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Indium-III-Labeled Antibody Heavy Metal Chelate Conjugates: A Potential Alternative to Radioiodination[1] (39196)

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During the past few years, nonradioactive methods have been developed to conjugate antibodies with various compounds to facilitate localization of antigen–antibody reactions at the cellular and intracellular levels. One method directly coupled diazotized anthranilic acid to the tyrosyl moieties in the antibody molecule (1). Electron-dense heavy metals were then chelated by the anthranilic acid portion of the conjugate. The coupling step reduced the antibody activity to less than one-tenth of its original activity. However, the subsequent addition of the metal ion did not further reduce the antibody's activity. Another more widely used method is the conjugation of ferritin to the antibody, generally by coupling the free amino groups in each with bireactive compounds such as *m*-xylylene diisocyanate, toluene-2, 4-diisocyanate, or glutaraldehyde (2–4). Moreover, these methods appear to not significantly alter the antibodies' binding capacity (5).

The most common method for labeling antibodies with a gamma-emitting radioisotope is by means of direct radioiodination with ^{131}I or ^{125}I. The radioiodine molecule reacts almost exclusively with the tyrosyl groups in the antibody. Radioiodination nearly always reduces the antibody's titer, and may destroy it completely, perhaps for the same reasons as the diazotized anthranilic acid method which also binds to the tyrosine groups. Recently a new method has been described (6) for labeling proteins with radioiodine to high activities utilizing 3-(4-hydroxyphenyl) propionic acid hydroxysuccinimide ester ^{125}I as an acylating agent to react with the free amino groups. This reaction does not require the tyrosyl moiety. The reagent has been used successfully with protein hormones such as human growth hormone, human luteinizing hormone, and human thyrotropin hormone, and may prove useful in antigen or antibody labeling.

This communication describes a method for labeling gamma globulin with a gamma-emitting radionuclidic marker other than radioiodine. By conjugating the IgG molecule to compounds containing free amino groups capable of chelating heavy metal ions, radionuclides such as indium 111 can be bound to the IgG–chelate conjugate without significantly altering its ability to participate in immunoreactions.

Materials and methods. Preparation of purified IgG. Pooled human serum was obtained from the VA Wadsworth Hospital Center, Nuclear Medicine Service Radioimmunoassay Laboratory. Goat anti-human IgG antiserum was obtained commercially. Human IgG was isolated from serum by the method of Baumstark *et al.* (7), which utilizes the chloride form of DEAE Sephadex A-50 at pH 6.5 and a 0.01 *M* phosphate buffer, pH 6.5. The goat antiserum IgG fraction was not satisfactorily isolated by this method alone, so an ammonium sulfate precipitation techniques preceding the DEAE Sephadex was used. After recovery of the dilute IgG fractions, each was dialyzed against distilled water, lyophilized, and stored at −10°.

Conjugation of IgG to compounds capable of chelating ^{111}In. Compounds such as transferrin, D-penicilamine, and deferoxamine that chelate many heavy metals including ^{111}In, and that also contain free amino groups, were chosen for conjugation to the IgG molecule. The conjugation procedure

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utilizing glutaraldehyde as the coupling compound is a modification of a procedure described by Avrameas (4) for coupling enzymes to proteins with glutaraldehyde. Glutaraldehyde has two active sites which bind the free amino groups on both the chelate and the IgG molecules. The procedure is technically simple and requires only a few hours for completion.

The lyophilized IgG is reconstituted in physiological buffered saline (PBS) in a concentration of 10 mg/ml. The transferrin, deferoxamine, and D-penicillamine are each reconstituted in PBS in a concentration of 4.0, 1.0, and 2.0 mg/ml, respectively. The conjugation is performed by mixing together thoroughly 1 ml of the IgG solution, 0.05 ml of the transferrin, deferoxamine, or D-penicillamine solutions, respectively, and 0.025 ml of a freshly prepared 0.5% glutaraldehyde solution obtained by dilution of a 50% by weight stock solution. The mixture is allowed to stand at room temperature for 2 hr. The resulting conjugates are dialyzed overnight against 1 liter of PBS at 4° to remove unreacted glutaraldehyde, and finally are centrifuged at 2300 rpm for 20 min at 4° to remove any precipitate that may have formed. These conjugates are stored at 4°.

