Studies on Antibody to Intrinsic Factor *

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Summary. Sera from a group of 79 patients with pernicious anemia were studied for the presence of antibody to intrinsic factor. Two general types of antibody activity were found, and it was possible to distinguish three groups of pernicious anemia sera on the basis of their content of these types.

Type I antibody blocks the binding of radioactive vitamin B_{12} to intrinsic factor when added to intrinsic factor before the B_{12}; it is not detected on intrinsic factor when added after B_{12}. This antibody blocks intrinsic factor-mediated B_{12} absorption *in vivo* when mixed in the sequence intrinsic factor + antibody I + B_{12}, but not when mixed in the sequence intrinsic factor + B_{12} + antibody I.

Type II antibody reacts with intrinsic factor when B_{12} is attached. This antibody prevents the absorption of B_{12} from intrinsic factor in pernicious anemia patients when mixed in the sequence intrinsic factor + B_{12} + antibody II, and is thereby distinguished from antibody I.

Introduction

The presence of antibodies to intrinsic factor in the sera of patients with pernicious anemia is well established (1–8). Two general types of test have been used to detect the antibody activity against human intrinsic factor (IF) *in vitro*. One type detects the ability of certain pernicious anemia sera to inhibit the binding of radioactive vitamin B_{12} (B_{12}) by IF as demonstrated by dialysis (5), gel filtration (5), charcoal adsorption (6), and zirconyl phosphate gel adsorption (7) techniques. These sera also inhibit IF–mediated absorption of B_{12} when IF, serum, and B_{12}, mixed in that sequence, are fed to a patient with pernicious anemia (5). The second type of test detects the combination of antibody with IF–radioactive B_{12} (IF–B_{12}) complex. This combination of antibody with IF–B_{12} may be demonstrated by electrophoretic (3), coprecipitation (4, 9, 10),

gel filtration (11), and immunodiffusion techniques (12, 13). Sera containing this antibody have been shown to inhibit IF–mediated B_{12} absorption when IF, B_{12}, and serum, mixed in that sequence, are fed to a patient with pernicious anemia (3, 14, 15). The sequences differ in that the antibody–containing serum is added to the IF before the B_{12} in the first sequence and after the B_{12} in the latter sequence.

For the purposes of this study, sera that block the binding of B_{12} by IF are said to contain antibody I activity, and sera that contain antibodies reactive with the IF–B_{12} complex are said to contain antibody II activity. Preliminary reports from this laboratory (11, 14, 15) and elsewhere (16) have indicated that these two activities are the result of the action of at least two different types of antibody molecules: one that will react with IF and block B_{12} binding, and one that will react with the IF–B_{12} complex.

If there are two different types of antibody molecules to IF, theoretically it should be possible to find four different classes of pernicious anemia sera based on their content of antibody to IF. One class would contain only type I antibody activity, a second would contain antibody I and II activity, a third would contain only antibody II activity, and a fourth would contain no antibody activity.

Furthermore, these different sera should have different *in vivo* effects depending on the order in which the serum is added to the mixture of IF and B_{12}. Thus, a serum that has only antibody 1 activity would be expected to inhibit IF–mediated B_{12} absorption only when added to the IF before the addition of the B_{12}, since this serum, by definition, does not react with the IF–B_{12} complex. Sera with antibody II activity might inhibit B_{12} absorption when added to the IF–B_{12} complex, if antibody were not digested off the IF–B_{12} molecule before reaching the ileum.

The present study reports *in vitro* and *in vivo* observations on three of the four classes of pernicious anemia sera described above and confirms and extends previous reports on the existence and behavior of the two types of antibody activity associated with these sera.

This report describes our observations on *in vitro* testing for antibody activity to IF in sera from a group of 79 patients with pernicious anemia and reports the effects of different sera in two *in vivo* testing procedures. In one procedure, serum is added to IF before the addition of B_{12}; in the other procedure, the serum is added after the B_{12}.

Methods

1) In vitro observations

Serum collected during the past 4 years from patients with pernicious anemia has been stored frozen and was thawed immediately before use. Three different tests were used to detect antibody to IF in these sera. The source of IF used in these tests was pepsin–inactivated neutralized normal human gastric juice collected and prepared as previously described (17). Throughout this report IF means neutralized normal human gastric juice.

