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Leakage of Immobilized IgG from Therapeutic Immunoadsorbents

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ABSTRACT

In developing therapeutic immunoadsorbents (IAs), antibodies (IgG molecules) covalently immobilized on porous carriers, a leak of IgG was determined both in the storage test with buffers at 25 and 4°C and in contact with plasma at room temperature (RT). The amount of antibody released from therapeutic IAs must be minimized to avoid side effects during treatment. The amount of IgG released was

a. Dependent on the amount of IgG immobilized

b. Much greater with CNBr-activated Sepharose 4B, or Formyl-Cellulofine as a support material than with aminopropyl CPG (controlled pore glass)

c. Found to yield again during another storage in buffers after the IAs were washed and their buffers replaced with fresh ones and

d. Decreased after the IAs were treated with glutaraldehyde (GA) solutions.

Whereas treating the IAs with GA solutions significantly reduced the amount of IgG released, it caused some deterioration of the adsorption characteristics of the IAs. An irradiation dose of 2.5 Mrad as a crosslinking procedure also reduced the amount of IgG released; its effect was comparable to that of 0.025% GA, the lowest concentration used.

Index Entries: Anti-IgE antibodies; gamma rays irradiation; glutaraldehyde; IgE; IgG; immobilization; immunoadsorbent; leakage; storage test.

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INTRODUCTION

On-line plasmapheresis involving plasma separation and selective removal of pathogenic substances from the separated plasma may be one of the ideal plasmapheresis therapies. Generally, the selective removal of substances can be accomplished by affinity chromatography, the components of which are solid-phase antibodies or antigens. We have reported a therapeutic immunoadsorbent (IA) for selectively removing human IgE, which plays an important role in type I anaphylactic hypersensitivity (*1*). Our therapeutic IA is anti-IgE antibody immobilized covalently on porous carriers; it effectively adsorbs human IgE from plasma. The requirements for therapeutic IAs are: high adsorption, high selectivity, good mechanical strength, resistance to sterilization (*2,3*), no activation of the complement and coagulation systems, and no leakage of immobilized ligands (e.g., antibodies) from the support materials. In particular, leakage of antibodies, for example, must be minimized; otherwise, they might act as antigens and trigger an undesirable immune response if they are animal products and present in quantity, because the plasma would be perfused over the IA column intensively several times during a given period. There are two cases of ligand leakage in therapeutic IAs: leakage in simple aqueous solution systems during the storing and transporting of IAs, and leakage during immunoadsorption treatment, where unknown enzymes and other substances might play a role in releasing the ligands. Although some reports presented leakage of ligands, which were covalently bound to support matrices (*4–8*), as a problem of affinity chromatography, from our viewpoint it is not. The present study is designed to learn how much antibodies (anti-human IgE antibodies: goat IgG) are released from supports in both aqueous media and plasma, and the effect of glutaraldehyde (GA) crosslinking for IAs on decreasing the leakage of IgG. Although there are some methods for determining minute amounts of anti-human IgE antibodies based on chemical and biological reactions, we determined them chemically as goat IgG.

MATERIALS AND METHODS

Reagents

Goat IgG: anti-human IgE antibody raised in a goat and purified by ammonium sulfate precipitation followed by DEAE cellulose-ion exchange chromatography was used as goat IgG. Anti-goat IgG: rabbit anti-goat IgG antibody $F(ab')_2$ fragment was purchased from Cappel Laboratories, Inc., USA. Anti-goat IgG-biotin conjugate, horseradish peroxidase (HRP)-avidin D conjugate (Vector Laboratories, Inc., USA), *o*-phenylenediamine, ethanolamine (Katayama Chemical Ind., Japan), Tween 20, gelatin, glutaraldehyde (Wako Pure Chemical Ind., Japan), human serum

albumin (HSA, Miles Laboratories, USA), were purchased commercially. All other reagents were analytical grade.

Microtiter Plate

Nunc-Immuno Plate I (96 wells, U-shaped; Nunc, Denmark) was used.

