamine on gastric secretion of HCl. An augmented histamine test. *British Medical Journal*, **ii**, 77–80.

LOWENSTEIN, L., COOPER, B.A., BRUNTON, L. & GARTHA, S. (1960) An immunological basis for acquired resistance to oral administration of hog intrinsic factor and vitamin B_{12} in pernicious anemia. *Journal of Clinical Investigation*, **40**, 1656–1662.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.

MARCOULLIS, G. & GRÄSBECK, R. (1977) Solubilized intrinsic factor receptor from pig ileum and its characteristics. *Biochimica et Biophysica Acta*, **496**, 36–51.

MARCOULLIS, G., SALONEN, E.-M. & GRÄSBECK, R. (1977) Isolation of vitamin B_{12}-binding proteins by combined immuno and affinity chromatography. Comparative studies on the isolated and unisolated

proteins. *Biochimica et Biophysica Acta*, **495**, 336–348.

MARCOULLIS, G., SALONEN, E.-M. & GRÄSBECK, R. (1978a) Porcine serum cobalophilin and transcobalamin. Identification, isolation and properties including electrofocusing patterns. *Biochimica et Biophysica Acta*, **534**, 48–57.

MARCOULLIS, G., PARMENTIER, Y., NICOLAS, J.P. & PERRIN, M.O. (1978b) Two antibody types against all vitamin B_{12} binders in a pernicious anaemia serum. *XVII International Congress of Haematology*, July 1978, Paris, France, no. 59/1181-4.

OUCHTERLONY, O. & NILLSSON, L.A. (1973) Immunodiffusion and immunoelectrophoresis. *Handbook of Experimental Immunology* (ed. by D. M. Weir), 2nd edn, vol. 1, p. 37. Blackwell Scientific Publications, Oxford.

RETIEF, F.P., GOTTLIEB, C.W. & HERBERT, V. (1967) Delivery of $Co^{57}B_{12}$ to erythrocytes from α and β globulin of normal, B_{12}-deficient, and chronic myeloid leukemia serum. *Blood*, **29**, 837–851.

ROSE, M.S. & CHANARIN, I. (1969) Dissociation of intrinsic factor from its antibody: application to study of pernicious anaemia gastric juice specimens. *British Medical Journal*, **i**, 468–470.

ROSE, M.S., CHANARIN, I., DONIACH, D., BROSTOFF, J. & ARDEMAN, S. (1970) Intrinsic-factor antibodies in absence of pernicious anaemia: 3–7-year follow-up. *Lancet*, **ii**, 9–12.

RUOSLAHTI, E. (1976) Immunoabsorbents in protein purification. *Scandinavian Journal of Immunology* (Suppl. 3), pp. 1–86.

SAMLOFF, I.M. & BARNETT, E.V. (1965) Identification of intrinsic factor autoantibody and intrinsic factor in man by radioimmunodiffusion and radioimmunoelectrophoresis. *Journal of Immunology*, **95**, 536–541.

SAMLOFF, I.M., KLEINMAN, M.A., TURNER, M.D., SOBEL, M.V. & JEFFRIES, G.H. (1968) Blocking and binding antibodies to intrinsic factor and parietal cell antibody in pernicious anemia. *Gastroenterology*, **55**, 575–583.

SCHADE, S.G., ABELS, J. & SCHILLING, R. (1967) Studies on antibody to intrinsic factor. *Journal of Clinical Investigation*, **46**, 615–620.

SCHADE, S.G., FEICK, P., MUCKERHEIDE, M. & SCHILLING, R.F. (1966) Occurrence in gastric juice of antibody to a complex of intrinsic factor and vitamin B_{12}. *New England Journal of Medicine*, **275**, 528–531.

SCHILLING, R.F. (1953) Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. *Journal of Laboratory and Clinical Medicine*, **42**, 860–866.

SCHARTZ, M. (1958) Intrinsic-factor-inhibiting substance in serum of orally treated patients with pernicious anaemia. *Lancet*, **ii**, 61–62.

SIMONS, K. (1964) Vitamin B_{12} binders in human body fluids and blood cells. *Commentationes Biologicae*, **27** (Fasc. 5), pp. 1–94. Societas Scientiarum Fennica.

SKOUBY, A.P., HIPPE, E. & OLESEN, H. (1971) Antibody to transcobalamin II and B_{12} binding capacity in patients treated with hydroxocobalamin. *Blood*, **38**, 769–774.

SPRAY, G.H. (1955) An improved method for the rapid estimation of vitamin B_{12} in serum. *Clinical Science*, **14**, 661–667.

STEINBUCH, M. & AUDRAN, R. (1969) The isolation of IgG mammalian sera with the aid of caprylic acid. *Archives of Biochemistry and Biophysics*, **134**, 279–284.

WAGER, O., RÄSÄNEN, J.A., HALTIA, K. & WASASTJERNA, C. (1971) M components with antibody activity. Anti-smooth muscle, anti-thyroglobulin and anti-streptolysin-O activity in five M component sera. *Annals of Clinical Research*, **3**, 86–97.

WHITTINGHAM, S., MACKAY, I.R., UNGAR, B. & MATHEWS, J.D. (1969) The genetic factor in pernicious anaemia. *Lancet*, **i**, 951–954.