Charcoal adsorption technique. Sera that block the attachment of B_{12} to IF were detected by the test described by Ardeman and Chanarin (6). A mixture containing 1 vol of gastric juice, 4 vol of serum, and 1 vol of B_{12}-^{60}Co (100 ng per ml, SA 1 nc per ng) was diluted to 4 ml. Then 0.6 ml of this mixture was removed as a standard. To two 1.5-ml samples of the remainder was added 1 ml of a mixture of bovine serum albumin and charcoal, prepared as described by Gottlieb, Lau, Wasserman, and Herbert (18), in order to adsorb free vitamin B_{12}. The samples were centrifuged, and the supernatant, containing IF-bound B_{12}, and the standard were counted. The per cent of B_{12} bound by the gastric juice was calculated. The serum: gastric juice ratio (vol/vol) in this test was 4:1.

Electrophoretic retention test. Alkaline starch borate gels were utilized for vertical electrophoresis to detect the presence of antibody to IF according to the procedure of Jeffries, Hoskins, and Sleisenger (3). Mixtures of IF and radioactive B_{12}, to which serum was added in a volume equal to the quantity of gastric juice used, were applied to the slots, and vertical electrophoresis was carried out for 18 hours at 115 v. In this system radioactive B_{12} attached to IF migrates to the anode, free B_{12} migrates to the cathode, and the IF-B_{12} antibody complex remains at the origin or moves only slightly towards the anode. When serum is added to the IF-B_{12} complex and radioactivity is demonstrated at or near the origin after electrophoresis, antibody is present.

Coprecipitation tests. Antibody that is attached to the IF-B_{12} complex may be detected by precipitation with antihuman globulin serum (4), a method we have found to be more sensitive and reproducible than precipitation with ammonium sulfate (9, 10). Rabbit antihuman globulin serum (RAHG) was prepared by injecting rabbits with ethanol-fractionated and DEAE-separated human γ-globulin.[1] The optimal quantity of this RAHG serum that will cause maximal precipitation of a given quantity of antibody II–IF-B_{12} was established by titration. The coprecipitation technique used was as follows: A quantity of gastric juice with a B_{12}-binding capacity of about 70 ng per ml was mixed with an equal volume of B_{12}-^{57}Co solution (100 ng per ml, SA 3 nc per ng) and the mixture dialyzed against running tap water for 48 hours. This was then further diluted with 1 vol of water. The content of gastric juice in the mix was then 1 part in 4 (vol/vol). To 0.05 ml of this mix was added 0.05 ml of the test serum and then 1.1 ml of saline. Two aliquots of 0.1 ml were withdrawn from the mix, and each was precipitated with 0.2 ml of the RAHG serum. The RAHG: serum ratio in the final mix equaled approximately 50:1. The serum: gastric juice ratio was 4:1. The centrifuged precipitate was washed once with saline and assayed for radioactivity.

2) In vivo observations

Experiments were performed to study the *in vivo* effect of different antibody-containing sera. The sera were grouped into two different classes: AbI designates those sera with only antibody I activity,[2] and AbI,II designates those sera with both antibody I and II activity, as determined *in vitro*. These sera were then tested for

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[1] Given to us through the kindness of H. L. Deutsch and R. L. Johnson.

[2] One serum, that of J.L., contained a trace amount of antibody II, but in this test was used as an antibody I serum. At the time of these tests, this serum had been tested for antibody II only by electrophoresis at a 1:1 ratio of serum to gastric juice, and at that ratio antibody II was not demonstrated. When tested later by coprecipitation with RAHG at a 4:1 ratio of serum to gastric juice, the presence of antibody II was revealed. However, even at a 4:1 ratio, this serum did not precipitate the amount of B_{12} precipitated by other more potent sera. This serum, having a low titer of antibody II, was, therefore, treated as an antibody I serum in these *in vivo* tests.

their *in vivo* activity when administered with IF and B_{12} in two different sequences to patients with pernicious anemia. In the first sequence, B_{12} was added to the IF and then the serum was added. In the second sequence, the serum was added to the IF and then B_{12} was added. These mixtures were then fed to patients with pernicious anemia, and an estimate of the B_{12} absorption was made by the urinary excretion test (19). The *in vivo* tests were done on patients in whom intrinsic factor lack (pernicious anemia or total gastrectomy) had been documented earlier by the urinary excretion test.