Labeling the IgG-chelate conjugate with $^{111}InCl_3$. Indium 111 chloride was obtained commercially in 0.05 *N* HCl with an activity of 2 mCi/ml. When desired, the $^{111}InCl_3$ is added to the prepared conjugate dropwise in a ratio of 1:5 by volume. This ratio is arbitrary and is chosen merely to prevent depletion of the buffer by the $^{111}InCl_3$ solution. Binding of ^{111}In by the chelate portion of the conjugate is essentially instantaneous.

Assay of immunologic activity. Standard Ouchterlony immunodiffusion plates were used to demonstrate immunologic activity of the human IgG-chelate conjugates and goat antihuman IgG-chelate conjugates. Autoradiographs utilizing standard X-ray film were made of the dried immunodiffusion plates to demonstrate the distribution of the radioactivity in the precipitin bands of the ^{111}In-labeled conjugates. Routine hemagglutination assay was performed to quantify the effect on the antibody of the conjugation procedure and subsequent labeling with ^{111}In. Type A human red blood cells was used as the antigen to determine and compare the titers obtained from the IgG fraction isolated as before from human anti-A serum and the anti-A IgG-chelate conjugate before and after labeling with ^{111}In.

Results. Electrophoretic analysis of the isolated IgG fraction on cellulose acetate strips using a Beckman R-101 electrophoresis cell showed a single discrete spot corresponding to the migration of IgG in the whole serum of both the human gamma globulin and the goat anti-human gamma globulin. After conjugation, the electrophoresis was performed on the IgG-transferrin, IgG-deferoxamine, and IgG-D-penicillamine conjugates, and unconjugated transferrin, deferoxamine, and D-penicillamine labeled with ^{111}In. Autoradiographs of the electrophoretic strip demonstrated essentially all the radioactivity to be confined to the conjugates and to be distinctly different from the unconjugated transferrin, deferoxamine, and D-penicillamine spots (Fig. 1).

Ouchterlony immunodiffusion plates readily demonstrated good retention of the immunologic activity of the conjugates before and after labeling with ^{111}In. The human IgG-chelate conjugates, unconjugated mixtures of human IgG and the respective chelators (mixtures that were carried throughout the entire conjugation procedure except that no glutaraldehyde was added prior to the incubation), and the purified human IgG reconstituted in the same

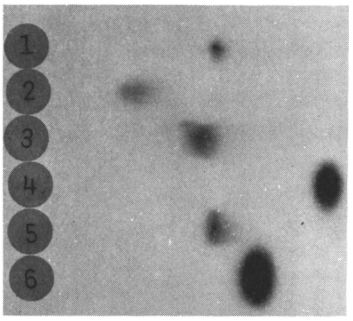

FIG. 1. Autoradiograph of an electrophoretic pattern showing ^{111}In-labeled IgG-chelate conjugates and unconjugate ^{111}In-chelates. 1, ^{111}In-IgG-deferoxamine conjugate; 2, ^{111}In-deferoxamine chelate; 3, ^{111}In-IgG-transferrin conjugate; 4, ^{111}In-transferrin chelate; 5, ^{111}In-IgG-D-penicillamine conjugate; 6, ^{111}In-D-penicillamine chelate.

concentrations as that used in the conjugation procedure were reacted against goat anti-human IgG antisera. The purified IgG and all the conjugates and their respective mixtures produced readily visible precipitate bands. Moreover, no apparent difference is visible in the conjugates before and after labeling with the ^{111}In (Fig. 2).

These results show that the conjugation of human IgG to the chelate and labeling with ^{111}In has not sufficiently changed the human IgG molecule to prevent it from participating as the antigen in the antigen–antibody reaction. To demonstrate the ability of the unlabeled and labeled IgG–chelate conjugates to participate as the antibody in the immune reaction, similar Ouchterlony immunodiffusion plates were made of goat anti-human IgG–chelate conjugates, and of the respective unconjugated goat anti-human IgG–chelator mixtures against human IgG. Again, all the conjugates produced similar precipitin bands after labeling with ^{111}In (Fig. 3).

