

Studies on the Optimal Immunization Schedule of the Mouse as an Experimental Animal. The Effect of Antigen Dose and Adjuvant Type

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This study was undertaken to establish the optimal immunogen dose for immunization of mice, using a viomycin-protein conjugate as a hapten immunogen. It was found that specific immunoglobulin G (IgG) formation depends on both the dose of antigen and the type of adjuvant: the optimal antigen dose for an immune response is quite different depending on whether the mice are being treated with Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FICA). The total IgG amount depends mainly upon the type of adjuvant used. FCA gave the double the level of IgG compared to that obtained with FICA. The antigen dose was found to have little influence on the total production of IgG. Mice given a primary immunization with 10 μ g of antigen emulsified in FCA and then given a booster with the same amount of antigen emulsified in FICA produced a strikingly high level of specific anti-viomycin antibody of over 2.5 mg/ml of the antiserum. It was also found that decreases in the weight of the mice were related to the kind of adjuvant used as well as to the level of the specific antibody formed.

Keywords antigen dose; Freund's adjuvant; total mouse IgG; specific mouse IgG; body weight; optimal immunization schedule

An antibody specific to a drug can be a key reagent for various medical studies. To obtain a specific antiserum containing a high level of the antibody with high specificity, some important conditions of immunization, such as the immunogen dose, the type of adjuvant and animal to be used, and the route and number of injections, must be taken into consideration.¹⁾ A wide variety of immunizing schedules has been used to generate specific antisera in various laboratories.^{2–5)} Immunogen doses ranging from ten nanograms to one milligram have been injected, but the adjuvants widely used have been limited to two kinds, FCA and FICA.^{6–8)} Many researchers have devoted themselves to the study of adjuvant activities,^{9–13)} while others have concentrated on antigen doses.^{14–20)} Most studies have used titer or the number of antibody-forming cells as an index of antibody formation, but few papers have reported a systematic quantitative analysis of the effect of interaction between antigen doses and adjuvant activities on the immunological response of an animal.^{21,22)}

Since our knowledge about the optimal conditions for immunization of experimental animals with a drug immunogen is meager, we undertook a series of studies to establish such optimal conditions. In previous papers, we reported the development of several enzyme immunoassay (EIA) methods for antibodies which proved to be useful tools for quantitative analyses of the immune responses of rabbits¹⁾ and mice.²³⁾ Some preliminary results of comparative studies of the adjuvant activities of Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) were reported.²³⁾ Further studies on the relationships between immunogen doses and the immune response of mice in relation to a comparative study of the adjuvant activities of FCA and FICA were undertaken using these analytical methods. In the present paper we report evidence that the immunogen dose and the selection of an appropriate type of adjuvant for both primary and booster injections are extremely important determinants of the antibody responses of mice.

Materials and Methods

Reagents Bovine serum albumin (BSA) and pig serum albumin (PSA) were bought from Miles Lab., Kankakee. Bovine milk casein, FCA and

FICA were purchased from Nakarai Chemicals, Kyoto; Amino-Dylark balls (diameter 6 mm)²⁴⁾ from Sekisui Chemicals, Osaka; and viomycin (VM) from Taito Pfizer Co., Tokyo. VM conjugates VM-(*m*-maleimido-benzoyloxy)succinimide (MBS)-BSA^{25,26)} and VM-(γ -maleimidobutyryloxy)succinimide (GMBS)-PSA²³⁾ were prepared according to the cited methods. A sandwich EIA for mouse IgG²³⁾ and an enzyme-linked immunosorbent assay (ELISA) for mouse anti-VM antibody²³⁾ were performed by the cited methods. Other chemicals used in this work were of reagent grade.

Animals Male BALB/C mice, 8 weeks old, weighing 18–24 g, were purchased from Otsubo Experimental Animal Lab., Nagasaki.

Buffers Buffer A, 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1 M MgCl₂, 0.1% (w/v) BSA, 0.1% (w/v) NaN₃; buffer B, 60 mM sodium phosphate buffer, pH 7.4, containing 10 mM ethylenediamine tetraacetate, 0.1% (w/v) BSA, and 0.1% (w/v) NaN₃; buffer C, the same constituents as buffer B except that casein was used instead of BSA.