Determination of Goat IgG in Buffer Solutions

An avidin-biotin microenzyme-linked immunosorbent assay (*9*) was modified and used to determine goat IgG in the buffer solutions. Anti-goat IgG was diluted with coating buffer to make a concentration of 0.5 μg/mL. Aliquots (100 μL) were added to microtiter wells. The plates were incubated for 60 min at 37°C. The solution was then discarded and the wells were rinsed 5 times. Three hundred μL of diluent buffer was added to each well and the plates were incubated at 37°C for 3 hr. After the solution was discarded and the plate was tapped upside down on sheets of paper, goat IgG standards or samples appropriately diluted with corresponding buffer were added to each well (100 μL). The samples were added in duplicate. Wells that did not contain goat IgG or a sample served as blanks. The plates were kept at 37°C for 60 min; the wells were rinsed 5 times with rinse buffer; 100 μL of anti-goat IgG-biotin conjugate (1.5 mg/mL solution freshly diluted 1:2250 with diluent buffer) was added to each well; and the plates were incubated at 37°C for 60 min. The solution was again discarded and the wells were washed 5 times with rinse buffer. A stock solution of HRP-avidin conjugate (5.0 mg/mL in 0.1*M* $NaHCO_3$, pH 8.5) was freshly diluted 1:1220 with HRP-avidin diluent buffer, 100 μL aliquots dispensed into each well, and the plates incubated at 37°C for 10 min. Then the contents of the plate were discarded, the wells were washed 5 times with rinse buffer, and any excess moisture was removed by tapping on sheets of paper. Finally, 100 μL of freshly prepared substrate solution (0.5 mg/mL *o*-phenylenediamine and 0.006% H_2O_2 in 0.2*M* potassium phosphate buffer, pH 7.0) was added to each well. The plates were allowed to stand at room temperature in the dark for 10 min. The enzyme reaction was terminated by adding 100 μL of 2*M* H_2SO_4 and the absorbance read within 15 min at 490 nm in an ELISA Analyzer ETY-96 (Toyo Sokki, Ltd., Tokyo, Japan). The lower limit of detection was 1 ng/mL and the upper 200 ng/mL. The buffers for the present enzyme immunoassay were

a. Coating buffer: 0.1*M* sodium carbonate-bicarbonate buffer, pH 9.6
b. Rinse buffer: phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween 20
c. Diluent buffer: PBS, pH 7.4, containing 1% HSA and 0.05% Tween 20, and

d. HRP-avidin diluent buffer: PBS, pH 7.4, containing 1% HSA, 0.5% gelatin, and 0.05% Tween 20.

Determination of Goat IgG in Human Plasma

Goat IgG in the plasma was determined by a slight modification of the procedure described above; it was tried to decrease the nonspecific background. The concentration of the anti-goat IgG-biotin conjugate was diluted to 1/10 of that used in the assay for IgG in the aqueous media. The other conditions were the same as those outlined above. The detection limit was almost the same as that for the buffer solutions.

Preparation of Immunoadsorbents

Porous bead carriers were used; Formyl-Cellulofine (250–350 μm; Seikagaku Kogyo, Japan), CNBr-activated Sepharose 4B (Seph. 4B; Pharmacia Fine Chemicals, Sweden) and Amino propyl CPG-1400 (CPG-1400; Electro-Nucleonics, Inc., USA) were purchased commercially. Immobilization of goat IgG by Formyl-Cellulofine and Sepharose 4B was carried out according to the manufacturer's instruction. Immobilization by CPG-1400 was reported previously (*10*). The amount of preparation immobilized on the support was determined from the difference in protein concentrations at the pre- and post-coupling stages; almost 100% of the goat IgG loaded was found to be immobilized on each support. After the immobilization process, some of the IAs were treated with GA (0.025–2.5% (v/v) in 0.2*M* phosphate buffer pH 7.0) for 1 h at RT.

Storage Test

Each filtered IA, the carrier of which was initially 0.25 g on a dry wt base, or 1 mL of gel of Formyl-Cellulofine was stored in a tightly sealed glass vial with 3 mL of saline or buffer at 25 and 4°C and was occasionally shaken gently during the storage. The volume of IA was approximately 30% of the total volume. At certain intervals the aqueous media were taken to determine the IgG concentrations, then the IA was washed with 50 mL of saline and was stored again with 3 mL of the fresh buffer.