After formation of the precipitin bands, the immunodiffusion plates were placed in 10% saline solution for 24 hr, in 95% ethanol solution for 2 hr. After drying in room air, autoradiographs were made of the immunodiffusion plates and significant concentration of the radioactivity is present along the precipitin bands of all the ^{111}In-labeled conjugates and none along the precipitin bands produced by the unconjugated mixtures (Fig. 4).

The hemagglutinin titers of the isolated IgG fraction obtained from human anti-A sera, reconstituted in PBS in the same con-

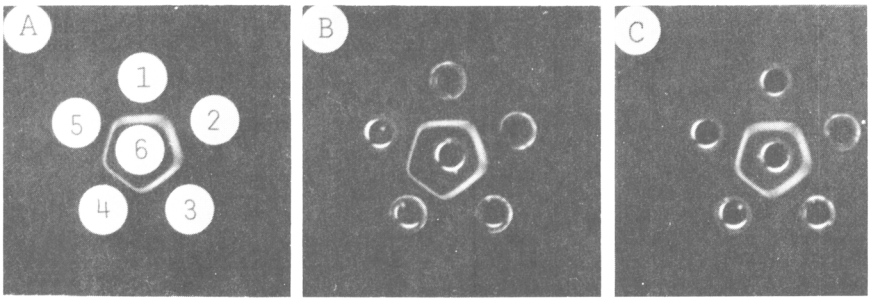

FIG. 2. Immunodiffusion precipitin patterns comparing the human IgG–chelate conjugates, before and after labeling, to unconjugated human IgG + chelate mixtures and the purified human IgG. A1, Human IgG; A2, human IgG + deferoxamine mixture; A3, ^{111}In–deferoxamine + human IgG mixture; A4, human IgG–deferoxamine conjugate; A5, ^{111}In–human IgG–deferoxamine conjugate; A6, goat anti-human IgG sera. B1, Human IgG; B2, human IgG + D-penicillamine mixture; B3, ^{111}In–D-penicillamine + human IgG mixture; B4, human IgG–D-penicillamine conjugate; B5, ^{111}In–human IgG–D-penicillamine conjugate; B6, goat anti-human IgG sera. C1, Human IgG; C2, human IgG + transferrin mixture; C3, ^{111}In–transferrin + human IgG mixture; C4, Human IgG–transferrin conjugate; C5, ^{111}In–human IgG–transferrin conjugate; C6, goat anti-human IgG sera.

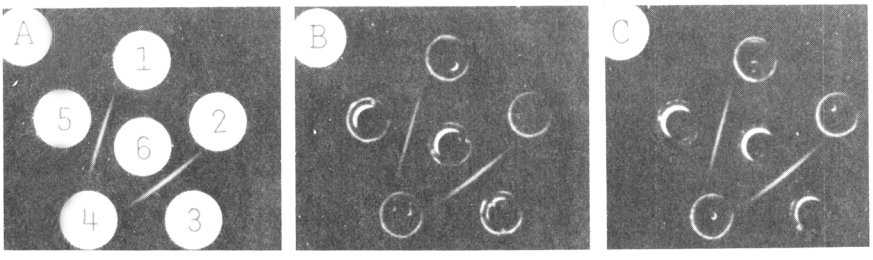

FIG. 3. Immunodiffusion precipitin patterns comparing the ^{111}In–goat anti-human IgG–chelate conjugates to the goat anti-human IgG + ^{111}In–chelate mixture. A1, Empty; A2, empty; A3, goat anti-human IgG + ^{111}In–deferoxamine mixture; A4, empty; A5, ^{111}In–goat anti-human IgG–deferoxamine conjugate; A6, human IgG. B1, Empty; B2, empty; B3, goat anti-human IgG + ^{111}In–D-penicillamine mixture; B4, empty; B5, ^{111}In–goat anti-human IgG–D-penicillamine conjugate; B6, human IgG. C1, Empty; C2, empty; C3, ^{111}In–transferrin + anti-human IgG mixture; C4, empty; C5, ^{111}In–anti-human IgG–transferrin conjugate; C6, human IgG.