Measurement of β -D-Galactosidase (GAL) Activity The GAL activity was measured by a modification of a published method.²⁵⁾ The Amino-Dylark balls were incubated with 0.2 ml of 0.1 mM 7- β -D-galactopyranosyloxy-4-methylcoumarin in buffer A at 30°C for 30 min to measure the bound-enzyme activity. The reaction was stopped by adding 2 ml of 0.2 M glycine-NaOH buffer, pH 10.6, and the 7-hydroxy-4-methylcoumarin liberated was measured at 365 and 448 nm as excitation and emission wavelengths, respectively, with a fluorometer. The amount of GAL-labeled anti-mouse IgG was expressed in units (U) of GAL activity, and 1 U of the enzyme activity was defined as the amount that hydrolyzes 1 μ mol of the substrate per min.

Immunizations Dose-Response Study Using Two Kinds of Adjuvant: Ten groups, each containing four BALB/C mice, were given primary immunization with 200 μ l of saline solution containing 0.01, 0.1, 1, 10, 100, 200, 400, 600, 800, or 1000 μ g of VM-MBS-BSA conjugate emulsified with 200 μ l of FCA. The same experiments were performed for 10 other groups of mice except that FICA was used instead of FCA. All the mice received a booster injection of 10 μ g of the antigen emulsified with FICA in a similar way 4 weeks after each primary injection. The mice were bled through the eye vein two, four, six, and eight weeks later. Every mouse was weighed during the first week after the primary injection. The antisera were kept at –30°C until use.

Study on the Effect of Adjuvant in Primary and Booster Injection: Four groups of mice were given a primary injection of 10 μ g of VM-MBS-BSA, and four weeks later, every member received one booster with the same amount of antigen. The adjuvant used in the primary and booster injections for the four groups were FCA and FCA, FCA and FICA, FICA and FICA, FICA and FCA. The mice were bled four, six and eight weeks after their primary injections.

Results

Effect of Immunogen Doses on the FCA-Aided Immunizations The effects of immunogen doses on elicitation

TABLE I. Total IgG Levels in the Serum Samples of Mice Immunized with Various Antigen Doses Using Either FCA (A) or FICA (B) as the Adjuvant for Primary Injection^{a)}

Antigen dose	Total IgG ^{b)}		
	15th day	29th day	43rd day
A. Total IgG contents in the serum samples of mice receiving FCA-aided immunizations			
10 ng	7.5 ± 0.7	9.5 ± 0.4	5.4 ± 1.0
100 ng	7.6 ± 0.7	13.4 ± 0.6	7.5 ± 1.5
1 µg	6.9 ± 0.7	10.9 ± 1.8	6.1 ± 0.9
10 µg	7.6 ± 0.7	9.3 ± 2.1	5.9 ± 0.5
100 µg	9.1 ± 1.5	10.0 ± 1.7	4.6 ± 0.7
1 mg	6.6 ± 0.9	9.0 ± 0.2	4.6 ± 0.2
B. Total IgG contents in the serum samples of mice receiving FICA-aided immunizations			
10 ng	5.1 ± 0.4	3.2 ± 0.4	3.6 ± 1.1
100 ng	7.3 ± 1.2	6.4 ± 0.5	3.3 ± 0.1
1 µg	5.4 ± 1.0	4.4 ± 0.2	3.7 ± 0.2
10 µg	3.0 ± 0.4	3.8 ± 0.2	2.2 ± 0.1
100 µg	4.7 ± 0.6	3.2 ± 0.2	ND ^{c)}
1 mg	3.4 ± 0.5	4.2 ± 0.5	ND ^{c)}

a) The BALB/C mice were given primary immunization with six doses of antigen using either FCA or FICA as the adjuvant, and given a booster injection with 10 µg of antigen four weeks later, using FICA as the adjuvant. b) The values are expressed as mg/ml of serum, mean ± S.E., *n* = 4. c) Not determined.

of total IgG and the antibody specific to VM in the serum samples were studied. Table I(A) summarizes the total IgG responses of mice two, four and six weeks after the primary immunization with various doses of the VM immunogen, VM-MBS-BSA conjugate, using FCA as the adjuvant. Total IgG responses of the mice of every group were the highest four weeks after the immunization and the maximal level of 13.4 mg/ml was observed at the immunogen dose of 100 ng.