Sampling to Determine Goat IgG from Supports

Aliquots of the supernatant in the vial were withdrawn through a 0.2-μm pore membrane filter (Minisart NML, Sartorius GmbH, FRG).

Leakage of Goat IgG into Plasma

After the aqueous media were taken to determine the IgG released, IA was washed with 50 mL of saline and filtered by a glass filter. The IA was put in a 10-mL glass tube together with 3 mL of plasma and the tube was sealed tightly. Then the tube was placed on the edge of a disk (approx 30 cm diam.) tilted at an angle of 45° and the disk was rotated at

one round/6 s at RT. The conditions of this rotary mixing procedure allowed the contents to be mixed sufficiently and did not destroy the supports. After 3 h of mixing the glass tube was set aside for a while, then the supernatant was taken to determine the soluble IgG.

Irradiation

Gamma rays from a ^{60}Co source were used to irradiate the IAs in saline or 0.2*M* phosphate buffer pH 6.8. The IAs were washed with 50 mL of saline, then stored in saline at 25°C.

IgE Adsorption Test

One mL or 0.25 g (initial dry wt) of IA was stirred in 10 mL of bovine plasma containing human IgE (Behringwerke AG, Marburg, FRG) for 1 h at RT. IgE concentrations before and after the 1-h incubation were determined by a commercial test kit, IgE EIA Kit "MITSUI" (Kainos, Ltd., Tokyo). The initial IgE concentration in the plasma ranged from 610 to 795 U/mL.

RESULTS

Goat IgG released into the buffers from the supports after various storage times was determined. Figure 1 shows the IgG release from IA in saline, where the IgG was immobilized on Sepharose 4B at 10 mg/g of the support or 25 mg, that is, 2.5 or 6.25 mg of IgG immobilized in the IA was stored at 25°C. First, after 2 wk of storage, the IgG released into the saline was determined, then the supernatant was discarded. The IAs were washed with 50 mL of saline, filtered by a glass filter and stored with 3 mL of fresh saline for 2 d; then IgG was determined again. The IAs were washed again in the same manner, then stored for an additional 2 wk, when IgG was determined again.

Release of IgG was found even after the IAs were washed and then stored; however, the amount of IgG released during 2 d was less than that during 2 wk (Fig. 1). The amount of IgG leaked for the first 2 wk was greater than that for the next 2 wk after the washing. This phenomenon is diagrammed in Fig. 2, where it is shown that the greater the number of washings, the lower the amount of IgG released, although the experimental conditions differed. Figure 2 shows the IgG leakage related to the period of storage and the washing frequency. The data in Fig. 2 were based on the immobilization level at 10 mg IgG/one g (2.5 mg/IA) of Sepharose 4B (#1–#5) and of CPG-1400 (a–c); the IAs were stored at 25°C with 3 mL of saline and pH 7.4-phosphate buffers (0.05, 0.1, and 0.2*M*). No significant differences in release of bound IgG among the buffers, including 0.1*M* borate and 0.1*M* Tris buffers of pH 7.4, and 0.1*M* phosphate buffer pH 6.5, were observed (not shown). However, IgG immobi-

