Studies on the Optimal Immunization Schedule of Experimental Animals. VI.[1] Antigen Dose–Response of Aluminum Hydroxide-Aided Immunization and Booster Effect under Low Antigen Dose

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The dose–response relationships of a viomycin (VM) immunogen for total immunoglobulin (Ig) G and anti-VM antibody response of mouse using aluminum hydroxide as adjuvant was studied. The condition required to absorb a protein on aluminum gel was first established. The effective immunogen dose for total and specific IgG response of mouse using aluminum hydroxide as the adjuvant was found to be in the narrow range of 5 to 20 μg, and 10 μg per mouse was optimal. The most effective number and intervals of booster injections were studied: when mice were immunized with a lower antigen dose than the optimal, both the number and interval period of booster injections greatly affected the immune response; the more boosters were given, the higher was the response level of specific IgG. The results are contrary to those obtained by immunizing with the optimal or a higher antigen dose.

Keywords optimal immunization schedule; aluminum hydroxide; booster injection; low antigen dose; optimal antigen dose

Although a number of immunization schedules have been reported, it has been difficult to show that any one scheme is better than another.[2] We have undertaken a series of studies of various immunizing conditions to establish an optimal immunization schedule using a viomycin (VM)-immunogen as the common antigen.[1,3–6] We developed two kinds of highly sensitive and accurate enzyme immunoassay methods for mouse immunoglobulin (Ig) G and mouse anti-VM antibody,[4] and these were successfully applied to evaluate the immune response of mice under various conditions.

In previous studies it was found that the optimal antigen dose changes depending on the type of adjuvant used.[5] The way of handling booster injections greatly affected the antibody response.[6]

A wide variety of adjuvants are known to have the property of promoting the antibody response of animals to injected immunogen. Among them, only inorganic adjuvants such as aluminum hydroxide and aluminum phosphate gels have been commonly utilized in human vaccine.[7]

The dose–response relationships of a VM-immunogen for total and anti-VM antibody response of mice using aluminum gel as the adjuvant was studied. In the present paper, the effects of trial times and interval periods of boosters were also reported when the mice were immunized with a lower antigen dose than the optimal, as a further study of booster conditions.[4,6]

Materials and Methods

Reagents Bovine serum albumin (BSA) and pig serum albumin (PSA) were bought from Miles Lab., Kankakee, Ill., and aluminum chloride, bovine milk casein, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) from Nakarai Chemicals, Kyoto. Microtiter plate (96 wells) from Nunc Co., Denmark, and viomycin (VM) from Taito Pfizer Co., Tokyo. VM immunogens, viomycin–(*m*-maleimidobenzydoxy)-succinimide–BSA (VM-MBS-BSA)[8,9] and VM–(γ-maleimidobutyryl-oxy)succinimidyl–PSA (VM-GMBS-PSA)[4] conjugates, and purified goat anti-mouse IgG and its β-D-galactosidase-label[4] were prepared according to cited methods. Amount of the label was expressed as the international unit (U) of galactosidase (GAL) activity measured according to the method reported.[4] Goat anti-mouse IgG antibody- and VM-GMBS-PSA-loaded microtiter plates were also prepared according to reported methods.[1] Other chemicals used in this work were of reagent grade.

Animals The groups of 3 BALB/c male mice aged 8 weeks weighing 18—24 g used for each experiment were purchased from Otsubo Experimental Animals Laboratory, Nagasaki.

Buffers Buffer A (60 mM phosphate buffer, pH 7.4, containing 0.01 M ethylenediamine tetraacetic acid, 1 mM $MgCl_2$, 0.1% (w/v) BSA, and 0.1% NaN_3); Buffer B (20 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1% BSA and 0.1% NaN_3) was used as the diluter of immunological reagents except as otherwise stated; Buffer C (the same composition as buffer B, except that casein was used instead of BSA); Coating buffer (10 mM Tris–HCl buffer, pH 8.5): Washing buffer (buffer A containing 0.05% (v/v) Tween 20); Substrate solution (0.1% *O*-nitrophenyl-β-D-galactopyranoside, dissolved in buffer A); Stop solution (0.2 M glycine–NaOH, pH 10.3).

