A RAPID METHOD TO PRODUCE ANTI-GENTAMICIN ANTIBODY

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The aminoglycoside antibiotic, gentamicin, was conjugated to erythrocytes or bovine serum albumin (BSA) by a simple procedure in which ECDI was employed as the coupling reagent. When rabbits were immunized by injecting gentamicin-goat erythrocyte conjugates, three kinds of antibody were produced: 1. anti-gentamicin antibody, 2. anti-ECDI antibody, 3. goat erythrocyte agglutinins. The interfering anti-ECDI antibody was easily neutralized by adding acidified ECDI solution to the immune serum. Goat agglutinins were avoided by employing rabbit erythrocytes as the carrier cell in the hemagglutination titration. Highly specific anti-gentamicin antiserum was produced in rabbits by first injecting an initial dose of gentamicin-BSA conjugate as an emulsion in incomplete Freund's adjuvant *via* the foot pad, followed by multiple intravenous injections of gentamicin-erythrocyte conjugates. The immunization took approximately 21 days. High titered anti-gentamicin antibody was also produced by foot pad inoculation of gentamicin-BSA conjugates; however, the time necessary to achieve comparable titers was considerably longer (55 days). The antibodies produced by both immunization procedures were mainly of the IgG class.

It is well known that low molecular weight compounds, when coupled as haptens to protein or polypeptide carriers, will elicit the production of antibodies capable of reacting specifically with the hapten used[1]. A variety of methods are available for coupling almost any small molecular weight hapten to carriers, the functional group of the hapten governing the selection of the method of conjugation. Conjugation procedures appropriate for different molecules have been reviewed by BEISER, *et al.*[2] The route selected for immunization can often affect the antibody titer attained in the animal. WEBSTER[3] studied the influence of route and schedule of vaccination on the time course of the immune response in a group of rabbits injected with aqueous suspensions of influenza virus antigens. He reported that the routes tested, ranked in the order of decreasing efficiency, were; intravenous, intraperitoneal, and subcutaneous. He also reported that a single intramuscular injection of the virus suspension emulsufied in Freund's adjuvant enhanced the antibody response to subsequent injections of the aqueous antigenic preparation. In most cases, antibodies to low molecular weight compounds have been produced by injecting hapten-protein conjugates in Freund's adjuvant intramuscularly.

Enhancement of antibody production to protein antigens has been achieved by coupling the protein molecule to a particulate carrier[4]. This procedure resulted in increased 19S antibody synthesis, primarily. We report here enhancement of anti-hapten antibody formation by immunization of rabbits with hapten-erythrocyte conjugates. The immunization procedure was considerably faster than those which utilized hapten-protein conjugates, and the antibodies produced were mainly IgG.

Materials and Methods

Conjugate Preparation

Conjugation of gentamicin to bovine serum albumin: Two hundred mg of bovine serum albumin (Mann Research Labs) and 640 mg of gentamicin (Sigma) were dissolved in 20 ml of 0.85% NaCl, 0.01 M

sodium phosphate buffer, pH 7.5. Coupling of the gentamicin to the carrier protein occurred following the dropwise addition of a solution of 6.2 mg 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (ECDI; Sigma) in 20 ml of distilled water. The mixture was incubated for one hour at room temperature followed by 3 days at 4°C. The mixture was then dialyzed at 4°C against physiological buffered saline for four days[5]. This procedure resulted in coupling of approximately 50 molecules of gentamicin to every molecule of albumin (as determined by incorporation of a trace amount of ^{125}I-labelled gentamicin into the antibiotic solution).

Conjugation of gentamicin to goat erythrocytes: Four ml of 50% washed goat erythrocytes were mixed with 240 mg of gentamicin in 120 ml of 0.85% saline. The coupling procedure was accomplished by dropwise addition of 2 g of ECDI in 20 ml of conjugation buffer. After 1 hour incubation at 4°C, the erythrocyte suspension was centrifuged and washed three times in conjugation buffer, washed once in 0.85% saline, and then resuspended in 0.85% saline at 10% final concentration. Following the coupling reaction approximately 100 molecules of gentamicin were bound to every erythrocyte.

