Separation of Intrinsic Factor Antibodies from Parietal Cell Antibodies in Pernicious Anemia Serum by Gel Filtration[1] (35553)

ELIZABETH JACOB AND G. B. JERZY GLASS
Gastroenterology Research Laboratory, Department of Medicine, New York Medical College, New York, New York 10029

The circulating intrinsic factor antibodies (IFA) which are found in a large number of patients with pernicious anemia (PA) (1–4) practically always coexist with parietal cell antibodies (PCA). This precludes the utilization of the IFA containing sera for the detection of intrinsic factor (IF) in the parietal cells of the gastric mucosa, by the indirect immunofluorescent reaction with fluorescein labeled anti-IgG. A positive immunofluorescence under these conditions could be due, not to the union of the circulating IFA and IF within the parietal cell but to a complex formed between the coexisting PCA in the serum and the microsomal parietal cell antigen.

Except for the recently described selective absorption of the PCA present in IFA containing PA serum by the mucosal extract from the corpus and fundus of the hog stomach (5), the only other method of obtaining IFA (without PCA contamination) has been the immunization of rabbits with human or hog IF, resulting in formation of circulating antibodies to IF in their serum (6–8).

In the current investigation we have succeeded in developing a new method to separate IFA from coexisting PCA in the sera of PA patients, using gel filtration on Sephadex G-200 and G-150 columns. The separated IFA was free of PCA, it reacted with IF as shown by zirconium gel and charcoal radioimmuno assays, and formed an immune complex within the parietal cell of man in the absence of PCA, which fixed serum complement (C′). The preliminary reports on these findings have been published earlier in the abstract form (9, 10).

Materials and Methods. Sera were obtained from patients with PA whose diagnosis was proven by hematological and isotopic methods, including radio-B_{12} absorption tests by double label hepatic uptake technic (11), and radioimmuno assay of IF in gastric juice by the Ardeman and Chanarin (12) charcoal method, as modified by Yamaguchi and Glass (13) and by the zirconium gel (Z-gel) technic of Hansen *et al.* (14).

Gastric mucosae were obtained at laparotomy from the fundus and body of the stomach in patients undergoing surgery for duodenal ulcer. The gastric specimens were frozen immediately and stored under refrigeration. Only histologically normal specimens were used. Gastric juices were obtained from normal individuals and patients with duodenal ulcer following augmented histamine stimulation (15). The specimens were collected on ice; and the juice, obtained 0–15 and 15–30 min after histamine stimulation, which is the richest in IF (16–18), was filtered through gauze. The pH of the juice was adjusted to 10.0 to inactivate the pepsin and after 0.5 hr it was brought back to pH 7.4. The juice was then centrifuged and the supernatant specimens were kept frozen at —20° until use.

Assays of PCA in PA sera and in the fractions eluted from Sephadex columns were done *in vitro*, using normal human or rat gastric mucosae, by the Coons' indirect immunofluorescent technic as applied to gastric mucosa (3). The fluoresceinated antihuman immunoglobulin G was obtained from Hyland Division Travenol Laboratories Inc. Los Angeles, California. This contained fluores-

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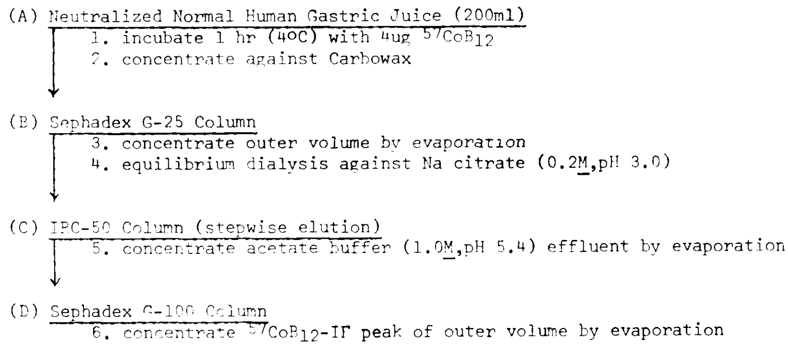

FIG. 1. Summary of the method used for purification of intrinsic factor from human gastric juice [after Ref. (21)].

ceinated protein in the ratio of 5–7 mg of fluorochrome to 1 g of protein.

Assay of IFA in PA serum and in the fractions eluted from the Sephadex columns was done by the zirconium gel technic. The "blocking antibodies" were determined by the method of Hansen *et al.* (14), at pH 5.0, while the "binding antibodies" were assayed by the method of Jacob *et al.* (19), at pH 6.25.