Sandwich Enzyme Immunoassay (EIA) for Mouse IgG The EIA was performed as follows according to the reported method[1]: each well of a microtiter plate coated with goat anti-mouse IgG was incubated at 28 °C for 1 h with 100 μl of a sample or buffer B as a control. After washing with the washing buffer, each well was incubated at 28 °C for 3 h with 500 μU of GAL-labeled goat anti-mouse IgG solution. The bound enzyme on each well was reacted with 125 μl of the substrate solution at 37 °C overnight. The enzyme reaction was stopped by adding 75 μl of the stop solution and the enzyme activity bound to the microtiter plate was measured at 414 nm using an enzyme-linked immunosorbent assay (ELISA) Analyzer (EAR 400AT, SLT-Labinstruments, Austria).

ELISA for Mouse IgG Specific to VM ELISA for mouse anti-VM antibody was assessed by the same procedures described for sandwich EIA for mouse IgG, except that VM-GMBS-PSA-loaded microtiter plates were used instead of goat anti-mouse IgG antibody-loaded ones.

Absorption Ratio of Protein on Al^{+3} To 5 ml of aluminum chloride solution was added 0.5 ml of BSA solution, and the pH of the solution was adjusted either with 1 N NaOH or saturated $NaHPO_4$ to form $Al(OH)_3$ or $Al_4(PO_4)_3$ precipitation, respectively (conditions for the absorption, such as concentration of the reagents, the reaction temperature, pH and time, are described in Table I). After gentle stirring, the suspension was centrifuged at $1000 \times g$ for 10 min. The amount of BSA left in the supernatant was assessed by optical density at 280 nm. The absorption ratio of BSA to the precipitate was expressed as the following equation:

$$\text{absorption ratio} = \frac{[\text{BSA}]_{\text{added}} - [\text{BSA}]_{\text{left}}}{[\text{BSA}]_{\text{added}}} \times 100$$

Preparation of the Absorbed Antigen in Aluminum Gel A solution of 44.5 mg of $AlCl_3 \cdot 6H_2O$ (molecular weight 241.43), in 5 ml of water was mixed with 0.2 ml of antigen solution, and pH of the solution was adjusted to 6.5—7.0 with 1 N NaOH. The solution was made up to 10 ml with water, and stirred gently for 30 min at room temperature.

Immunization 1. Dose–Response Relationship of Antigen Using Aluminum Hydroxide as the Adjuvant: Eight groups of mice were used: the 3 members of each group were i.p. injected with 0.5, 1, 5, 10, 20, 50, 250 or 650 μg of the VM immunogen, VM-MBS-BSA, which was adsorbed on a 200 μg aliquot of Al^{+3}. Four weeks later, each mouse received a booster injection of 10 μg of antigen adsorbed on 200 μg of Al^{+3}. All the mice were bled through the eye vein two, four, six and eight weeks after priming.

2. Influence of the Interval between Priming and Booster on the Immune Response of Mice: Three groups of BALB/c mice were given a primary injection of 3 μg of VM-MBS-BSA emulsified with 200 μl of FCA, and

each group received one booster injection with the same amount of antigen emulsified with 200 μl of FICA, 2, 4, or 6 weeks later. The mice were bled four, six and eight weeks after the priming.

3. Influence of the Number of Booster Injections: Three groups of BALB/c mice were given the same primary injection described above. Then, 1, 2 or 3 boosters were given at biweekly intervals in the same way as for the booster described above. The mice were bled four, six and eight weeks after primary injection.

Results

Conditions for Absorption of Protein on Al^{+3} BSA was absorbed on the surface of aluminum gel under various conditions (Table I). More than 98% of the albumin was adsorbed under the conditions tested, regardless of factors such as the concentration of Al^{+3} or BSA, the temperature, and the absorption time.

The pH of solutions, however, had significant effect on the absorption. As shown in Fig. 1, aluminum phosphate absorbed the protein under acidic conditions. At pH above 7.0, little protein was absorbed. In contrast, aluminum hydroxide absorbed protein quantitatively at pH above 6.5.

Effect of Immunogen Dose on Aluminum Hydroxide-Aided Immunization The effect of immunogen dose on elicitations of total IgG and the antibody specific to VM in mouse serum samples was studied. Figure 2 shows the dose–response curves of total IgG in mouse serum samples collected two, four and six weeks after priming. The total IgG level was highest in the samples collected 2 weeks after priming and decreased thereafter. The total IgG response largely depended upon antigen dose, with the optimal dose being 10 and 20 μg.

