Studies on the Optimal Immunization Schedule of Experimental Animals. IV.[1] The Optimal Age and Sex of Mice, and the Influence of Booster Injections

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To establish the optimal condition for preparing mouse antiserum specific to a drug, the optimal age and sex of mice for the immune response were studied by measuring the mouse serum levels of total immunoglobulin G (IgG) and specific antibody to viomycin, as well as the changes in weight of mice immunized with a viomycin immunogen. It was observed that age was a more important factor than sex, and strongly affected productions of both total and specific IgGs of mice. The mice aged 8 weeks yielded the highest levels of both total IgG and the specific antibody. In the study on the influence of booster schedule, the number of boosters given had a larger influence on the immune response than the interval between priming and boosters. The greater the number of booster shots given, the less was the production of total and specific antibodies. The decrease in the weight of mice after immunization was also studied in more detail; it was found that it only occurred in the first week after priming but not after a booster injection. The mice aged eight weeks showed the largest weight loss.

Keywords optimal immunization schedule; mouse age; mouse sex; booster number; booster interval; total IgG; specific IgG

Immunization schedule of animals have not as yet been optimized.[2,3] Claims for a particular method are usually based on anectodal evidence which can not be reproduced in the hands of others. Despite the uncertainties, there are certain specific factors which can be related to the success of an immunization procedure, such as the nature and dose of the immunogen,[4–6] the use of adjuvant,[7–9] the animal species and strains,[10–17] and the route of immunization,[18–20] and so on. One of the most difficult problems in establishing the optimal conditions is to develop a reproducible method of analysis for the study of an immune response.

We have been undertaking a series of studies to establish the optimal conditions.[1,21,22] Viomycin (VM) was chosen as the hapten for the common immunogen, and inbred mice as the experimental animals. A sandwich enzyme immunoassay (EIA) for mouse immunogloblin G (IgG) and an enzyme linked immunosorbent assay (ELISA) for mouse antibody specific to VM were developed and used to evaluate the humoral immunological response.[22] Studies to establish the optimal combination of two kinds of adjuvants[1] and the optimal immunogen dose[1] were reported.

As an extension of these studies, the optimal age and sex of mice, as well as the effect of the method used to inject the immunogen were studied. In the present paper, we report evidence showing that both the age of mice and the number of booster injections given have a very strong influence on the production of both total and specific IgG. The sex of mice and the length of the interval between priming and boosting had little influence on the immune response of mice, under the conditions studied.

Materials and Methods

Reagents Bovine serum albumin (BSA) and pig serum albumin (PSA) were bought from Miles Lab., Kankakee. Bovine milk casein, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) were purchased from Nakarai Chemicals, Kyoto; Amino-Dylark balls (diameter 6 mm)[23] were from Sekisui Chemicals, Osaka; and VM was from Taito Pfizer Co., Tokyo. VM conjugates, named VM-MBS-BSA[24,25] and VM-GMBS-PSA,[22] were prepared according to the cited methods. A sandwich EIA for mouse IgG[22] and ELISA for mouse anti-VM antibody[22] were performed by the cited methods. Other chemicals used in this work were of reagent grade.

Animals Both male and femal BALB/c mice aged 2, 4, 6, 8, 12, and 16 weeks were purchased from Seiwa Research Institute for Experimental Animals, Yoshitomi-cho, Fukuoka.

Buffers Buffer A, 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1 M $MgCl_2$, 0.1% BSA (w/v), 0.1% NaN_3; buffer B, 60 mM sodium phosphate buffer, pH 7.4, containing 10 mM ethylenediamine tetraacetate, 0.1% BSA (w/v), and 0.1% NaN_3 (w/v); 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.[24] 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 (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 μmol of the substrate per min.

Immunizations 1. Study on the optimal age and sex of mice for the immune response.

