# An Approach for Immunoradiometric Assay with Metallic Radionuclides: Gallium-67-Deferoxamine-dialdehyde Starch-IgG

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Radiogallium (Ga) labeling of an immunoglobulin-G-deferoxamine conjugate (DF-IgG) to a high-specific radioactivity was performed to allow the development of a radiometallic immunoradiometric assay (IRMA) system. To increase the specific radioactivity of Ga-DF-IgG, we used dialdehyde starch (DAS) as a multi-site spacer for the binding of DF to IgG. Six DF molecules bound to each IgG molecule after DAS conjugation. DF-DAS-IgG was then labeled with the previously reported $^{67}$Ga labeling solution, producing labeled IgG with a specific radioactivity of 11,766 MBq/mg IgG. Using this method, we labeled an anti-CA125 tumor-associated antigen monoclonal antibody (130-22), allowing the first application of $^{67}$Ga-DF-DAS-IgG to an IRMA system. With this system, a higher sensitivity could be obtained than with $^{125}$I IRMA. In addition, a very high correlation ($r = 0.995$) was obtained between serum CA125 levels as determined by $^{67}$Ga IRMA and $^{125}$I IRMA. Gallium-67-labeled antibodies with a high-specific radioactivity appear to hold promise for use in highly sensitive radioassay systems.

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Metal radionuclide labeling of proteins is a rapid and simple method that achieves a high labeling stability as well as offering a wide choice of radionuclides with a wide range of half-lives and various patterns of disintegration (*1–7*). These characteristics provide this method with a good applicability to various fields of the biosciences. The labeling of IgG with metal nuclides has been extensively studied in clinical medicine, but these labeled IgGs have only been used for in-vivo diagnosis because of their low specific radioactivity. To extend the uses of metal-labeled IgGs, e.g., to allow their use in immunoradiometric assay (IRMA) systems, an improvement in the specific radioactivity of the labeled immunoglobulin is required.

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In the present study, two tactics were used to achieve a high-specific radioactivity of metal-labeled IgG: the use of a radionuclide with a high disintegration rate (short half-life) and an increase in the number of radionuclide atoms bound to each IgG molecule. To achieve the former objective, radiogallium ($^{67}$Ga: $T_{1/2} = 78$ hr or $^{68}$Ga: $T_{1/2} = 68$ min) was selected. The use of $^{67}$Ga in combination with deferoxamine (DF) as a bifunctional chelating agent has become an accepted method for the metal labeling of proteins (*7*). To achieve the latter objective, a multi-site spacer (dialdehyde starch, DAS) was introduced during the binding of IgG and DF so as to increase the number of DF molecules bound to each IgG molecule while preserving the immunoreactivity of IgG (Fig. 1). The DAS method has already been successfully applied to $^{67}$Ga-labeled fibrinogen, a thrombus-seeking radiopharmaceutical (*6*). Using these tactics, application of a $^{67}$Ga-labeled antibody to IRMA was achieved using the monoclonal antibody 130-22 (*8*), which detects the tumor-associated antigen CA125.

## MATERIALS AND METHODS

### Preparation of the $^{67}$Ga Labeling Solution

A Fe-free $^{67}$Ga labeling solution was prepared as reported previously (*9*). Gallium-67-citrate (Nihon MediPhysics, Takarazuka, Japan), 0.1 *M* ascorbic acid, and concentrated HBr were mixed at a volume ratio of 4:1:7 and then extracted with butyl acetate by vigorous mixing for 30 sec. After the butyl acetate layer was separated and evaporated, the residue was dissolved in 0.5 N HCl (prepared from iron-free HCl, Nacalai Tesque, Tokyo, Japan) containing 0.5 *M* ascorbic acid.