Figure 1 summarizes the anti-VM antibody responses of the same mice mentioned above four and six weeks after the immunization. The specific antibody responses of the mice varied greatly depending on the immunogen doses used and the optimal dose for immune response was found at 10 µg at a specific antibody level of 2.1 mg/ml of the serum.

Effect of Immunogen Doses on the FICA-Aided Immunizations Table I(B) summarizes the total IgG responses of mice two, four and six weeks after the primary immunization with various doses of the immunogen and FICA as the adjuvant. Total IgG levels were maximal for most doses two weeks after the immunization and then decreased in subsequent weeks. Though the level of normal mice before immunization was about 0.8 mg/ml, the maximal level was 7.3 mg/ml at the immunogen dose of 100 ng.

The anti-VM antibody responses of the same mice mentioned above determined four and six weeks after the immunization are shown in Fig. 2. The specific antibody response six weeks after the immunization was higher than that four weeks after the immunization. The FICA-aided immunization required larger amounts of the immunogen for the optimal dose and gave a lower response of the specific antibody compared with the corresponding FCA-aided immunizations. The maximal level of anti-VM antibody was only 0.15 mg/ml at the immunogen dose of 200 µg.

The Study of the Optimal Booster Injection Four groups

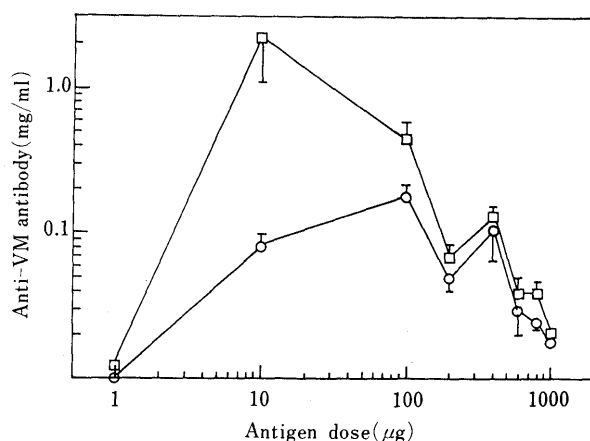


Fig. 1. The Dose-Response Curves of Anti-VM Antibody Elicited in the Mice Four (Circles) and Six (Squares) Weeks after the Primary Immunization Using FCA as the Adjuvant

The mice received primary immunizations with various antigen doses and one booster injection was given to each mouse four weeks later with 10 µg of the antigen using FICA as the common adjuvant. Log doses of antigen and anti-VM antibody responses are given on the horizontal and vertical axes, respectively. Data are displayed as mean ± S.E.

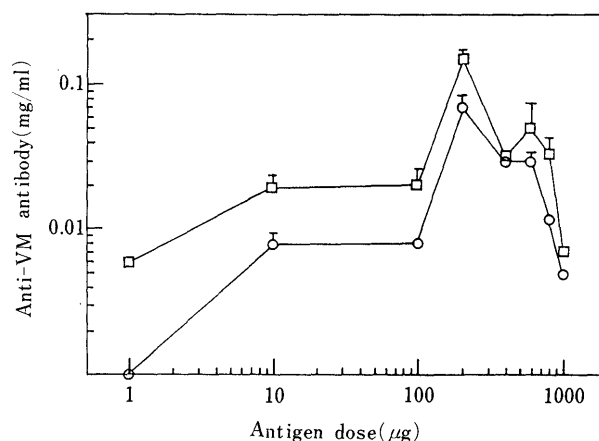


Fig. 2. The Dose-Response Curves of Anti-VM Antibody Elicited in the Mice Four and Six Weeks after the Primary Immunization Using FICA as the Adjuvant

The mice were immunized as described in the legend of Fig. 1, except that FICA was used instead of FCA. The figures are drawn in the same way as in Fig. 1.

of mice were given a primary immunization with a 10 µg aliquot of the VM immunogen, using FCA as the adjuvant for two groups and FICA for the others. One booster injection of a 10 µg aliquot of the immunogen was given to each mouse four weeks after the primary immunization using either FCA or FICA as the adjuvant and the anti-VM antibody responses are shown in Fig. 3.