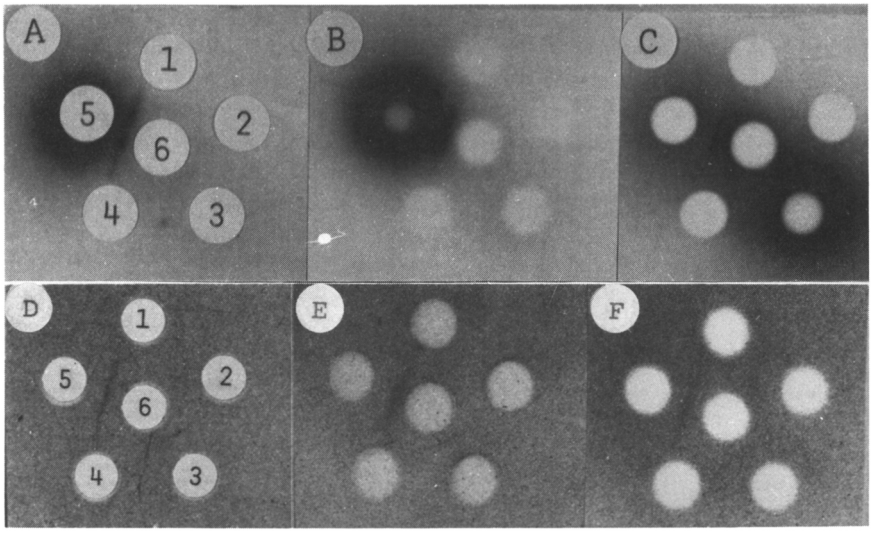

FIG. 4. Autoradiographs of the immunodiffusion precipitin patterns produced by the ^{111}In-IgG-chelate conjugates and the ^{111}In-chelate + IgG mixtures. A1, Empty; A2, empty; A3, ^{111}In-deferoxamine + human IgG mixture; A4, empty; A5, ^{111}In-human IgG-deferoxamine conjugate; A6, Goat anti-human IgG sera. B1, Empty; B2, empty; B3, ^{111}In-D-penicillamine + human IgG mixture; B4, empty; B5, ^{111}In-human IgG-deferoxamine conjugate; B6, Goat anti-human IgG sera. C1, Empty; C2, empty; C3, ^{111}In-transferrin + human IgG mixture; C4, empty; C5, ^{111}In-human IgG-transferrin conjugate; C6, goat anti-human IgG sera. D1, Empty; D2, empty; D3, ^{111}In-deferoxamine + anti-human IgG mixture; D4, empty; D5, ^{111}In-anti-human IgG-deferoxamine conjugate; D6, human IgG. E1, Empty; E2, empty; E3, ^{111}In-D-penicillamine + anti-human IgG mixture; E4, empty; E5, ^{111}In-anti-human IgG-D-penicillamine conjugate; E6, human IgG. F1, Empty; F2, empty; F3, ^{111}In-transferrin + anti-human IgG mixture; F4, empty; F5, ^{111}In-anti-human IgG-transferrin conjugate; F6, human IgG.

centration as that used in the conjugation procedure, and from the ^{111}In-labeled human anti-A IgG-chelate conjugates were identical (Fig. 5).

Discussion. Although gamma globulin and other proteins are commonly labeled with radioiodine, radioiodination has several drawbacks. The procedure requires radioiodine of high specific activity. Consistent high quality labeling requires careful control and cannot be performed easily in the average clinical laboratory. Radioiodinated compounds have a limited shelf-life. Furthermore, if *in vivo* use is contemplated, the radionuclidic characteristics of both ^{131}I and ^{125}I prohibit injection of millicurie amounts that are desirable for imaging procedures.

The ^{111}In-labeled IgG-chelate conjugate overcomes many of these objections. The conjugation procedure is relatively simple, and the conjugate is prepared with nonradioactive materials and does not require special equipment or precautions. Although we have not specifically evaluated the conjugate's shelf-life, Avrameas reported no significant loss of either the enzymatic or immunologic activity of enzyme-antibody conjugates for at least 3 months when such conjugates were stored at 4° (4). The basic reagents can easily be incorporated into "kit forms" which are widely used in clinical nuclear medicine and in radioassay laboratories. Labeling with ^{111}In or potentially other metal radionuclides can then be performed whenever needed, simply and instantaneously.