### Preparation of DF-DAS-IgG

DF-DAS-IgG was prepared according to the method reported by Takahashi et al. (*6*) with some modifications. DAS (100 mg, 14 μmole, Sigma, St. Louis, MO) was dissolved in 5 ml of phosphate-buffered saline (PBS) (0.01 *M*, pH 7.4) by heating to 90°C, following which DF (Ciba Geigy, Basel, Switzerland) was dissolved in this solution (48–128 mg/ml, 70-190 m*M*) to make a DF-DAS solution. Human IgG (10 mg, $6.3 \times 10^{-8}$ mole, Sigma) was dissolved in 1 ml of PBS and mixed with 100–400 μl of DF-DAS solution. Then, 9.6–19.2 μl (93-186 μmole) of triethylamine (TEA) was added as a neutralizing agent to transform DF-mes-

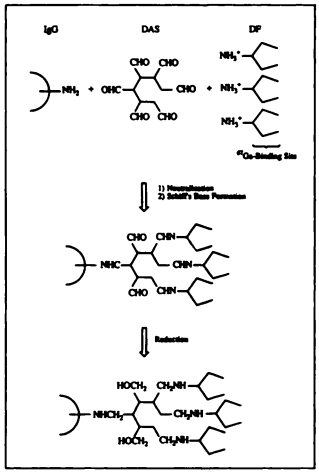

**FIGURE 1.** The synthesis of DF-DAS-IgG.

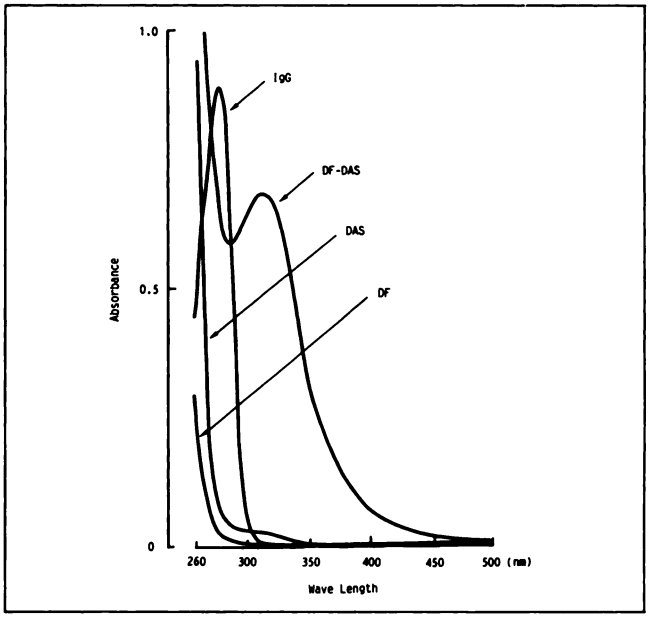


**FIGURE 2.** Photoabsorbance profiles of the DF-DAS conjugate, DF, DAS, and the IgG solution. The concentrations of DF, DAS, and IgG were 2.1 mg/ml, 0.53 mg/ml, and 0.67 mg/ml, respectively. The DF-DAS conjugate containing 21 mg/ml of DF and 6.3 mg/ml of DAS was diluted ten times to measure the absorbance.

ylate to reactive DF-$NH_2$ and also as a basic catalyst for Schiff's base formation. The reaction mixture was gently stirred for 2 hr at 15–18 °C. Following this, 1 mg (26 μmole) of $NaBH_4$ was added and stirring was continued for another hour. The DF-DAS-IgG conjugate was separated from free DF, DAS, and DF-DAS by passage through a Sephadex G-50 column (17 × 250 mm). For further purification, the DF-DAS-IgG fraction was then applied to an Affi-Gel Protein A column (Bio-Rad, Hercules, CA) equilibrated with 1/15 *M* phosphate buffer (pH 8.0). After washing with PBS, the IgG fraction was eluted from the column with 0.1 *M* citrate buffer (pH 3.5). The DF-DAS-IgG fraction was collected and the buffer was changed using a Sephadex G-50 column. The number of DF molecules per DAS-IgG molecule was calculated as described by Takahashi et al. (*6*). Briefly, the DAS-IgG concentration was determined by measuring the absorbance at 280 nm, and the DF concentration was determined by measuring the absorbance of $Fe^{3+}$-DF at 430 nm after the addition of $Fe^{3+}$. To estimate the number of DF-DAS clusters per IgG molecule (DF-DAS/IgG conjugation level), the specific absorption of the DF-DAS conjugate was determined after chromatography (Fig. 2). An aliquot of the DF-DAS-IgG reaction mixture was applied to a Sephadex G-50 column (1 × 25 cm, PBS) and the elution profile was determined at 311 nm. The DF-DAS/IgG conjugation level was then calculated as follows:

DF-DAS/IgG conjugation level =

$$\frac{\text{DF-DAS (mole) in the mixture}}{\text{IgG (mole) in the mixture}} \times \%\ \text{DF-DAS in the IgG fraction,}$$

where the molecular weights of DAS and IgG used were 7,000 and 160,000, respectively, and molecular weight was calculated from the total amount eluted from the column, determined by measuring the absorbance of DF-DAS at 311 nm.

### Labeling of DF-DAS-IgG with $^{67}$Ga

One hundred microliters of DF-DAS-IgG solution were mixed with 10 μl of $^{67}$Ga labeling solution and let stand for 1 hr at room temperature. An aliquot of the mixture was applied to a Sephadex G-50 column (17 × 250 mm), the fractions were collected, and the radioactivity of each fraction was counted. The labeling efficiency was calculated as the ratio of the radioactivity associated with DF-DAS-IgG to the sum of all eluted radioactivity.

### Stability of $^{67}$Ga-labeled IgG

Gallium-67-labeled IgG (50 μg/ml) was diluted 10,000 times with PBS containing 200 μg/ml of IgG and allowed to stand for 24 hr at 4°C. Then the labeling efficiency was analyzed by the method previously described.

### Preparation of $^{67}$Ga-labeled 130-22 Antibody

Conjugation was carried out as previously described. The mouse monoclonal antibody 130-22 (10 mg/ml, 63 μ*M* in PBS), 400 μl of DF-DAS solution (80 mg DF/ml, 0.12 *M* as DF) and 14.4 μl (140 μmole) of TEA were stirred for 2 hr at room temperature, reduced with 1 mg (26 μmole) of $NaBH_4$, and then applied to a Sephadex G-50 column. For further purification, the DF-DAS-130-22 antibody conjugate was passed twice through another Sephadex G-50 column (2 × 40 cm) with 10 m*M* phosphate buffer (pH 7.4) containing 1 *M* NaCl as the eluant. Following this, the conjugation level of DF-DAS-130-22 was checked. Then, the DF-DAS-130-22 conjugate was labeled with the $^{67}$Ga labeling solution as described for the DF-DAS-human IgG conjugate and diluted with HSA solution for use in the IRMA.

### IRMA Using $^{67}$Ga-labeled 130-22 Antibody

The "CA130 Kit Daiichi" used as provided by Daiichi Radioisotope Laboratory (Tokyo, Japan) and the $^{67}$Ga IRMA was performed following the manufacturer's instructions but using $^{67}$Ga-labeled 130-22 antibody instead of $^{125}$I-labeled 130-22 antibody. Fifty microliters of a standard CA125 antigen solution (0, 1, 3, 10, 30, 100, 300 or 500 U/ml) or human serum, 100 μl of radiolabeled 130-22 antibody solution ($^{67}$Ga: 3,108–7,955 MBq/mg antibody, 80,000–160,000 cpm/tube), and antibody-coated beads were mixed and incubated for 4 hr at room temperature. The supernatant was aspirated and the beads were washed twice

**TABLE 1**
Effect of the Volume of TEA Added to the Reaction Mixture on the Conjugation Level of DF-DAS-IgG

| Added TEA (μmole) | Level of conjugation (DF/DAS-IgG) mean (range) |
|---|---|
| 0 | N.D.* |
| 50 | 3.41 (3.35–3.47) |
| 100 | 3.51 (3.31–3.64) |
| 140 | 5.60 (5.21–5.88) |
| 190 | 3.73 (2.68–4.63) |

A volume of 200 μl of DF-DAS solution (80 mg DF/ml, 0.12 *M* as DF) was reacted with 10 mg ($6.3 \times 10^{-8}$ mole) of IgG (n = 3).
* Not detectable.

with distilled water, after which the $^{67}$Ga radioactivity bound to the beads was measured. The standard curve and assay data were compared with those obtained by simultaneously performing the original CA130 IRMA using $^{125}$I-labeled antibody.