Immunoelectrophoresis of the effluents from the Sephadex G-200 column was performed against antihuman goat serum by the micromethod of Scheidegger (20).

Purification of IF from human gastric juice was done according to the method of Yamaguchi *et al.* (21) of our laboratory, and described elsewhere in detail. The steps used in this purification are shown in Fig. 1.

Separation of IFA from PCA in the PA Serum. Rationale. IFA-IF complex which has a molecular weight of over 200,000 is separated from the parietal cell antibody which has a molecular weight of immunoglobulin G, *i.e.,* 160,000 by gel filtration on Sephadex G-200 column. The IFA-IF complex after elution in the void volume of the Sephadex G-200 column is then dissociated by treatment with a diluted HCl. The dissociated IFA, which has a mol. wt. of immunoglobulin G of 160,000 is then separated by gel filtration on Sephadex G-150 column from the IF which in its monomer or dimer form has a mol wt of 55,000–60,000 or 110,000–120,000, respectively and is retarded on the column.

Separation technic. Several milliliters of PA sera were added to normal human gastric juice, or the purified human IF-$^{57}CoB_{12}$ complex. These sera were positive for parietal cell antibodies and contained about 700 units of the "binding type" of IFA and 800–1200 units of the "blocking type" of IFA according to the nomenclature of Garrido-Pinson *et al.* (22). In each case IF was present in excess of the IFA.

The mixtures of PA sera with gastric juice or with purified IF fraction were then put on a G-200 Sephadex column (2.5×100 cm) and eluted with 0.15 *M* NaCl at 4°. Three peaks of optical density at 280 mμ were obtained (Fig. 2). The effluent fractions were concentrated by dialysis against Carbowax 20,000 and assayed for IFA and PCA by methods described above. The PCA was found in the effluent fractions forming peak II only, but was absent from all the effluent fractions of peaks I and III. The assays for IFA were initially negative in all the effluent fractions of peaks I, II and III. However, after addition of $^{57}CoB_{12}$ and incubation of each effluent fraction with 0.1 *N* HCl, which is known to dissociate the IF-IFA complex, the IFA was detected in most of the fractions of peak I, and in the initial fractions of the ascending limb of peak II. No IFA was detected in the fractions of the descending limb of peak II nor in those of peak III.

When the effluents from Sephadex G-200 column were subjected to immunoelectrophoresis against goat antihuman immuneserum, the fractions corresponding to peak I gave only a very slight precipitin line at the application point. This suggested that the fractions of high optical density in peak I

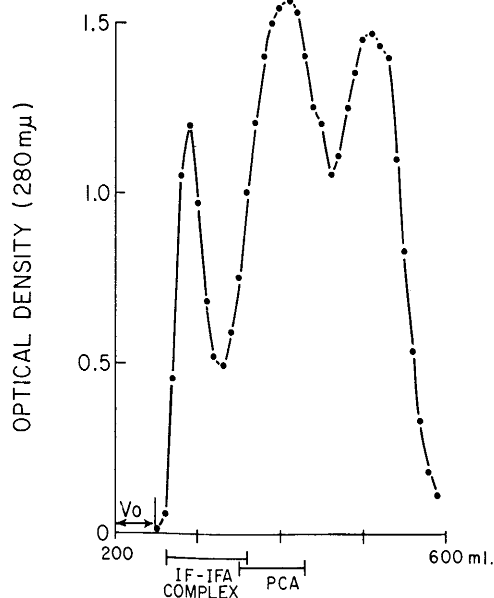

FIG. 2. Gel filtration on Sephadex G-200 of a mixture of intrinsic factor containing gastric juice and pernicious anemia serum containing parietal cell antibody and intrinsic factor antibody.

contained mostly gastric glycoproteins of mol wt over 200,000 and not serum proteins. These were eluted here together with the IF-IFA complex. Peak II, on immunoelectrophoresis, showed the presence of proteins of beta and gamma globulin mobility, while peak III contained proteins of serum albumin and alpha globulin mobility.