Routes and Schedules of Immunization

Three rabbits were injected with an initial dose of gentamicin-BSA conjugate (approximately 0.4 mg gentamicin and 10 mg BSA per injection) given as an emulsion in incomplete Freund's adjuvant, in the foot pad. The rabbits were rested for 4 days and then injected intravenously with 1 ml/kg body weight of a 10% gentamicin-sensitized goat erythrocyte suspension on the following days: 5, 6, 7, 8, 11, 13, 15 and 18.

Six rabbits were immunized in the same manner as the rabbits in the first group with the exception that the initial dose of antigen in adjuvant was omitted. In addition, three rabbits were given 6 foot-pad injections of gentamicin-BSA emulsion, each containing approximately 0.4 mg gentamicin and 10 mg BSA, in incomplete Freund's adjuvant. The injections were spaced 10 days apart.

Determination of Immune Serum Titers by Passive Hemagglutination

Prior to assay of anti-gentamicin antibody, an acidified ECDI solution (1 mg/ml) was added to antisera in equal volumes to neutralize any anti-ECDI antibody which may have interferred with the assay. This was necessary because anti-ECDI antibodies were present in the sera of animals immunized with the ECDI-erythrocyte complex. Preliminary experiments had shown that this procedure removed anti-ECDI antibody more efficiently than absorption of the sera with ECDI-treated erythrocytes. Other procedures (bis-diazo benzidene or tannic acid-treated blood cells) which might have been used to conjugate gentamicin to SRBC did not allow linkage of gentamicin to the erythrocyte membrane. In the hemagglutination test 0.05 ml of a 10% suspension of gentamicin-coated rabbit erythrocytes were added to each tube of 0.5 ml two-fold serial dilutions of ECDI-neutralized immune sera. Hemagglutination was observed after 3 hours of incubation at room temperature. Hemagglutination was evaluated by the presence of cell clumps after gently tilting the tubes.

Determination of the Antibody Class in Immune Serum

The class of immunoglobulin produced by the immunization procedures was determined according to the method of LANGONE *et al.*[6] Various concentrations of Con A Sepharose (Pharmacia) or Protein A Sepharose (Pharmacia) were added to an equal volume of limiting concentration (8 fold higher than the end-point titer) of antibody which had been previously treated with acidified ECDI. Following absorption at room temperature for 30 minutes, the Con A or Protein A Sepharose beads were removed by centrifugation at $1000 \times g$ for 10 minutes. The supernate was then tested for its ability to agglutinate gentamicin coated rabbit erythrocytes or rabbit red cells which had been treated with ECDI only.

Results and Discussion

Antibody Levels in Immune Sera

The results of antibody responses are shown in Table 1. It was found that two rabbits immunized by foot pad inoculation followed by i.v. injections produced high titered anti-gentamicin antibody.

Table 1. Antibody responses of rabbits immunized by foot pad (fp) and/or intravenous routes.

Rabbit number	Immunization route	Time required for immunization[a]	Anti-gentamicin antibody titer[b]	Goat erythrocyte agglutinin titer[b]
1	i.v.	21	7.5	10.5
2	i.v.	21	6.0	11.0
3	i.v.	21	7.0	12.0
4	i.v.	21	5.5	11.5
5	i.v.	21	5.0	10.5
6	i.v.	21	7.5	11.0
1	fp	55	9.3	none
2	fp	55	9.5	none
3	fp	55	9.0	none
1	fp-i.v.	21	9.0	12.0
2	fp-i.v.	21	9.3	13.3
3	fp-i.v.	21	7.0	11.3

[a] Days after immunization begun.
[b] Titer of immune sera expressed as $\log_2$; average of duplicate titrations of 3 separate serum samples drawn 2 days apart.