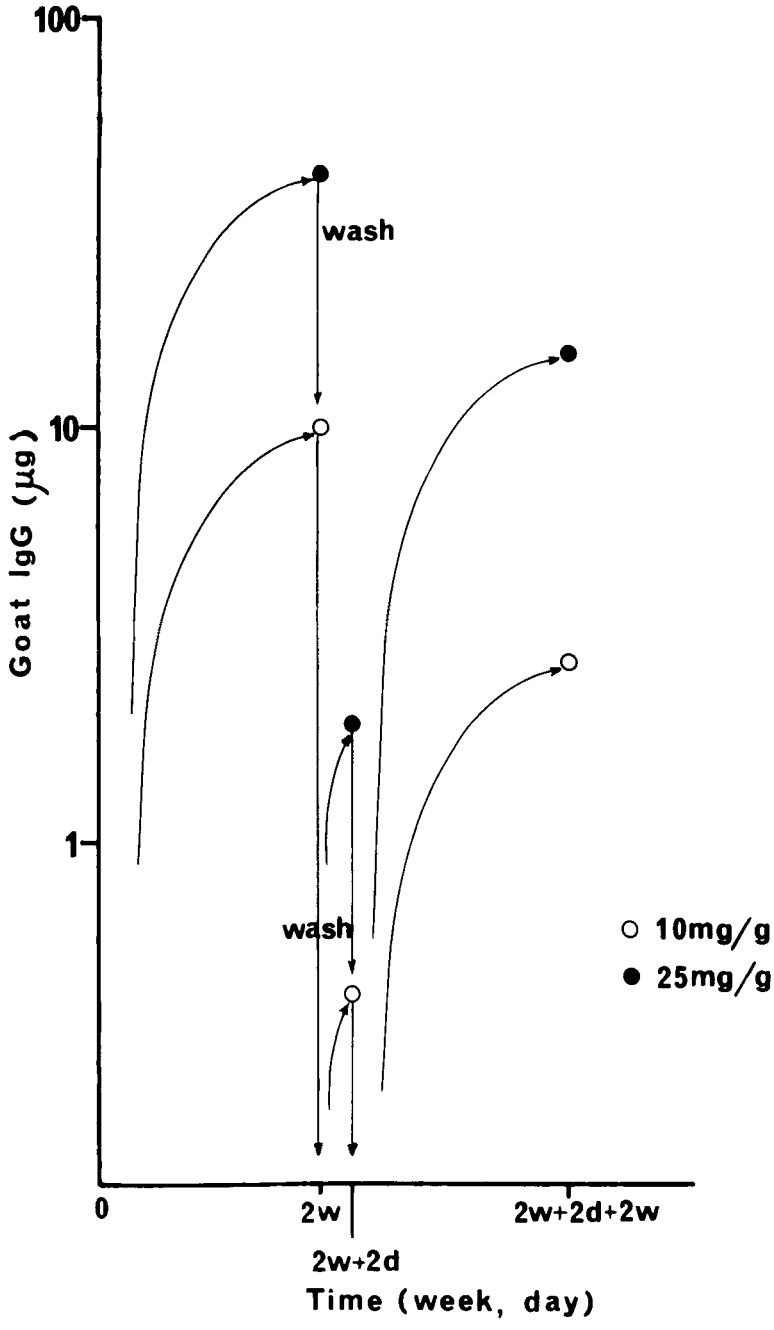

Fig. 1. Amounts of goat IgG released from Sepharose 4B-based IAs into saline in the storage test at 25°C. Sepharose 4B immobilized goat IgG at 10 (open circles) and 25 (closed circles) mg/g of the carrier; 0.25 g (initial dry wt) of Sepharose 4B was used, consequently, 2.5 and 6.25 mg of goat IgG covalently bound to the IAs were stored with 3 mL of saline. First, the IAs were stored for 2 wk (w), then washed with 50 mL of saline and again stored with 3 mL of fresh saline for 2 d (d), and then treated in the same manner as the above, followed by being stored for 2 wk. A symbol, +, in time indicates the washing and replacement of saline.