Figure 3 summarizes the anti-VM antibody response of the same mice four and six weeks after immunization. Four weeks after priming, the mice showed very weak response of anti-VM IgG, but six weeks after, they showed significant response depending on the immunogen dose used. The optimal dose was found to be 10 μg with a specific antibody level of 167 μg/ml of serum.

Effect of the Number of Boosters on Production of Total and Specific IgG Mice were primed using an emulsion of 3 μg of the antigen and FCA, and then one, two or three biweekly booster injections were given to three groups of

TABLE I. Conditions Studied for the Adsorption of BSA on Aluminum Hydroxide Gel and the Absorption Percentages

$[Al^{+3}]$ (mg/ml)	[BSA] (mg/ml)	pH	Temp. (°C)	Time (h)	Absorption (%)
1	10	7.0	20	12	>98
1	10	7.0	20	3	>98
1	10	7.0	20	1	>98
1	10	7.0	20	0.5	>98
1	10	7.0	4	1	>98
1	10	7.0	37	1	>98
1	10	7.0	60	1	>98
30	10	7.0	20	0.5	>98
10	10	7.0	20	0.5	>98
0.2	10	7.0	20	0.5	>98
1	30	7.0	20	0.5	>98
1	1	7.0	20	0.5	>98
1	10	5.0	20	0.5	42
1	10	6.0	20	0.5	94
1	10	8.0	20	0.5	>98

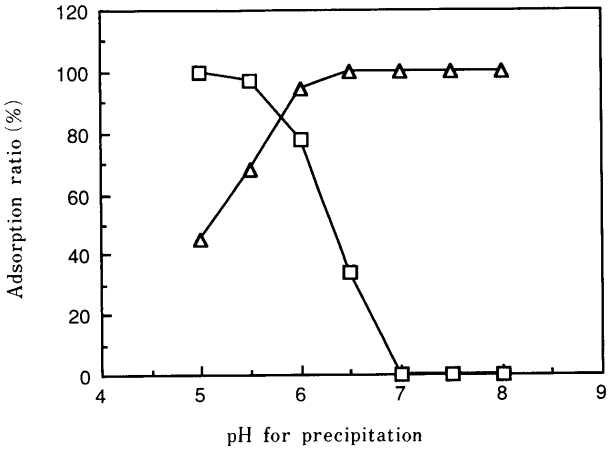

Fig. 1. Effect of pH on Absorption of BSA on Aluminum Gel

5 mg of BSA in 0.5 ml of a solution was absorbed and precipitated by adding 5 ml of aluminum hydroxide (triangles) or aluminum phosphate (squares) solution containing 1 mg/ml of aluminum ion under various pH. After centrifugation, the amount of BSA remaining in the supernatant was assessed by optical density at 280 nm.

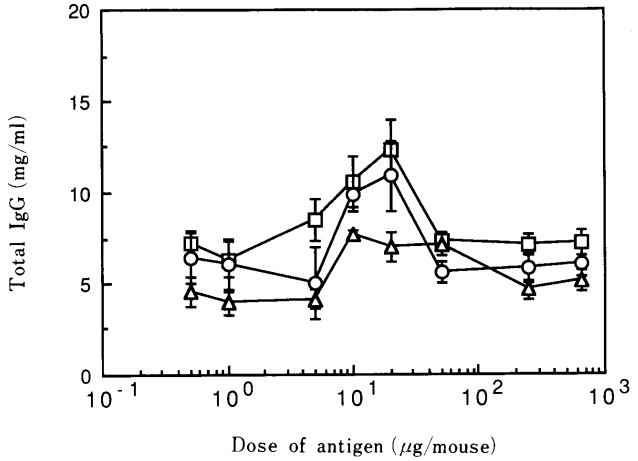

Fig. 2. Dose–Response Curves of Immunogen Dose for Total IgG Response of Mice Using Aluminum Hydroxide as Adjuvant

Mice received a primary immunization with various antigen doses and then one booster injection was given to each animal four weeks after priming using the suspension of a mixture of 10 μg of the antigen and 200 μg of aluminum hydroxide as the common primary and boosting adjuvant. The amount of IgG in the sera collected 2 (squares), 4 (circles) and 6 (triangles) weeks after priming are displayed as mean ± S.E.