## RESULTS AND DISCUSSION

Our preliminary studies concerning the direct application of the method reported by Takahashi et al. (*6*) to IgG only resulted in the production of DF-DAS-IgG conjugate with a very low level of conjugation (less than 3, data not shown). This problem seemed to be attributable to the structural differences between IgG and fibrinogen, so accordingly we investigated the factors related to raising the conjugaton level of DF-DAS-IgG.

Tables 1–3 show the respective effects of TEA, DF, and DF-DAS on the level of conjugation (DF/DAS-IgG ratio) of DF-DAS-IgG. Reaction conditions are described in the table legends. As shown in Table 1, the conjugation level of DF-DAS-IgG initially increased to 5.60 with the addition of TEA (range: 5.21–5.88 at 140 μmole of TEA), but after reaching this peak the further addition of TEA resulted in a decrease of the level of conjugation and enlarged its variability. TEA acted as a neutralizing agent and a catalyst in our reaction system and might have had some unknown effects at higher concentrations. For DF (Table 2) and DF-DAS (Table 3), the level of conjugation increased with the increased addition of either DF or DF-DAS, until it reached a plateau. This plateau effect might have been related to the limited availability of aldehyde residues for conjugation of DAS.

**TABLE 2**
Effect of the DF Concentration of the DF-DAS Solution on the Conjugation Level of DF-DAS-IgG

| DF concentration (m*M*) | Level of conjugation (DF/DAS-IgG) mean (range) |
|---|---|
| 70 | 3.44 (3.21–3.62) |
| 100 | 4.37 (4.08–4.53) |
| 120 | 5.60 (5.21–5.88) |
| 150 | 5.13 (4.97–5.29) |
| 190 | 5.37 (5.09–5.75) |

A volume of 200 μl of DF-DAS solution was reacted with 10 mg ($6.87 \times 10^{-8}$ mole) of IgG plus 140 μmole of TEA (n = 3).

**TABLE 3**
Effect of the Volume of DF-DAS Solution Added on the Conjugation Level of DF-DAS-IgG

| DF/DAS solution (μl) | Level of conjugation (DF/DAS-IgG) mean (range) |
|---|---|
| 100 | 2.34 (2.12–2.68) |
| 200 | 5.60 (5.21–5.88) |
| 300 | 6.20 (5.98–6.35) |
| 400 | 6.22 (5.90–6.45) |

DF-DAS solution (80 mg DF/ml, 0.12 *M* as DF) was reacted with 10 mg ($6.3 \times 10^{-8}$ mole) of IgG plus 140 μmole of TEA (n = 3).

Under the conditions used, the level of DF/DAS-IgG conjugation reached 6.2 (Table 3), with a DF-DAS/IgG conjugation level of 1.1. Thus, approximately one molecule of DAS with 6 DF molecules attached was conjugated to each IgG molecule.

Next, $^{67}$Ga-labeling was performed (Fig. 3) using the DF-DAS-IgG conjugate. Under the conditions studied, the labeling efficiency increased as more DF-DAS-IgG was added and reached more than 95% at 50 μg/ml. In the case of DF-IgG [glutaraldehyde method (*3*)], a higher concentration of DF-IgG (200 μg/ml, conjugation level: 0.81) was required to obtain a similar labeling efficiency (*10*). This difference is considered to reflect the different DF/IgG conjugation levels of DF-DAS-IgG and DF-IgG.