In one group of experiments, one binding unit of gastric juice was used to provide the intrinsic factor. A "binding unit" is defined here as that quantity of gastric juice which will bind 1 μg of B_{12} by the dialysis method. That this unit gives a measure of IF activity is supported by previous publications from this laboratory (20, 21). In each test 1 μg of B_{12} and a quantity of serum equal in volume to that of the gastric juice were used.

In a second group of experiments, quantities of antibody I were measured in AbI sera and in AbI,II sera by adding increasing quantities of serum to a constant quantity of gastric juice. The B_{12}–binding capacity of the gastric juice and antibody mixture was then determined by the charcoal technique. The binding capacity was plotted against the quantity of antibody serum used, and the region of the curve where the line became horizontal was chosen as the equivalence region. An example is given in Figure 1. A series of four tests on consecutive days was done on each of three patients with one AbI serum and one AbI,II serum. The tests were as follows: *1*) IF + B_{12} + AbI, *2*) IF + AbI + B_{12}, *3*) IF + B_{12} + AbI,II, and *4*) IF + AbI,II + B_{12}. B_{12}-⁵⁷Co and B_{12}-⁶⁰Co were used on alternate days. The tests were done in random order. In these tests, the quantity of serum was adjusted so that in each of the series of four observations, the same quantity of antibody I activity was present.

Results

1) In vitro observations. Figure 2 shows the frequency distribution of the results of the charcoal adsorption tests recorded as per cent of added B_{12} that was bound to gastric juice. Because the B_{12} was added to give a final concentration of 100 ng per ml of gastric juice before treatment with charcoal, the per cent figures also are nanograms of B_{12} bound per milliliter of gastric juice.

For this test, any value below 60% B_{12} bound was considered to indicate the presence of type I antibody to IF. This value is outside 3 SD from the mean of a study of 43 sera from patients without pernicious anemia. The number is, nonetheless, arbitrary, but in most cases a clear distinction between presence and absence of antibody can be made.

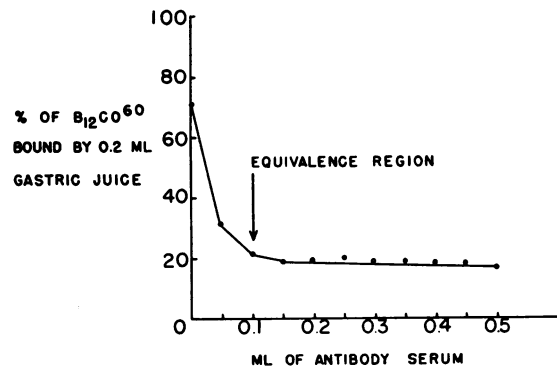The frequency distribution of the results of

FIG. 1. A REPRESENTATIVE TITER CURVE FOR ANTIBODY I. On the horizontal axis is plotted the amount of pernicious anemia serum added to 0.2 ml of normal human gastric juice. This amount was brought to 1 ml with normal serum to equalize the protein content in each instance. Twenty ng of B_{12}-⁶⁰Co was then added and the mixture treated with the bovine serum albumin and charcoal mix as described in the text. The vertical axis records the per cent of the added B_{12} bound by the gastric juice, i.e., remaining in the supernatant fluid after removal of charcoal. The titer is taken as the point where the slope of the curve appears to change maximally. The type of antibody designated "I" blocks the binding of B_{12} to intrinsic factor (IF) when added to IF before B_{12}, as distinguished from antibody "II," which reacts with IF when B_{12} is attached.

the coprecipitation test plotted as picograms of B_{12}-⁵⁷Co precipitated is depicted in Figure 3. The distinction between positive and negative results is clear, and the results were recorded as being either positive or negative for the presence of antibody II. A diagrammatic summary of these results is tabulated in Table I.