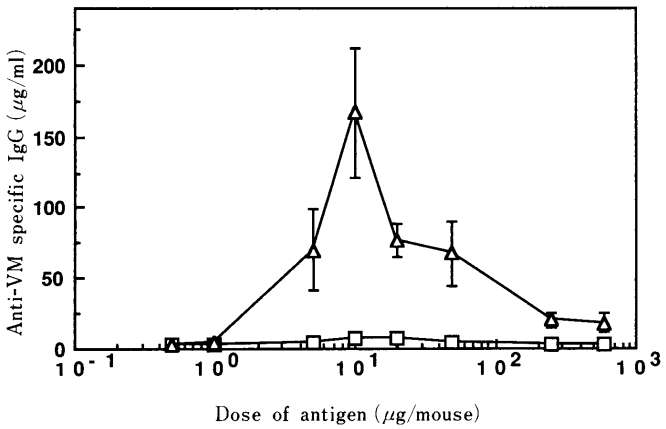

Fig. 3. Dose–Response Curves of Immunogen Dose for Anti-VM IgG Antibody Response of Mice Using Aluminum Hydroxide as Adjuvant

The amount of specific antibody to VM in sera of the same mice as described in Fig. 2 collected 4 (squares) and 6 (triangles) weeks later. The values are displayed as mean ± S.E.

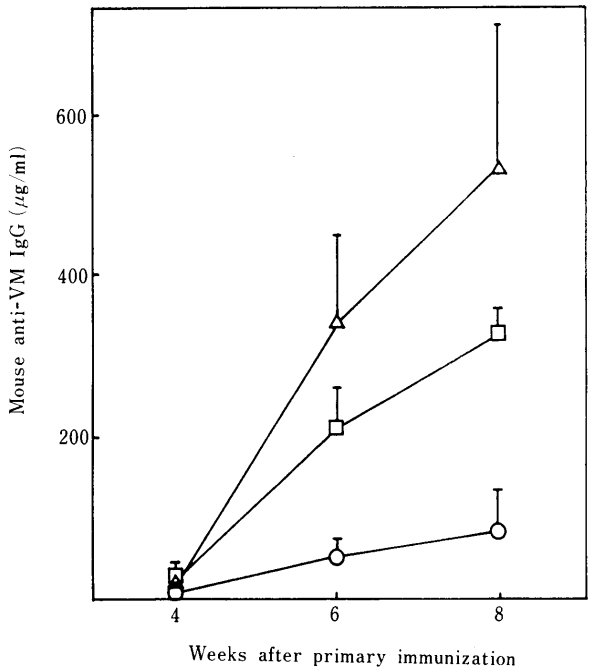

Fig. 4. Influence of the Number of Booster Injections on the Production of Mouse Antibody Specific to VM Using Low Antigen Dosage

8 week old male BALB/c mice were injected with 3 μg of antigen emulsified with FCA, followed by 1 (circles), 2 (squares), and 3 (triangles) biweekly boosters using the same amount of antigen emulsified with FICA. The specific IgG level in the serum samples collected 4, 6, and 8 weeks after the primary injection are displayed as mean±S.E.

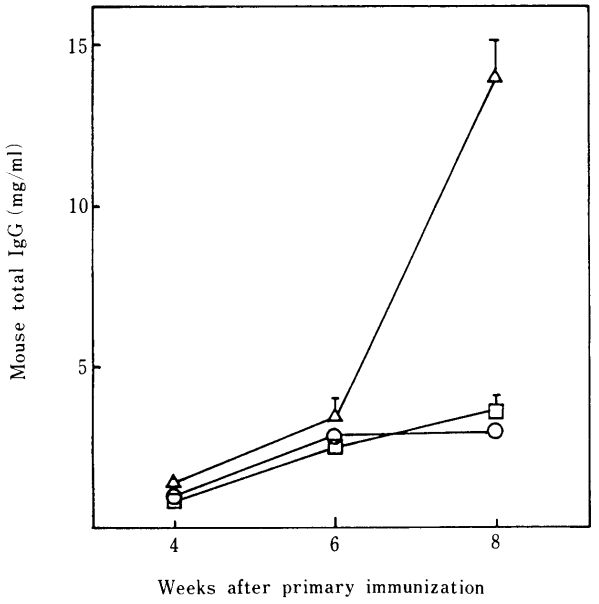

Fig. 5. Influence of the Number of Booster Injections on the Production of Mouse Total IgG Using Low Antigen Dosage

The total IgG level in the serum samples of the same mice are displayed in the same way as in Fig. 4.