Groups of male or female mice, aged 2, 4, 8, 12, and 16 weeks, were used; each group contained three BALB/c mice. Every mouse was given primary immunization with 200 μl of a saline solution of 10 μg of VM immunogen, VM-MBS-BSA conjugate, emulsified with 200 μl of FCA, excepting that mice aged 2 weeks received immunization with half of the amount. Each mouse received one booster 4 weeks after the primary injection using 10 μg of the antigen emulsified with FICA. The mice were bled through the eye vein four and six weeks later. Every mouse was weighed during the first week after the primary and booster injections. Mice were bled occasionally and antisera were kept at −30 °C until use.

2. Study on the influence of the interval between priming and booster inoculation on the production of mouse IgG.

Four groups of BALB/c mice, each containing three male animals aged 8 weeks, were given a primary injection of 10 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, 6, or 8 weeks later. The mice were bled four, six, eight and ten weeks after the primary injections.

3. Study on the influence of number of booster injections.

Four groups of BALB/c mice, each of which contained three male mice aged 8 weeks, were given the same primary injection described above. Then, 1, 2, 3, or 4 booster(s) were given at biweekly interval(s), using the same method and amount of the immunogen mentioned above for the booster. The mice were bled four, six, eight and ten weeks after primary injection.

Results

Influence of Mice Age on the Production of Total and

Specific IgG The effect of the age of mice was studied in terms of both total IgG and the antibody specific to VM in the serum samples. Figure 1 summarizes the total IgG raised in the mice six weeks after the primary immunization with VM-MBS-BSA conjugate. Total IgG of the mice showed a peak of over 15 mg/ml in the serum of the mice aged 8 and 12 weeks, but the amount was low for mice aged 2 and 16 weeks.

Figure 2 summarizes the anti-VM antibody response of the same mice four and six weeks after priming. The responses of specific anti-VM antibody in sera of mice also varied largely depending on age. Both male and female mice aged 4 and 8 weeks raised in their sera over 1000 μg/ml of specific IgG six weeks after priming; this is twice the level in mice aged 2 or 16 weeks. The serum collected four weeks after priming showed similar results.

Influence of Sex on the Production of Total and Specific IgG In contrast to the influence of age, no obvious difference between male and female mice was observed in the production of total IgG or specific antibody, as shown in Figs. 1 and 2.

Influence of Age and Sex of Mice on the Changes of Body Weight after Priming and Booster Injections The percentage change in body weight of mice within a week after the primary injections was calculated based on the body weight before injections as 100%, and the obtained values are given in Table I. Generally, all the mice lost weight after priming, showing the minimum values at the fourth day. Female mice decreased in weight less than the corresponding male mice. The decreases of young mice were smaller than those of older ones. The mice aged 8 weeks showed the largest decrease. Recoveries of body weight began on the fifth day, but the recoveries did not reach 100% within one week, except for mice aged 2 and 4 weeks.

As shown in Table II, when the mice received a booster four weeks later, hardly any changes in their body weights were observed before and after the booster.

Influence of the Number of Boosters on the Production of Total and Specific IgGs One, two, three, or four biweekly booster injections were given to four groups of mice, respectively. The contents of anti-VM antibody as well as total IgG in the serum samples collected at four different time periods are shown in Figs. 3 and 4, respectively. The number of boosters given strongly influenced the responses of both total and specific IgG.

In the production of specific IgG, Fig. 3 shows that the more the number of boosters, the less amount of the IgG produced in serum samples collected at the eighth week. The specific antibody response to one booster was 1760 μg/ml, while two and three boosters gave 1150 and