Fifty-two sera were tested by both electrophoresis and coprecipitation; all 79 were tested by the coprecipitation technique. The electrophoretic test

TABLE I

The distribution of antibody I and antibody II activity in the 79 patients tested*

	+ Antibody I	− Antibody I
+ Antibody II	27 (34%)	0
− Antibody II	15 (19%)	37 (47%)

* Antibody I blocks the binding of radioactive vitamin B_{12} to intrinsic factor when added to intrinsic factor before B_{12}; antibody II reacts with intrinsic factor when B_{12} is attached.
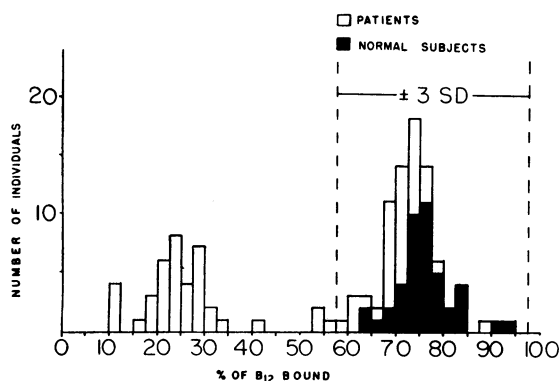
FIG. 2. DISTRIBUTION FREQUENCY OF ANTIBODY I. On the horizontal axis is plotted the percentage of 20 ng of B_{12}-^{60}Co bound by 0.2 ml of normal gastric juice when 0.8 ml of pernicious anemia serum or normal serum is added to the gastric juice before the B_{12}. The number of sera allowing the indicated uptake of B_{12} by gastric juice is recorded on the vertical axis. Antibody to IF was said to be present in those sera allowing less than 60% uptake by the gastric juice. Details of the test are given in the text. The mean of the 43 normal sera tested was 78% with a SD of 6.5%.

gave a negative result in three instances and a questionably positive result in five instances when the coprecipitation test gave a clearly positive result. This is probably due to two factors: 1) The electrophoretic test was performed with a serum: gastric juice ratio of 1:1, whereas the coprecipitation test was performed with a serum:gastric juice ratio of 4:1; and 2) the electrophoretic retention test was at times difficult to interpret because of small amounts of radioactivity left at the origin when normal control serum was used.

Fifteen sera were found that had only antibody I activity at a 4:1 ratio of serum to gastric juice. No sera were found with only antibody II activity, although there was one serum with no detectable antibody I activity when tested at a serum:gastric juice ratio of 1:1, whereas antibody II was easily detectable at that ratio. The data are summarized in Figure 4.

2) *In vivo observations.* In both sets of *in vivo* experiments (see Methods) Figures 5 and 6 demonstrate that AbI sera inhibit IF–mediated B_{12} absorption only when added to IF before B_{12}. AbI,II sera inhibit IF–mediated B_{12} absorption when added to IF either before B_{12} or after B_{12}.

It was conceivable that the data from the first set of *in vivo* observations (Figure 5) were due

simply to a larger quantity of antibody I in the AbI,II sera than in the AbI sera. Therefore, in the second set of *in vivo* observations (Figure 6) the quantities of sera were adjusted so that in each series of four observations the quantity of antibody I was constant. Under these conditions the *in vivo* inhibitory activity of AbI,II sera is clearly different from AbI sera.

Discussion

The *in vivo* activity of these sera corresponds with the *in vitro* activity. It is likely that the *in vivo* effects of AbI sera are due to their preventing the binding of B_{12} to IF, a necessary prelude to IF–mediated absorption of B_{12}.

The *in vivo* activity of antibody I and II molecules indicates that they cannot be readily digested off intrinsic factor by the intestinal system of exo-
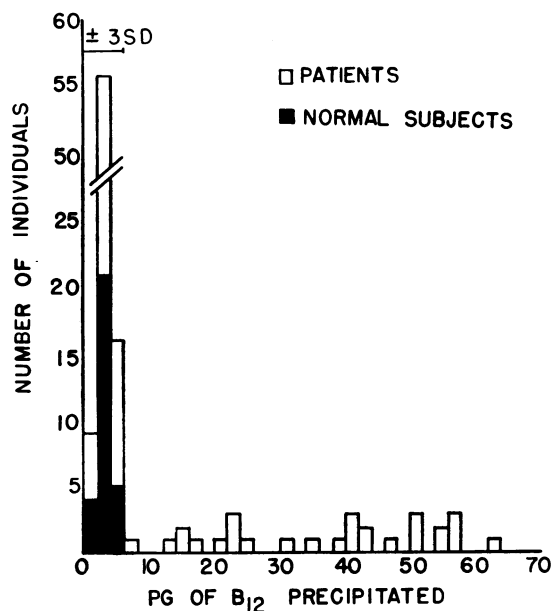