Anti-VM antibody responses of the mice using FICA as the primary adjuvant were very low regardless of whether FCA or FICA was used as the booster adjuvant as shown in Fig. 2. For the mice using FCA as the primary and the booster adjuvants, the maximal level of anti-VM antibody response was 1.2 mg/ml two weeks after the booster. The level, however, decreased to 0.84 mg/ml a further two weeks later. When FICA was used as the booster adjuvant, the level increased from 0.25 to 1.8 mg per ml of serum two weeks after the booster and a further increase was observed to 2.55 mg/ml another two weeks later.

The Relationship between the Weight of Mice and the

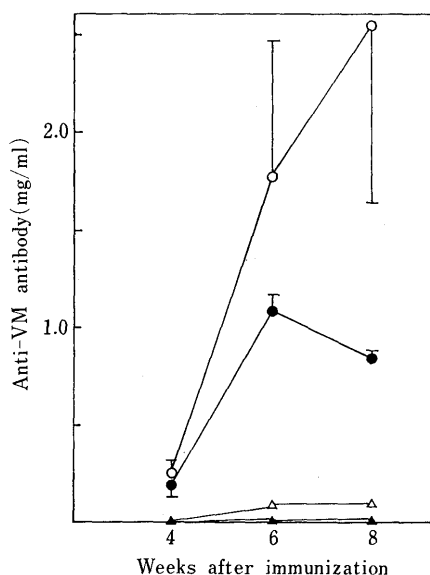


Fig. 3. Serum Anti-VM IgG Levels of Mice before and after Booster Using Two Kinds of Adjuvants for the Primary and Booster Injections

The booster was given four weeks after the primary injection. Both primary and booster injection used $10 \mu\text{g}$ of antigen. The adjuvants used for the primary and booster injections were FCA and FICA (open circles), FCA and FCA (closed circles), FICA and FCA (open triangles), FICA and FICA (closed triangles), respectively. Data are displayed as mean \pm S.E.

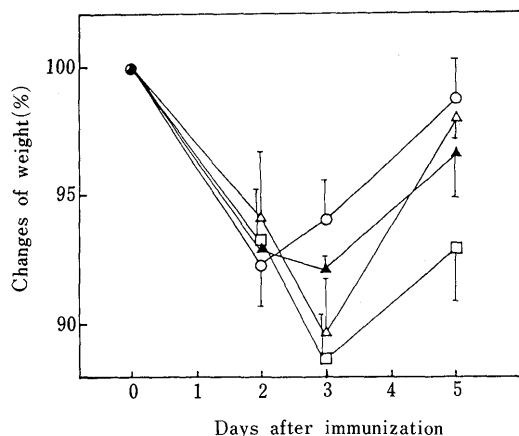


Fig. 4. Percentage Changes in the Weight of Mice after Receiving Primary Injection of $200 \mu\text{g}$ (Open Circles), $400 \mu\text{g}$ (Open Squares), $600 \mu\text{g}$ (Open Triangles) or $800 \mu\text{g}$ (Closed Triangles) of Antigen Emulsified with FCA

The weight of each mouse before injection was calculated as 100%.

Immune Response Increases or decreases of the weights of the mice immunized using FCA as the adjuvant are summarized in Fig. 4. Every mouse lost weight in the first week. The mice of the group receiving $200 \mu\text{g}$ of immunogen decreased in weight to the minimum, 93%, two days after the primary injection. The mice of the other three groups decreased in weight to the minimum on the third day and the group receiving $400 \mu\text{g}$ of antigen showed a minimum of 88%. On the fifth day, the weight of all the groups began to recover, though that of group 3 remained the lowest.