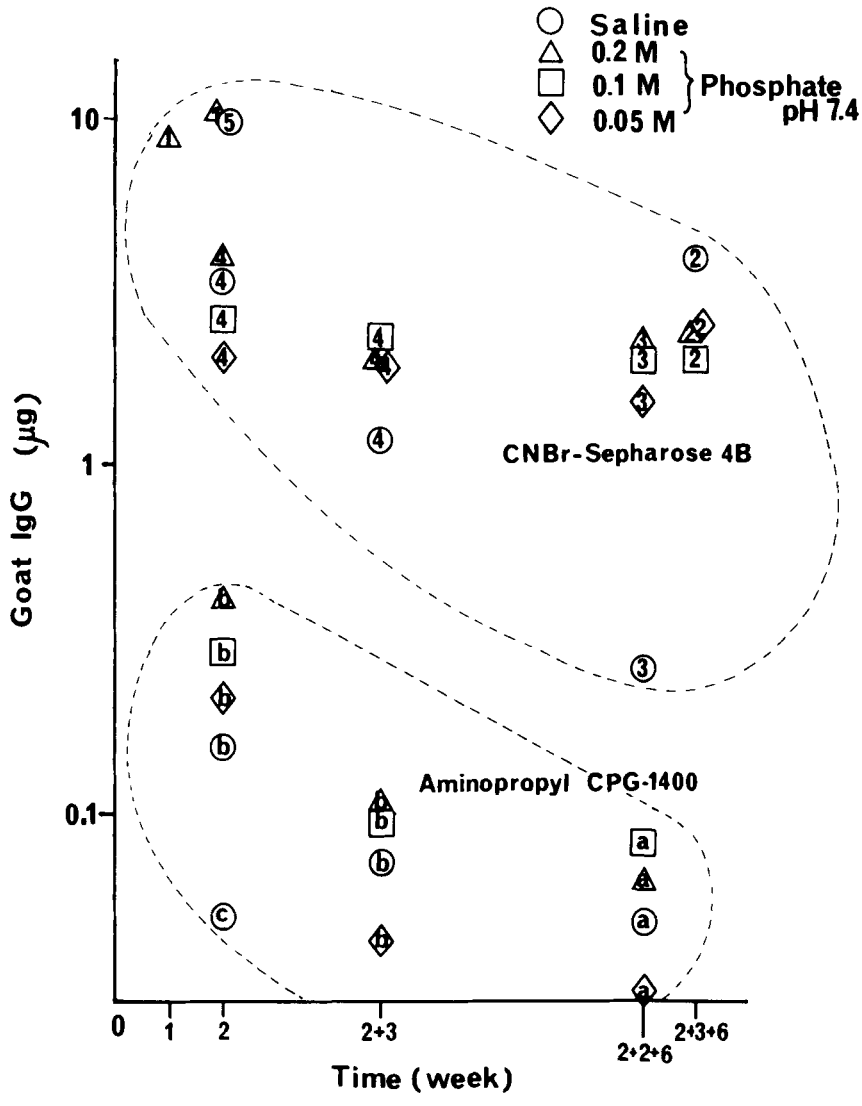

Fig. 2. Amounts of goat IgG released from Sepharose 4B- and CPG-1400-based IAs into 3 mL of aqueous media in the storage test at 25°C. Some lots of the IAs were stored (lot numbers 1–5 for Sepharose 4B-based IA and lot numbers a–c for CPG-1400-based IA), with 3 mL of saline and different molar phosphate buffers (pH 7.4). The indication in time (x-axis) is the same as in Fig. 1. Each IA immobilized 2.5 mg of goat IgG.

lized on Sepharose 4B was detached approximately 10-fold more than that immobilized on CPG-1400.

In the most representative examples, the amount of IgG released in saline for 2 wk at 25°C from the Sepharose 4B-based IA immobilizing 2.5 mg of IgG was 6.51 μg (n = 28), and from Formyl-Cellulofine immobilizing 2.5 mg of IgG was 3.83 μg (n = 4).

The effect of storage temperature on the amount of IgG released from the Sepharose 4B-based IA was studied. The IA immobilizing 2.5

mg of IgG was stored at 4 and 25°C for 4 wk. The amount of IgG released was 1.8 μg at 4°C and 8.8 μg at 25°C.

The amounts of IgG released were proportional to the amounts of IgG immobilized on Sepharose 4B. Similar dose response-like curves were obtained in the two circumstances (Fig. 3), although the absolute values were different; the IgG detached in the saline for 2 wk was more than 10 times the amount of IgG detached in the 3-h rotary mixing system using plasma.

The effect of human IgE in plasma on IgG (anti-human IgE antibody) leakage from the Sepharose 4B-based IA was examined with the rotary mixing procedure; human plasmas containing IgE at 1300 U/mL (3 μg/mL) and at 56 U/mL (129 ng/mL; within the normal range), and rat serum containing no human IgE were used as media. No significant difference in the IgG leakage was observed between the human plasmas (Table 1), however, less IgG was released into the rat serum containing