FIG. 3. DISTRIBUTION FREQUENCY OF ANTIBODY II. On the horizontal axis is plotted the picograms of B_{12}-^{57}Co precipitated by rabbit antihuman globulin acting on a mixture of pernicious anemia or normal serum and gastric juice–B_{12}-^{57}Co. The number of sera giving the indicated precipitation is recorded on the vertical axis. Sera causing precipitation of more than 10 pg of B_{12}-^{57}Co were recorded as positive for the presence of antibody to the IF–B_{12} complex. Testing was done at a ratio of 4 parts of serum to 1 part of gastric juice. Details are given in the text. The mean of the 30 normals was 3 pg with a SD of 1 pg. Seventy-nine sera from patients with pernicious anemia were tested.

and endopeptidases. At least enough remains in the case of antibody I to prevent the attachment of B_{12}; and, in the case of antibody II, a sufficient fragment remains attached to the $IF-B_{12}$ complex to block the biologic activity of IF at the ileal absorption site. The inferred resistance to digestion of these proteins when attached to IF appears remarkable, and it may be that their attachment somehow protects them from complete digestion. Alternatively, the antibody II on IF may render the IF susceptible to destruction by digestive enzymes in the gut.

The finding of a set of sera with antibody I activity and a second set of sera with both antibody I and antibody II activity supports the conclusion that in pernicious anemia sera at least two types of molecules acting as antibody to IF may be found (14, 16). Molecules of antibody in the sera with only antibody I activity do not react with the $IF-B_{12}$ molecule. Sera with antibody I and antibody II activity could have two types of molecules, one that reacts with IF only and blocks the attachment of B_{12} and one that can react with $IF-B_{12}$; or they might contain a single type of molecule
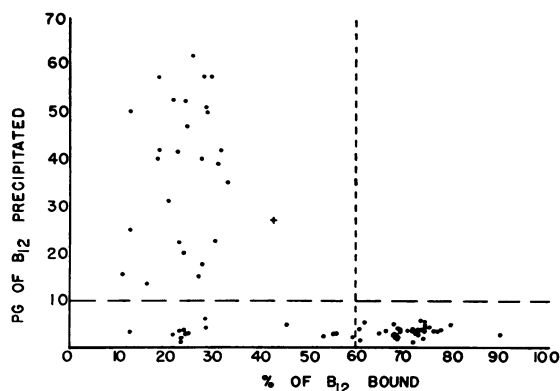

FIG. 4. SCATTERGRAM ILLUSTRATING THE INCIDENCE AND ACTIVITY OF ANTIBODIES TO IF AND THE $IF-B_{12}$ COMPLEX. Each one of the 79 points represents a pernicious anemia serum tested by charcoal adsorption for IF antibody I, i.e., antibody to the B_{12}-binding site (horizontal axis: positive at less than 60% B_{12} bound), and by immunocoprecipitation for IF antibody II, i.e., antibody to the $IF-B_{12}$ complex (vertical axis: positive at greater than 10 pg B_{12} precipitated). Note that antibody II activity as a rule coincides with strong antibody I activity, but that the latter can occur without detectable antibody II. Antibody activity was tested for at a 4:1 serum: gastric juice ratio. + = serum in which antibody I activity was not detected at a 1:1 serum:gastric juice ratio.
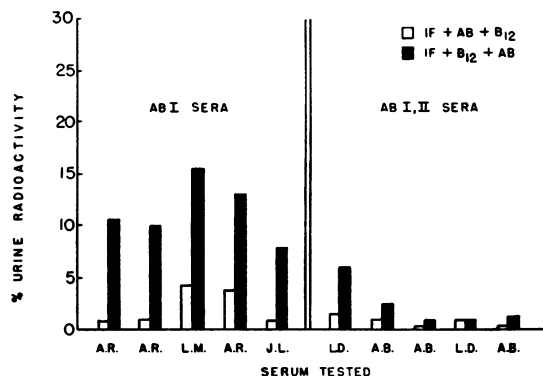