The efficiency of the radiolabeling process appears equal for all the antibody-chelate conjugates. However, if *in vivo* use is contemplated, it is best to use transferrin as the chelator portion since transferrin binds indium very strongly and has been shown to remove indium from other labeled compounds after intravenous injection (8). In experiments performed in this laboratory, it has been shown that transferrin will remove the ^{111}In label from deferoxamine and D-

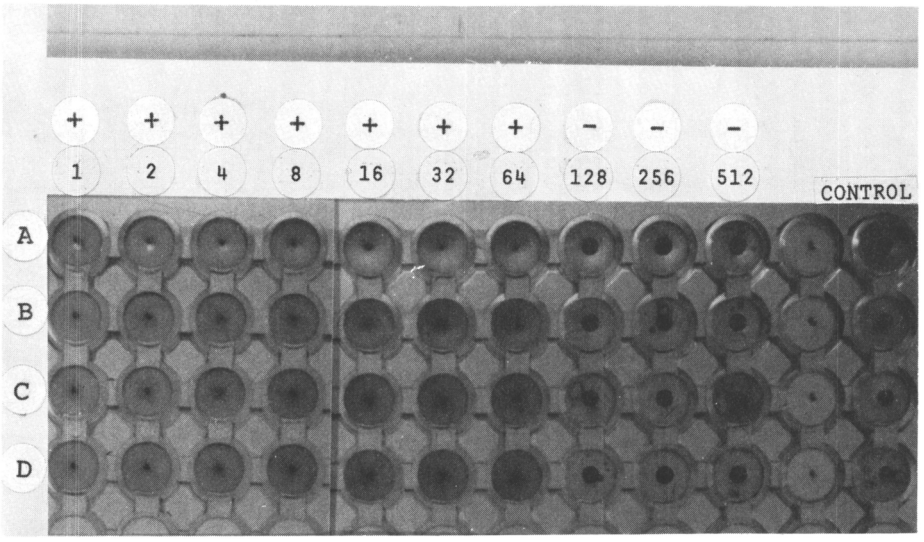

FIG. 5. Hemmagglutination using ^{111}In-labeled human anti-A IgG–chelate conjugate and human anti-A IgG against human type-A red blood cells. A, Human anti-A IgG; B, ^{111}In–human anti-A IgG–deferoxamine conjugate; C, ^{111}In–human anti-A IgG–D-penicillamine conjugate; D, ^{111}In–human anti-A IgG–transferrin conjugate.

penicillamine when these compounds are incubated in whole serum. Several other chelating agents exist which either contain, or can be modified to contain, a free amino group that may bind indium more tightly than transferrin. The efficacy of some of these compounds is being investigated currently, especially for *in vivo* use where competition with transferrin is intense.

In the conjugation procedure it is advantageous to use chelating compounds of low molecular weight since any unconjugated excess can be easily removed by dialysis. Removal of excess chelate is desirable to reduce nonspecific radioactivity when the conjugate is subsequently labeled with the radionuclide. This feature would be especially useful *in vitro* where no significant competition with transferrin exists. Moreover, other radionuclides such as ^{59}Fe that are more tightly bound by deferoxamine and have a longer half-life may be a more suitable label than ^{111}In.

Another potential advantage of this procedure is its less destructive effect on antibody. Methods which react with the tyrosyl groups in the antibody seem invariably to reduce antibody titer. This is certainly true after radioiodination or with anthranilic diazo-coupled gamma globulin. On the other hand, our procedure as well as others that utilize the free amino groups have been shown not to reduce significantly the antibody's titer (5). Furthermore, the subsequent addition of ^{111}In does not react with the antibody portion of the conjugate, and therefore does not appear to further reduce the antibody's titer.

Summary. An alternative method to radioiodination is outlined for labeling antibodies with a radioactive marker. The method requires the conjugation of the antibody molecule to chelating agents that contain a free amino group and are capable of binding heavy metal ions. Glutaraldehyde is the coupling agent used to bridge the free amino groups on both the chelate and the antibody molecules. ^{111}In is then added to the antibody–chelate conjugate and is bound instantaneously by the chelating portion. Electrophoretic, autoradiographic, immunodiffusion, and hemagglutination techniques were used to confirm the integrity of the ^{111}In-labeled antibodies.

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