Improvement of the specific radioactivity of radiolabeled IgG was the major aim of this study. When 100 μg/ml of DF-DAS-IgG (DF/DAS-IgG conjugation level: 6.45) was used for $^{67}$Ga-labeling, the radioactivity obtained was

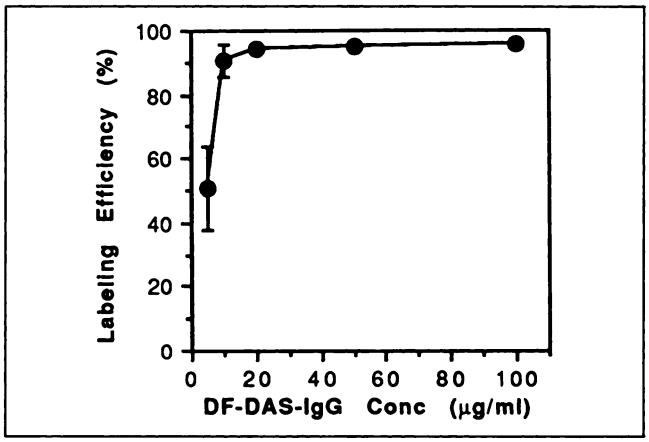


**FIGURE 3.** Labeling efficiency of DF-DAS-IgG with $^{67}$Ga under the conditions studied. DF-DAS-IgG (100 μl, DF/DAS-IgG = 6.45, IgG conc. = 5, 10, 20, 50, and 100 μg/ml) was labeled with 10 μl of $^{67}$Ga labeling solution (37–74 MBq/ml) and applied to a Sephadex G-50 column. Labeling efficiency was calculated by dividing the radioactivity bound to the DF-DAS-IgG by the total radioactivity in the eluate. Each point represents the mean and s.d. (n = 4).

11,766 (11,067–12,425) MBq/mg IgG (mean and (range) of three experiments) and a high labeling efficiency of 92.5% (91.9–93.3) was achieved. This specific radioactivity was 20 times higher than the maximum theoretical specific activity of $^{125}$I-IgG (calculated as one $^{125}$I atom per IgG molecule) (*11*). In addition, the labeling stability of $^{67}$Ga-DF-DAS-IgG was good. The %$^{67}$Ga associated with DF-DAS-IgG remained very high, even after incubation for 24 hr at a 10,000-fold dilution (preincubation: 95.14% ± 0.85%, postincubation: 92.80% ± 1.08%, mean ± s.d. of four experiments). The high specific radioactivity and excellent stability of $^{67}$Ga-DF-DAS-IgG encouraged us to perform further studies for the development of a new IRMA system.

The IRMA system for a CA125 tumor-associated antigen, which was originally developed at our department (*7*), was selected for the first application of $^{67}$Ga-DF-DAS-IgG. Under the best conditions that we obtained for human IgG, DF-DAS conjugation with the mouse monoclonal antibody 130-22 (anti-CA125) was performed and a DF/DAS-130-22 conjugation level of 6.5 was achieved. Gallium-67-labeling of the DF-DAS-130-22 conjugate resulted in a specific radioactivity of 3,108–7,955 MBq/mg IgG (range of five experiments). However, the labeling efficiency (63%–84%) was not so high as that of human IgG. This was probably because of differences in the purification procedure performed after the conjugation reaction. In this study, molecular-size fractionation by G-50 column chromatography was performed to purify the DF-DAS-130-22 conjugate, because of the insufficient binding of the antibody to a Protein-A Sepharose column. To improve the labeling efficiency, further IgG-specific purification would be needed; e.g., ion-exchange column chromatography might be useful. However, even without further purification, this $^{67}$Ga-DF-DAS-IgG was adequate for use in the IRMA.

Figure 4 shows the standard curve obtained for the CA125 IRMA using $^{67}$Ga-DF-DAS-130-22 (3,108 MBq/mg) and $^{125}$I-130-22 (commercially supplied). With the $^{67}$Ga system, a good linearity was obtained between the percent bound/total (%B/T) and the CA-125 concentration. The standard curve of the $^{67}$Ga IRMA system appeared to suggest that it would be more useful in the lower range of CA125 concentrations (<10 U/ml) than the $^{125}$I IRMA system.