FIG. 5. URINE RADIOACTIVITY TESTS DEMONSTRATING EFFECTS OF ANTIBODY ON VITAMIN B_{12} ABSORPTION. The vertical axis records urine radioactivity as per cent of a 1-μg dose of radioactive B_{12} given to pernicious anemia patients with a quantity of normal gastric juice sufficient to bind 1 μg of B_{12}. The quantity of gastric juice used ranged from 15 to 20 ml. Antibody I serum or antibody I,II serum was given in a volume equal to that of the gastric juice. The results are grouped in sets of two. Each set gives the results of a single serum tested in a single pernicious anemia patient after mixing in two different sequences. The open bar indicates the sequence gastric juice + antibody + B_{12}, and the closed bar indicates the sequence gastric juice + B_{12} + antibody. The vertical double bar separates the results using antibody I serum from the results using antibody I,II serum.

that can both react with IF and block the attachment of B_{12}, or can react with $IF-B_{12}$.

If there are different molecules in pernicious anemia sera, one carrying antibody I activity and one carrying antibody II activity, four classes of serum should be found. One type would contain only antibody I, a second would contain antibody I and II, a third would contain only antibody II, and a fourth no antibody. We have found a serum that has no detectable antibody I activity at a 3:1 ratio of serum to gastric juice, but does have antibody II activity detectable already at a 1:1 ratio. However, at a 4:1 ratio of serum to gastric juice, some antibody I activity does become detectable. This is the serum that was previously reported as having only antibody II activity (14). This finding alone indicates that, at least in this serum, there are two different molecules, one with antibody I and one with antibody II activity, and the antibody II molecules significantly outnumber the antibody I molecules. The fact that a serum with only antibody II present has not been found suggests that this type is quite rare.
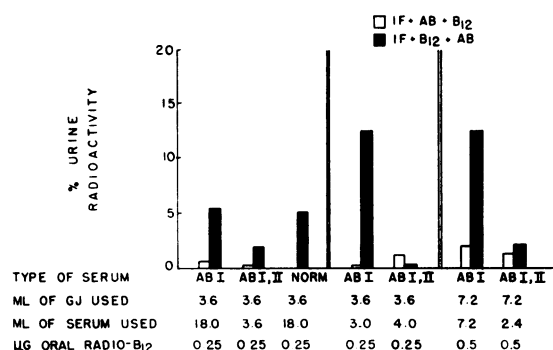
TYPE OF SERUM	AB I	AB I,II	NORM	AB I	AB I,II	AB I	AB I,II
ML OF GJ USED	3.6	3.6	3.6	3.6	3.6	7.2	7.2
ML OF SERUM USED	18.0	3.6	18.0	3.0	4.0	7.2	2.4
UG ORAL RADIO-B_{12}	0.25	0.25	0.25	0.25	0.25	0.5	0.5

FIG. 6. URINE RADIOACTIVITY TESTS DEMONSTRATING EF-
FECT OF ANTIBODY ON VITAMIN B_{12} ABSORPTION. The verti-
cal axis records urine radioactivity as per cent of the oral
dose of radioactive B_{12} given to pernicious anemia pa-
tients. The dose of B_{12} was 0.25 μg or 0.5 μg and was
given with a quantity of normal human gastric juice
sufficient to bind the amount of B_{12} used. There are
three groups of results separated by double vertical lines.
Within each group, a single pernicious anemia patient
was tested with two different sera. In the first frame,
normal serum was also used. One serum had only anti-
body I activity, and one serum had antibody I and II ac-
tivity. The quantities of sera used were adjusted so that
within each group an equal amount of antibody I ac-
tivity was used for each test. The sera were mixed with
gastric juice and B_{12} in two different sequences as indi-
cated by the open and closed bars. The quantities of
gastric juice, the amount of B_{12}, and the amount of serum
used are recorded in the Figure.

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