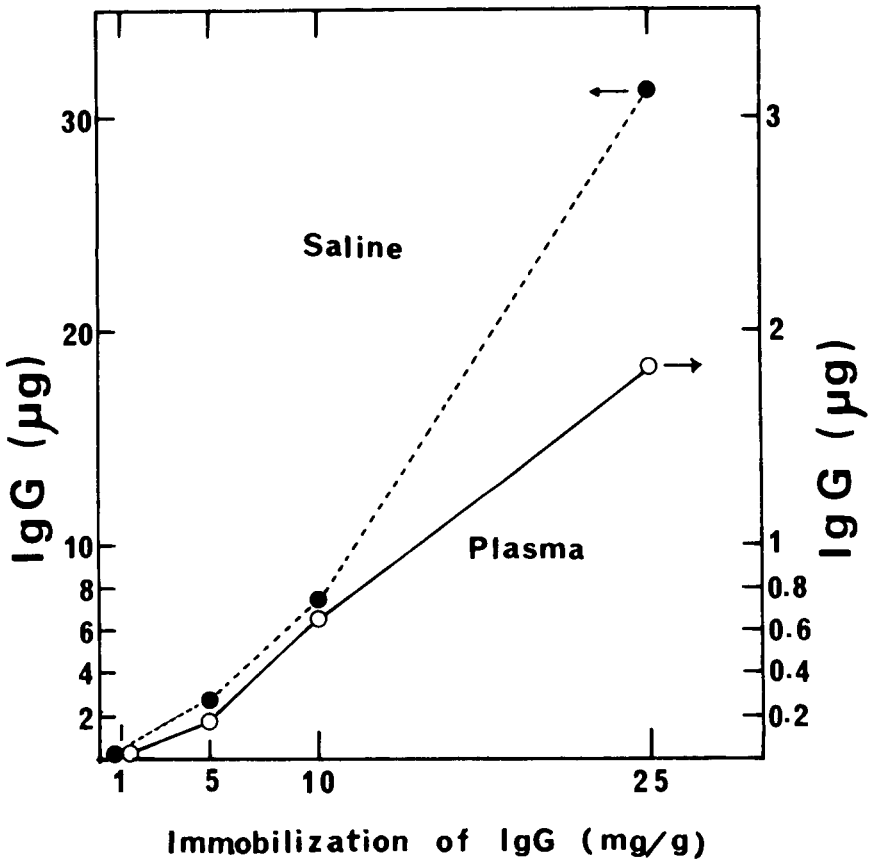

Fig. 3. Relationship between amounts of goat IgG released from Sepharose 4B-based IAs into 3 mL of saline and human plasma and amount of immobilization of goat IgG on Sepharose 4B. The amount of 0.25 g (initial dry wt) of Sepharose 4B was used. The IgG leak was determined both in the storage test of 2 wk with saline at 25°C and in the 3-h rotary mixing procedure.

Table 1
Effect of Human IgE on IgG Leakage from IA[a] into Plasma

	Human plasma		Rat serum
IgE conc. (U/mL):	1300	56	0
IgG release, ng	240	286	123

IA was Sepharose 4B immobilizing 2.5 mg of goat IgG (anti-human IgE antibodies).

no human IgE than into the plasmas. Each standard calibration curve was made using the corresponding plasma or serum as the solvent.

Table 2 shows the effect of GA on IgG leakage from IAs into buffers and plasma. The amount of IgG released from IAs treated with 2.5% GA was less than that from the intact IAs, in particular, a significant effect was observed with IAs using Sepharose 4B. A remarkable reduction of the IgG leakage was observed by treating the IAs with GA in both the aqueous media and plasma. Similar results were obtained with the Formyl-Cellulofine-based IA reacted with the GA solutions ranging 0.025–2.5% (Table 3). Tables 2 and 3 show the results of IgE adsorption test with the IAs treated with GA. IgE removal, defined as a percentage of an amount of IgE adsorbed to that applied, was affected by the GA treatment, but the reduction rate was acceptable.

To compare the effect of crosslinking with GA with that by radiation, the effect of gamma rays on the IgG leakage from the IAs was studied (Table 4). The gamma rays reduced the IgG leakage, but not drastically.

DISCUSSION

In developing therapeutic IAs consisting of antibodies immobilized on carriers, the ligand leakage should be minimized to avoid side effects. Ligand leakage is classified into two types: a leakage during storage or transportation of the IAs in aqueous media if the IAs are manufactured in a wet state, and leakage in plasma while being used, which may involve effects of serum proteins. The present study was carried out to examine both types. Absolute amounts of the ligand leakage in an actual clinical setting involving interactions of IA with plasma are most important from the viewpoint of side effects; however, such studies in vivo accompanied by animal toxicological studies are not presented here.