On the other hand, all the mice receiving FICA-aided immunizations increased in weight up to 108% during the first week as shown in Fig. 5, except for those injected with $200 \mu\text{g}$ of the immunogen, the optimal dose for the FICA-aided immunization (Fig. 3). The mice showed a slight weight loss on the second day, but recovery in their weights

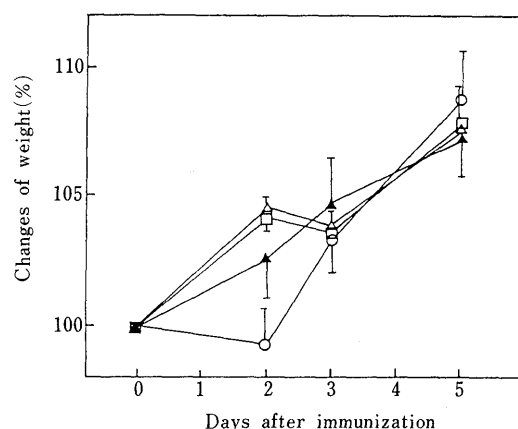


Fig. 5. Percentage Changes in the Weight of Mice after Receiving Primary Injection of $200 \mu\text{g}$ (Open Circles), $400 \mu\text{g}$ (Open Squares), $600 \mu\text{g}$ (Open Triangles) or $800 \mu\text{g}$ (Closed Triangles) of Antigen Emulsified with FICA

The weight of each mouse before injection was taken as 100%.

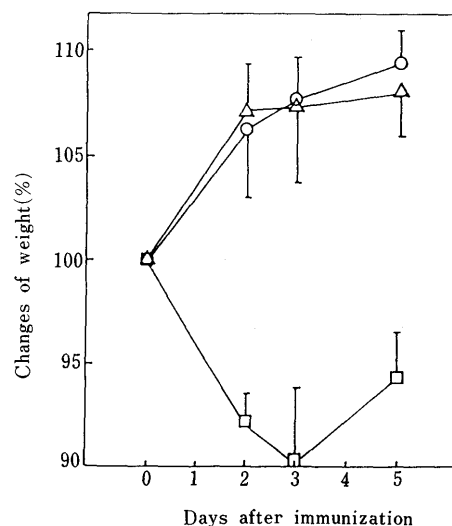


Fig. 6. Percentage Changes in the Weight of Mice after Receiving an Injection of Saline Solution (Open Circles), or FICA (Open Triangles) or FCA (Open Squares) Emulsified with Saline

was very fast, and little difference was observed between them at the fifth day.

For controls, two groups of mice were immunized with either a saline solution or FICA emulsified with saline. Both members showed similar increases in weight. A group of mice injected with FCA emulsified with saline decreased in weight to a minimum of 90% as shown in Fig. 6.

Discussion

We prepared various antisera specific to drugs^{25,27-29} and during the course of the studies, we observed that the titers of antisera varied markedly, depending upon the immunizing schedule of the animals, including factors such as immunogen doses, the intervals between booster injections, and the kinds of adjuvants used. Since the optimal conditions for immunization of experimental animals with drug immunogens have not been established, we undertook a series of studies to find the optimal conditions.^{1,23} In a previous paper we reported evidence that the adjuvant activities of FCA and FICA were similar for the anti-VM

antibody responses of mice, when 400 and 200 μg of a VM immunogen were given as the primary and booster injections, respectively. One interesting observation was that a single booster injection elicited a larger amount of the specific antibody than multiple biweekly boosters in mice.²³⁾

The immunogen doses used for the primary and booster injections should be very important for the immune response of animals, but our knowledge is meager as regards the optimal doses. Our efforts were therefore focused on finding a relationship between the dose and the specific antibody response of mice in relation to the adjuvant activities of FCA and FICA. Two methods of analysis, a sandwich EIA for mouse IgG and an ELISA for mouse anti-VM antibody, developed recently,²³⁾ were used for the present study.