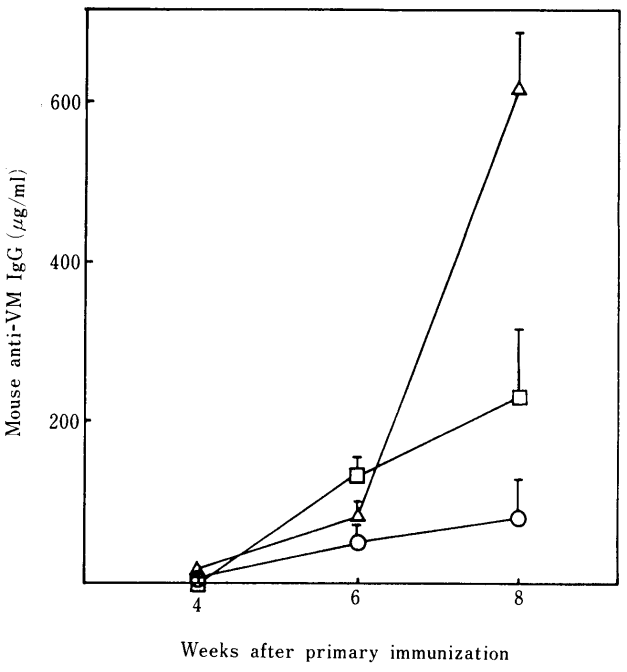

Fig. 6. Influence of Different Intervals between Priming and Booster Injections on the Production of Mouse IgG Specific to VM Using Low Antigen Dosage

Four groups of male BALB/c mice 8 weeks old received a priming immunization of 3 μg of antigen emulsified with FCA, followed by a booster immunization with the same amount of antigen emulsified with FICA at different intervals, namely, 2 (circles), 4 (squares), and 6 (triangles) weeks later. The specific IgG levels in the serum samples collected 4, 6, and 8 weeks after the priming injection are displayed as mean±S.E.

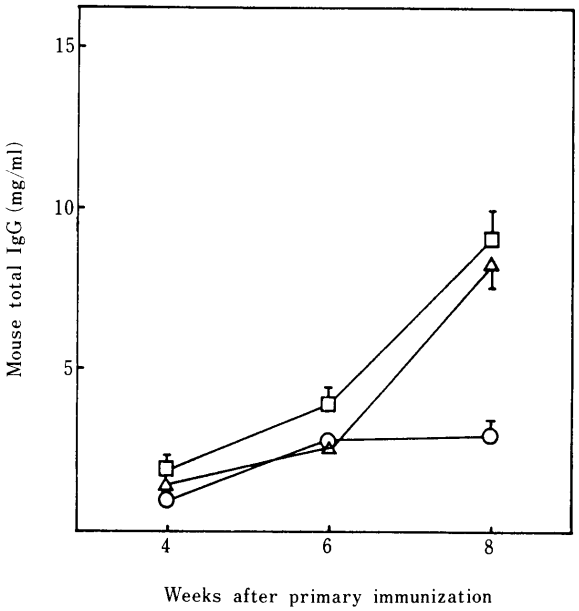

Fig. 7. Influence of the Intervals between Priming and Booster Injection on the Production of Mouse Total IgG Using Low Antigen Dosage

The total IgG level in the same samples as described in the legend of Fig. 6 were measured. The figures are drawn in the same way as in Fig. 6.

the animals, respectively. The contents of anti-VM antibody as well as total IgG in the serum samples collected at three different periods are shown in Figs. 4 and 5, respectively.

The more boosters were given, the larger was the amount of specific IgG elicited in serum samples collected eight weeks after priming under the conditions studied. The specific antibody responding in animals receiving one booster was only 80 μg/ml, while in those receiving three boosters it was 530 μg/ml, about 6.6 times more.

The highest serum levels of total IgG of the same mice were also determined: in those given three boosters the level was 13.5 mg/ml, while in mice given one or two boosters it was about 2 mg/ml, only one-fifth of that value.