Tesser, et al. (*6*) found that the ligand was slowly detached from the CNBr-activated supports by solvolytic processes above pH 5 and the free ligand, R-NH_2, appeared in the solution. They proposed mechanisms for ligand release through CNBr activation and amine substitution (covalent bond); hydrolysis between pH 5–10 might operate through protonation of the strongest base, RNH-, releasing the free ligand. We also found considerably large amounts of IgG released from the Sepharose 4B-based IA.

Table 2
Effects of GA on IgG Leakage from IAs into Buffers and Plasma and IgE Adsorption

Carriers of IAs	Immobilization amount of IgG, mg	GA treatment[a]	Amounts of released IgG in		IgE removal,[d] %
			Buffers, ng[b]	Plasma, ng[c]	
Seph. 4B	2.5	no	10260	405	99
		yes	21	3	98
	6.25	no	36360	1500	100
		yes	36	3	98
CPG-1400	2.5	no	45	NT[e]	NT
		yes	21	NT	NT
	6.25	no	87	NT	99
		yes	12	NT	70

[a]IAs were treated with 2.5% GA for 1 h at RT.
[b]They involved saline, 0.2*M* phosphate buffers pH 7.4 and 6.5. IAs were stored in 3 mL of fluids at 25°C for 2 wk.
[c]Rotary mixing for 3 h.
[d]Percentage of the amount of IgE adsorbed to that of IgE loaded (7300 U:16.8 μg).
[e]Not tested.

Table 3
Effects of GA on IgG Leakage from Formyl-Cellulofine Immobilizing 5 mg of IgG into Saline and Plasma, and IgE Adsorption

GA treatment, %	Amounts of released IgG into		IgE removal,[a] %
	Saline, ng	Plasma, ng	
None	6500	375	96
0.025	800	96	93
0.25	10	12	90
2.5	1.5	3	85

[a]Percentage of the amount of IgE adsorbed to that of IgE loaded (6100 U:14.0 μg).

A great difference in the amounts of IgG liberated from Sepharose 4B-based and from CPG-1400-based IAs was observed. Comparatively large amounts of IgG were released into saline and plasma from the intact Formyl-Cellulofine-based IA than from the CPG-1400 in spite of utilizing the same covalent bonding. This difference might be attributed to the support materials, but this remains unclear. CPG-1400 was found to be better than Sepharose 4B from the viewpoint of ligand leakage. The amount of IgG liberated during the first 2 wk at 25°C from Sepharose 4B was large, estimated to be 0.1–0.25% of the immobilized IgG, and, according to the findings of Lash and Koelsch (*7*), would be expected to increase if the storage period was extended further. These authors also suggested that the enzyme (ligand) leakage from CNBr-activated Sepharose 6B was high enough to prohibit long-term use of such preparations in routine work at room temperature.

The storage of the Sepharose 4B-based IA at 4°C for 4 wk yielded a certain amount of IgG release. This is important, even though IAs are stored at cold temperatures in spite of the fact that the amount at 4°C was less than at 25°C. Washing the IA just before it was used would effectively reduce the liberation.

The amounts of IgG released from the support into saline and plasma were almost linearly dependent on the amounts of IgG immobilized onto Sepharose 4B. This finding suggests that the amount of antibody to be immobilized must be minimized; to attain this the antibody

Table 4
Effect of Gamma Irradiation on IgG Leakage from IAs into Saline and Plasma

Carriers	Immobilized IgG, mg	Irradiation dose, Mrad	Amounts of IgG released into	
			Saline, ng	Plasma, ng
Seph. 4B	2.5	0	5300	252
		2.5	2800	33
Formyl-Cellulofine	5.0	0	6500	375
		2.5	500	90

should be purified extensively, and the smallest amount possible immobilized.