Using this $^{67}$Ga IRMA system, serum CA125 levels were measured in 40 patients and compared with those obtained by the commercial $^{125}$I IRMA system. As shown in Figure 5, the $^{67}$Ga IRMA system values correlated excellently with those obtained by the $^{125}$I IRMA system (r = 0.995).

These results also indicate that the immunoreactivity of DF-DAS-130-22 was maintained satisfactorily. As described above, it was calculated that about one DF-DAS cluster was bound to each IgG molecule. In the case of DF-glutaraldehyde, no marked change in immunoreactivity has been reported at this level of conjugation, but

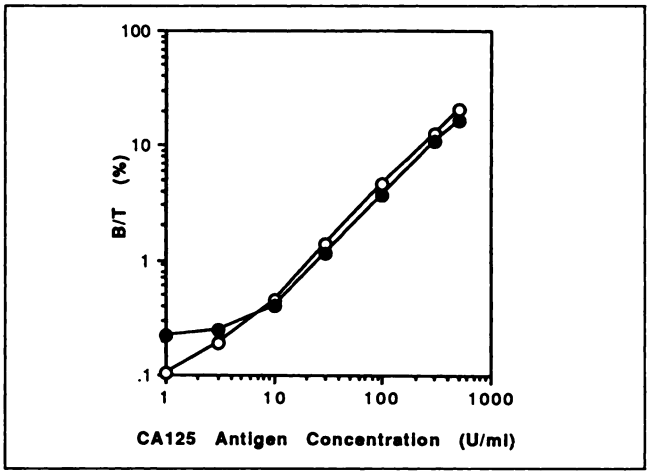


**FIGURE 4.** A typical standard curve for the CA125 IRMA using $^{67}$Ga-labeled antibody (○) or $^{125}$I-labeled antibody (●), with B/T% plotted against antigen concentration (1–500 U/ml) in a log-log plot. The specific radioactivity of the $^{67}$Ga-labeled antibody was 3,108 MBq/mg and the labeling efficiency was 84%.

immunoreactivity was reduced at higher conjugation levels (*10*). Thus, it might be that the size of the attached molecules has less effect on the immunoreactivity of IgG than the number of molecules that are attached.

The present study indicates strong potential applicability of radiogallium-labeled DF-DAS-IgG to IRMA systems. Gallium-67-DF-DAS-IgG had a better standard curve than $^{125}$I-IgG and was still applicable at low antigen concentrations. In addition, the simple $^{67}$Ga labeling procedure and high stability of the radiopharmaceutical should allow both the long storage of IRMA kits and on-site labeling if required, provided that purified $^{67}$Ga can be produced commercially. Moreover, the short half-life of radiogallium ($^{67}$Ga, $^{68}$Ga) should contribute to the reduction of

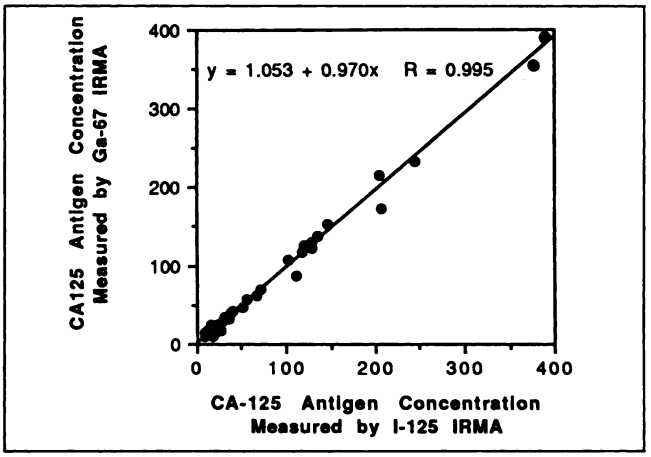


**FIGURE 5.** The serum CA125 antigen levels in 40 serum samples. The results obtained by IRMA using the $^{67}$Ga-labeled antibody are compared with those obtained using the $^{125}$I-labeled antibody.

radioactive waste disposal problems. Thus, the new Ga-IRMA system appears likely to provide a solution to some of the problems inherent in the current $^{125}I$ assay systems.