Experiments were designed to elucidate the dose-response relationships of the VM immunogen for anti-VM antibody elicitation in mice. Two sets of mice, each of which were divided into ten groups, were immunized with various doses of a VM-immunogen, ranging from 10 ng to 1 mg. Either FCA or FICA was used as the adjuvant for each set. Only one booster was given for all the mice four weeks after their primary immunization, since our previous study suggested that a single booster gave a higher antibody response in the mice than multiple boosters.²³⁾ We chose a 10 μg aliquot of the immunogen for all booster injections, since it was thought to be a sufficient dose for a booster and also simplified evaluation of the booster effects.

The changes in the total IgG amount of mice in relation to immunogen doses were first studied, since increases in the total IgG vary depending on immunization schedules as reported in previous papers.^{1,23)} However, a clear relationship between the immunogen dose and the total IgG elicited was not observed, for either the FICA- or the FCA-aided immunization. The kind of adjuvant but not the immunogen dose used was found to be most important for total IgG formation, since the highest total IgG level after FCA-aided immunization was twice that of the FICA-aided one, but, in both cases, the immunogen doses corresponding to their maximal levels were only 0.1 μg . Since the doses were so small, it was assumed that the immunogen dose did not have a large effect on the total IgG formation.

The changes in the anti-VM antibody levels of mice were then studied. FCA- and FICA-aided immunizations resulted in completely different dose-response relationships of the specific antibody. In the FICA-aided ones, the optimal dose before (4 weeks) and after (6 weeks) the booster injection was the same at the dose of 200 μg , eliciting 0.15 mg/ml of the specific antibody. However the optimal doses of the FCA-aided immunizations were different before and after the booster injections; 100 μg was optimal before (4 weeks) the booster, eliciting 0.12 mg/ml of the specific antibody, while 10 μg was optimal after (6 weeks) the booster with the elicitation of 2.1 mg/ml. Immunogen doses of either 0.1 or 0.01 μg did not elicit a detectable anti-VM antibody for either the FICA- or the FCA-aided immunization using the highly sensitive EIA method (data not shown in Fig. 2).

In a previous paper we reported that the FCA- and FICA-aided immunizations elicited similar doses of specific antibody in mice using 400 μg of the immunogen for the

primary immunization and 200 μg for the booster. As shown in the present study, the dose of 200 μg for the FICA-aided immunization and 400 μg for the FCA-aided one yielded similar anti-VM antibody responses (about 0.15 mg/ml; Figs. 1 and 2). The fortuitous choice of the immunogen doses for the primary and booster injections could be responsible for our previous conclusion on the similar adjuvant activities of FCA and FICA.

Selection of the optimal kind of adjuvant for booster injection was studied next. The optimal immunizing dose of 10 μg of immunogen in FCA-aided immunizations, as reported above, was used for the first immunization of mice and either FICA- or FCA-aided booster injection was given to the mice. FICA-aided primary injection with 10 μg of immunogen followed by similar booster injection was also performed for comparison.

It was found that using FCA as the primary injecting adjuvant was the key point for immunization, while in booster injection, FICA was more effective than FCA.

Concerning the relationships between body weight and immune responses of animals, we have observed several times that the animals showing high titers of specific antibodies, sometimes decreased in weight during the immunization processes (unpublished data). The relation between weight and immune responses of mice was therefore studied.

The body weights of the mice at the age of eight weeks, tended to increase on treatment with either a saline solution or FICA alone (Fig. 6). FCA contains immunogenic *Mycobacteria* and the mice immunized with FCA alone also decreased in weight. Consequently, it was concluded that the mice showing higher specific antibody levels, especially for the FICA-aided immunizations, tend to decrease in weight soon after their primary immunization, compared to mice showing a lower response.

Although it was found that dosage and the kind of adjuvant for both primary and booster injections are extremely important in the immunization of mice, other probably important conditions for the immunization of experimental animals such as booster interval, choice of mouse, route of immunization, etc. are also under study.

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