Influence of the Interval between Priming and Booster Injection on the Production of Total and Specific IgGs Three groups of mice were primed with 3 μg of the antigen and FCA, and then one booster was given them at different intervals. Figures 6 and 7 show the specific and total IgG levels in the sera collected four, six and eight weeks after priming; small differences were observed in the levels of total IgG of the three groups (Fig. 7). Remarkable differences in the production of specific IgG were observed: the mouse given a booster six weeks after priming responded with more than 600 μg/ml of specific IgG, which was about 3 and 6 times as much antibody as that of animals given a booster 4 weeks and 2 weeks later, respectively.

Discussion

We reported that the dose of immunogen was one of the most important factors in immune response of mice[5] and that optimal immunogen dose varies depending upon the kind of adjuvant used[5]: with the FCA-aided immunization of a VM immunogen, the optimal dose was 10 μg and the response with more than 2 mg/ml of specific IgG, while the optimal dose with the FICA-aided immunization was 200 μg and yielded only 150 μg/ml of specific IgG. Before immunization, average mouse serum contained no anti-VM antibody and 0.8 mg/ml of total IgG.[4]

Aluminum hydroxide and aluminum phosphate are the most popular adjuvants of human vaccines[10-15]: the immunogen was absorbed on aluminum gel for preparation of the vaccines. A few papers reported the necessary conditions for absorption of antigens on an aluminum gel,[16-19] but the dose–response relationship of an antigen in immune response of an animal using aluminum gel as the adjuvant has not been elucidated.

Various conditions under which BSA can be absorbed on aluminum hydroxide gel were first studied (Table I). Among those tested, pH was the most important: aluminum phosphate completely absorbed antigen when pH was acidic, while the optimal pH of aluminum hydroxide was neutral. Aluminum hydroxide was chosen for the adjuvant in the present study.

Experiments were designed to elucidate the dose–response relationships of the immunogen for total IgG and anti-VM antibody responses. Eight groups of mice were immunized with various doses of a VM-immunogen, ranging from 0.5 to 650 μg, using 200 μg aliquots of aluminum gel as the adjuvant. One booster injection of a 10 μg aliquot of the immunogen was given to all mice, and this proved to be the optimal immunogen-dose (Fig. 3). This is the same dose as used in FCA-aided immunization.[4] The highest level of specific IgG response was 165 μg/ml; this is a level similar to the FICA-aided one described above.

It was found that the effective immunogen-dose to respond to the specific IgG when Al^{+3} was used as adjuvant is in the narrow range of 5 to 20 μg under the conditions studied, suggesting that an immunogen dose should be carefully chosen.

We reported that mice receiving four biweekly boosters of a high dosage, *e.g.* 200 μg of antigen, produced only two-fifths the amount of a specific IgG compared to those receiving one booster.[4] It was also found that when mice were immunized with the optimal dosage of 10 μg of antigen using FCA and FICA as primary and booster adjuvants, respectively, the greater was the number of booster shots given, the less was the production of total and specific antibodies.[6] Change in the intervals of boosters had no noticeable difference on the specific antibody response.[6]

The effect of booster times on immune response of mice using a dosage lower than the optimal was studied in the following ways: a 3 μg aliquot of the antigen was used in primary and booster injections using FCA- and FICA-aided immunizations, respectively. Groups of mice received the primary injection, and then one, two, or three biweekly boosters, respectively, while other groups received only one booster but at different intervals, namely two, four or six weeks after priming.

Different from the cases given optimal or higher dosages, the number of boosters was in parallel with the production of both total and specific IgG: the more the boosters were given, the higher the total and specific IgG responded (Figs. 5, 4) under the conditions studied.

Also different from the cases of using either optimal or higher dosages, when mice received an immunization of 3 μg antigen, the interval between the primary and the booster injections greatly affected the production of specific IgG. A booster injected six weeks after priming produced the largest amount of specific IgG, while animals boostered two weeks later had the least response (Fig. 6). Differences in the interval of booster injection did not show a remarkable influence on total IgG level, however.

In conclusion, the effect of boosters was greatly influenced by the antigen dose. With optimal antigen dose, the more boosters that were given the weaker was the response observed; but with low antigen dosage, the more boosters that were given, the more the total and specific IgG produced. The reason for this has not been clarified. The injection of a slightly smaller dose than the optimal followed by several boosters could be better than an overdose in an immunization schedule, though the immune response dose range of mice was wider for an overdose than for a smaller dose (Fig. 3).

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