After a large amount of peak I was processed on Sephadex G-200 column the effluent fractions containing IFA-IF complex (but no PCA) were pooled together, $^{57}CoB_{12}$ was added and the IFA-IF complex was dissociated by addition of 0.1 N HCl. The whole of the dissociated material was concentrated on Carbowax 20,000 and then placed on a Sephadex G-150 column (2.5 $\times$ 100 cm) and eluted with 0.15 M NaCl. Two peaks of optical density at 280 mμ and two radioactivity peaks were obtained (Fig. 3). For the purpose of identification, three markers were also run on the same Sephadex G-150 column, namely blue dextran, albumin and $^{57}CoB_{12}$. The dextran was eluted in the region of the first optical density peak, which contained the IFA and gastric mucosubstances. Albumin was eluted in the area of the first radioactivity peak, corresponding to the IF-$^{57}CoB_{12}$ complex. Finally $^{57}CoB_{12}$ was eluted in the area of the second radioactivity peak, thus corresponding to the free radio-B_{12}. Examination of the fractions from the first optical density peak confirmed the

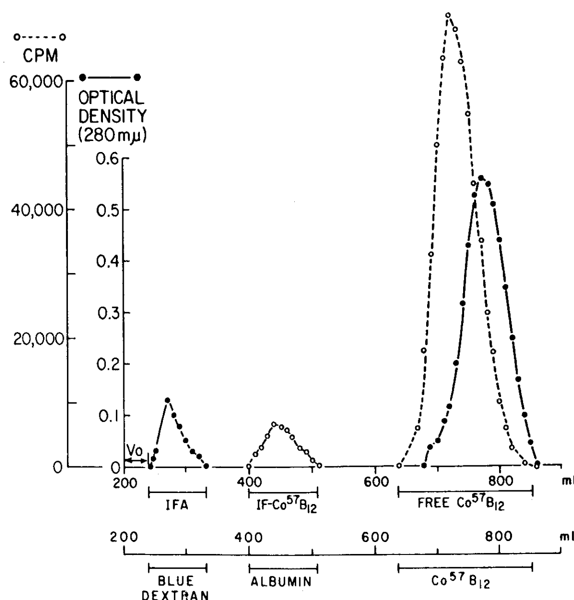

FIG. 3. Gel filtration on Sephadex G-150 of HCl-dissociated intrinsic factor–intrinsic factor antibody complex.

presence of both "blocking" and "binding" types of IFA by the Z-gel technic. When sections of normal human gastric mucosa were incubated with this isolated IFA the addition of fluoresceinated antihuman IgG produced fluorescence localized to the periphery of the parietal cell under the cell membrane, which was similar to that produced by serum of rabbits immunized with human IF-B_{12} complex in the same system (10, 23).

Results and Discussion. The findings reported here indicate that the IFA's can be separated from the PCA in PA serum by gel filtration on Sephadex G-200 column after they have been coupled to the IF of the gastric juice. The IF-IFA containing fraction does not contain PCA and does not produce immunofluorescence of the parietal cell on indirect Coons' test. After treatment of this fraction with diluted HCl, which causes dissociation of the IFA from the IF, the separation of the IFA from the IF was achieved by gel filtration on Sephadex G-150 column. By this technic it is possible to obtain IFA from PA sera free from PCA, which is much simpler than the method of obtaining IFA in the rabbit by prolonged immunization of the animals with IF or IF-B_{12} complex.

Our method of separation of IFA is well applicable to the demonstration of IF within the parietal cell of the stomach of man. When IFA was separated from PCA by this method and applied in an indirect Coons' test to human gastric mucosa in the presence of fluoresceinated antihuman IgG, a fluorescence on the periphery of the parietal cell occurred indicating that an IFA-IF complex formed within the parietal cell (10, 23). This was similar to that obtained with antihuman IF rabbit immune serum in the same system (10, 23) and which was also localized at the periphery of the parietal cell. This fluorescent reaction appeared to be more distinct than that reported with the method of the selective absorption of PCA from PA serum (5) with an extract from hog fundic mucosa.

When IFA separated by our method from PA sera was applied in an indirect Coons' test to human gastric mucosa in the presence of normal serum, containing complement, and the fluorescein labeled antihuman complement (C′) serum, immunofluorescence of the entire parietal cell developed (10, 23) indicating that the IF-IFA complex was fixing complement. This reaction was similar in appearance to that produced in a similar system with a rabbit antihuman IF immune serum (10, 23) and to that obtained in our earlier work (24) with the use of complement, fluorescein labeled antihuman complement (C′) serum and PCA containing serum.

Summary. The separation of IFA from PCA in PA serum was achieved by coupling the IFA with the partially purified human IF and eluting this relatively large complex in the void volume on a Sephadex G-200 column. The dissociation of the IF-IFA complex was achieved, thereafter, by treatment with 0.1 *N* HCl and elution of the IFA in the void volume of the G-150 Sephadex column, while IF was retarded on this column.

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