El Habib et al. studied a leakage problem of their therapeutic IA consisting of collagen membranes immobilizing single- and double-stranded DNA for treating systemic lupus erythematosus (*11*). They concluded that no leakage of DNA from the IA occurred when 250 mL of serum was passed through a module containing the DNA-coated collagen membrane for 3 h and after the membrane was stored in serum at 4°C for up to 76 d. However, although they used radiolabeled DNA and counted the radioactivity retained on the membrane, the sensitivity and accuracy of their assay is suspect. According to our calculations, the assay does not seem to be able to detect as much as 235 ng of DNA. Solal-Celigny et al. (*12*) used protein A immobilized on Sepharose as an immunoadsorption system for treating acute leukemia; they detected as much as 600 μg on the average in 1500 mL of the efferent plasma (405 ng/mL); however, no relation between the amounts of protein A in the plasma and either the intensity of side effects or the drop in blast counts was reported. Although we cannot criticize their analyses because the immunoadsorption procedures were inadequately described, it would seem that the level of foreign protein leached is roughly that at which no particular abnormal biological response occurs in clinical uses.

Although our methodology used to determine the IgG liberated from the support carriers may be criticized because both soluble IgG and still-immobilized IgG on smaller carriers, which can go through the 0.2-μm pore filter, may be determined as a total, we believe that our results are still valid in terms of avoiding leakage of undesirable foreign matters into the therapeutic systems.

The release of protein A from protein A-Sepharose (CNBr-activated) was studied by other researchers (*13*), where the amount of released protein A after incubation with rabbit plasma was more than that after incubation without plasma. The released protein A from the insoluble matrix reacted with IgG of the plasma and formed a complex; a small amount of protein A was also released as fragments as a consequence of the proteolytic action of plasmin in the plasma. In our studies with plasma having a high human IgE level—3 μg/mL, much greater than the level of released IgG—no significant difference in leakage was observed between the high and low IgE plasma (Table 1). The released anti-human IgE antibodies (goat IgG) must be bound to human IgE in the plasma to form a complex. Further experiments will be needed on this subject and on the problem of fragments of the immobilized antibodies. The rotary mixing of IA with rat serum having no human IgE yielded a lower amount of released IgG. At present the mechanism of this remains unknown.

We demonstrated the usefulness of GA as a crosslinking agent to reduce the amount of IgG liberated from support carriers. The greatest reduction of IgG liberation was observed during storage after IAs consisting of Sepharose 4B and Formyl-Cellulofine were crosslinked with GA (Tables 2 and 3). GA treatment provided a hundred times better pro-

tection against leaking of IgG from the supports. The ratio of IgG released to IgG immobilized was calculated to be 2.4×10^{-6} to 1.7×10^{-5} in the aqueous storage system. Soluble intact goat IgG was used as a standard to calibrate the assay. Anti-goat IgG seems to bind intact goat IgG but does not necessarily bind crosslinked IgG perfectly. Thus, if crosslinked IgG leaked from the supports, our assay would not detect all of it. Consequently, the amount of IgG released from GA-treated IAs might be somewhat less than the total amount of all leaked IgG. Kowal and Parsons (*8*) developed a technique to prevent leakage of proteins immobilized on Sepharose. Their procedure involved the use of GA at concentrations ranging from 0.015 to 0.25% (v/v). They reported that treating IA with GA produced a concentration-dependent decrease in ligand leakage. They might have adopted 0.25% at maximum because their iodination procedure employed was capable of iodinating proteins in amounts as low as 0.1 μg. In our study, treating Formyl-Cellulofine with 0.25% GA satisfactorily prevented leakage. Kowal and Parsons also used lactoperoxidase as a ligand and stated that reduction of leakage was significantly greater than the reduction of enzyme activity. Our experience with the antibody activity agrees with their finding on enzyme activity.

Because gamma irradiation makes proteins crosslinked, its use is expected to decrease the amount of IgG released. However, a dose of 2.5 Mrad, which is thought to be a standard dose for sterilizing medical items, prevented the IgG leakage (Table 4), but the effect was no greater than that with GA of more than 0.25%. This quantitative difference may be speculated as being a result of a whole crosslinking of immobilized IgG molecules, linking an adjacent molecule to another with GA, as a bifunctional reagent, whereas the radiation first partially breaks sidechains of IgG molecules, resulting in an intermolecular crosslinking.

The rotary mixing method with plasma used in this study does not necessarily represent an actual clinical situation, but it is useful to know what kinds of factors affect interactions: mainly, leakage of ligands between IAs and plasma, more easily, reproducibly, and conveniently than a column-flow method. A study on leakage of antibodies from IA in vivo associated with animal toxicological tests is the next step.

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