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*(continued from p. 824)*

# SELF-STUDY TEST
# Gastrointestinal Nuclear Medicine

## ANSWERS

### ITEM 2: Colonic Bleeding

ANSWER: C

The images in Figure 1 demonstrate a faint focus of abnormal $^{99m}Tc$-red blood cell accumulation in the left lower quadrant, which is first seen at 1 minute but becomes more intense with time and conforms with the descending colon. A bleeding diverticulum in the descending colon was found at colonoscopy.

There is no gastric, duodenal, or proximal jejunal activity present to suggest a bleeding duodenal ulcer, nor are small bowel loops seen on the images to suggest a bleeding leiomyoma. The activity in the left upper quadrant is the spleen, which is always seen normally, but may have unusually prominent activity if the patient has been transfused recently. This splenic activity remains stable with time and should not be confused with bleeding from the splenic flexure, which generally should increase with time and move distally.

The $^{99m}Tc$-red blood cell scan demonstrates an abdominal aortic aneurysm, but there is no evidence for a bleeding aortoenteric fistula. Spontaneous aortoenteric fistulae are rare, occurring in approximately seven of 10,000 autopsy cases. The most common area of fistula formation is between the aorta and duodenum, which is the case in 80% of patients. Atherosclerotic aneurysms of the iliac arteries can erode into the bowel, as well. In the current case, no aneurysm is seen in either iliac artery.

**Reference**


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### ITEM 3: Symptomatic Alkakine Reflux Gastritis

ANSWER: C

No esophageal reflux of tracer is identified in any of the images in Figures 2A–B to suggest that the patient's symptoms are due to reflux esophagitis. Furthermore, the patient's symptoms following vagotomy, hemigastrectomy, and Billroth II gastrojejunostomy are not typical of those experienced in patients with simple reflux esophagitis.

Clinical symptoms associated with the postgastrectomy syndrome of "alkaline reflux gastritis" include epigastric pain, nausea, vomiting, bilious vomiting, and weight loss. Ritchie has reported that, in general, it is not possible on clinical grounds alone to predict which patients will benefit from surgical procedures to divert bile flow. In his series of patients, the frequency of symptoms was no different between patients with documented "excessive" reflux compared to those with "normal" reflux. Bilious vomiting was the only symptom found more frequently in the "excessive" reflux group. However, bilious vomiting was absent in some symptomatic "excessive" refluxers who responded to surgical diversion. The absence of bilious vomiting cannot be used, therefore, to conclude that the patient does not have alkaline reflux gastritis.

While there is some early "pooling" of the hepatobiliary agent in the afferent loop of bowel, there is no significant retention in the later images to suggest obstruction of the afferent loop of the Billroth II reconstruction.

It is not abnormal to see bile reflux in patients following Billroth II reconstruction. In fact, the presence of reflux is a typical postoperative finding. Symptoms, however, are best correlated with the amount of reflux, which needs to be determined quantitatively and not merely by visual assessment. In this case, there is excessive reflux quantitated by an enterogastric reflux index of 64%. Values elevated into this range have been best correlated with the patient's symptoms. In Ritchie's study, asymptomatic postgastrectomy patients without excessive reflux had a mean reflux index of 16% ± 9%, while for symptomatic, "excessive" refluxers the mean value was 81% ± 15%.

Ritchie also reported surgical correction of reflux documented by objective criteria (e.g., measurement of gastric bile-acid concentrations, net bile-acid reflux, scintigraphic enterogastric reflux, and histologic evidence of gastritis) in all of 14 patients with excessive reflux following diversion with a 45-cm Roux-en-Y limb. This patient, therefore, has excessive bile reflux that is the likely cause for her symptoms. She would be expected to obtain relief of her symptoms following surgical diversion.

**Reference**


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