The third rabbit in the group responded significantly to immunization, although the titers were lower. Three of the six rabbits in the group immunized only by the intravenous route also responded well to immunization (*i.e.*, had a titer greater than 1: 128); however, their antigentamicin titers were slightly lower than that seen in rabbits which also received one foot pad injection. Three rabbits receiving only foot pad injections of BSA-gentamicin produced high titered anti-gentamicin antibody. The main advantage to the i.v. injection procedure was that high titered sera could be obtained in 21 days as opposed to 55 days when BSA-gentamicin was injected *via* the foot pad only.

Specificity of Immune Serum

Passive hemagglutination inhibition was employed to demonstrate the specificity of the immune serum. In one set of tubes, two fold dilutions of gentamicin were mixed with an antiserum dilution containing 4 hemagglutinating units. In another set of tubes, two fold dilutions of streptomycin were mixed with the same serum dilution. Similar dilutions of kanamycin and neomycin were also used. Gentamicin-conjugated rabbit erythrocytes in acidified ECDI buffer were added to all tubes. It was found that as little as 0.24 μg/ml gentamicin inhibited the hemagglutination reaction (Table 2). However, when streptomycin, kanamycin or neomycin were used, positive hemagglutination appeared throughout. These results showed that the antibodies produced by immunization with an erythrocyte-

Table 2. Antibody specificity as indicated by hemagglutination inhibition.

Antibiotic	500[a]	250	125	64	32	16	8	4	2	1	0.5	0.24	0.12
Gentamicin	−[b]	−	−	−	−	−	−	−	−	−	−	−	+
Streptomycin	+	+	+	+	+	+	+	+	+	+	+	+	+
Neomycin	+	+	+	+	+	+	+	+	+	+	+	+	+
Kanamycin	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Antibiotic concentration in μg/ml.
[b] +=Hemagglutination; −=Hemagglutination inhibition.

gentamicin complex were specific to gentamicin.

Immunoglobulin Class of Immune Serum

The ability of concanavalin A to absorb IgM antibodies and of protein A to absorb IgG antibodies was used to determine the immunoglobulin class of the antibodies present in immune sera. The results are shown in Table 3. Protein A treatment of immune sera could effectively remove anti-gentamicin antibodies from the immune sera regardless of the immunization procedure (*i.e.* i.v. or foot pad). Appropriate controls showed that Con A or Protein A treated sera still failed to agglutinate rabbit erythrocytes which were treated with ECDI in the absence of gentamicin (data not shown). Con A Sepharose treatment did not remove anti-gentamicin antibodies. These results indicated that the i.v. route, as well as the foot pad inoculations, elicited antibodies which were mainly of the IgG immunoglobulin class.

Table 3. Effect of protein A and Con A treatment of anti-gentamicin antibodies in immune sera.

Antiserum (route of immunization)	Treatment	Hemagglutination (+ or −)
Intravenous	Saline	+
	Protein A 0.2 μg	−
	Protein A 0.1 μg	−
	Protein A 0.05 μg	±
	Protein A 0.025 μg	+
	Con A 0.2 μg	+
	Con A 0.1 μg	+
	Con A 0.05 μg	+
	Con A 0.025 μg	+
Foot pad	Saline	+
	Protein A 0.2 μg	−
	Protein A 0.1 μg	−
	Protein A 0.05 μg	+
	Protein A 0.025 μg	+
	Con A 0.2 μg	+
	Con A 0.1 μg	+
	Con A 0.05 μg	+
	Con A 0.025 μg	+

In summary, we have developed a procedure for the production of high titered antisera to a hapten (gentamicin) which shortens the duration of the immunization procedure as compared to standard methods. Although TORRIGIANI and ROITT[4] reported heightened antibody synthesis when particulate carriers were used, their responses were not directed toward a haptenic determinant, but rather to a protein molecule. In addition, the procedure of TORRIGIANI and ROITT resulted in an enhancement of the IgM response primarily. The method which we have described elicited an antibody response which was mainly due to antibodies of the IgG class.

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