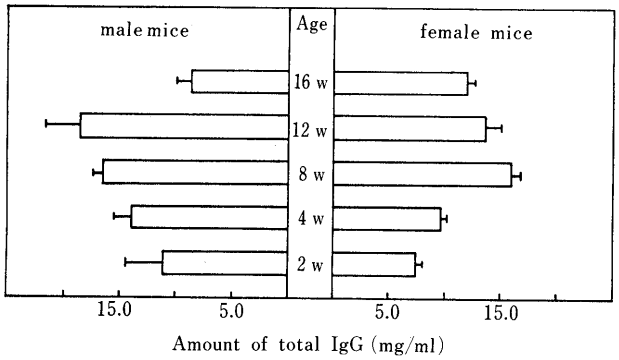

Fig. 1. The Influence of Age and Sex of Mice on the Production of Total IgG

Male and female BALB/c mice, aged 2, 4, 8, 12, and 16 weeks, received primary immunization with 10 μg of an antigen emulsified with FCA, and one booster injection was given to each mouse four weeks later with 10 μg of the antigen using FICA as the adjuvant. The total IgG level in the sera collected 6 weeks after the primary immunization are displayed here as the means ± S.E.

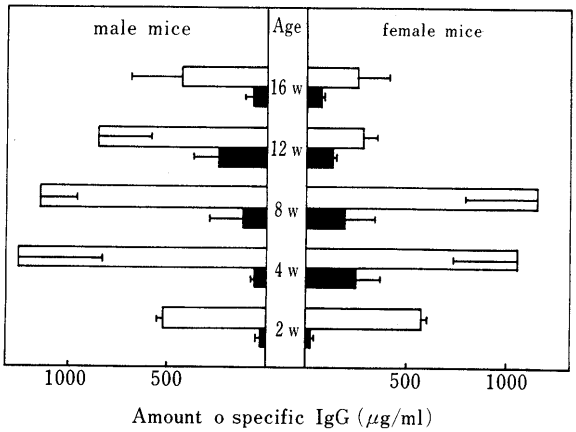

Fig. 2. The Influence of Age and Sex of Mice on the Production of IgG Specific to VM

The specific IgG level in the serum samples collected 4 (black bar) and 6 weeks (white bar) after priming of the same mice mentioned in the legend to Fig. 1 are displayed as the means ± S.E.

TABLE I. Changes in Body Weights of Mice after Priming

Mice[a]		Changes of body weight (%)[b]						
Age (w)[c]	Sex	1	3	4	5	6	7	8 (d)[d]
2	M	94.3	92.1	90.7	91.4	95.7	111.4	120.0
2	F	99.0	94.8	91.4	92.9	98.6	108.6	116.7
4	M	96.5	87.3	81.2	88.8	95.9	100.8	108.4
4	F	97.1	88.5	87.3	98.5	103.8	105.4	108.1
8	M	85.5	79.6	78.2	79.0	80.9	83.4	87.3
8	F	90.4	81.7	77.8	78.5	83.3	88.3	95.1
12	M	91.6	84.8	81.1	80.7	82.5	87.4	90.7
12	F	91.9	82.9	78.8	81.1	83.1	88.5	93.8
16	M	86.1	78.9	78.5	84.9	90.6	95.9	98.3
16	F	86.5	78.4	80.1	86.8	93.3	97.8	98.2

a) The BALB/c mice, male and female, aged from 2 weeks to 16 weeks were given primary immunization with 10 μg of antigen using FCA as an adjuvant, and given a booster injection with the same amount of antigen four weeks later, using FICA as the adjuvant. *b*) The percentage was calculated on the basis that the body weight before immunization is 100%. *c*) Weeks old. *d*) Days after priming or booster immunization. M: male. F: female.

TABLE II. Changes in Body Weights of Mice after the First Booster

Mice[a]		Changes of body weight (%)[b]					
Age (w)[c]	Sex	1	3	4	5	6	7 (d)[d]
2	M	100.9	99.1	100.9	105.3	107.1	111.1
2	F	101.1	98.9	99.6	102.6	105.2	108.1
4	M	98.5	100.5	101.3	98.9	102.3	104.8
4	F	105.1	104.0	104.9	104.4	104.2	104.7
8	M	105.3	102.4	104.9	101.6	101.6	103.5
8	F	104.2	100.6	101.4	102.0	100.8	102.2
12	M	101.9	103.2	103.5	104.9	105.2	108.1
12	F	98.9	98.1	96.7	97.8	98.0	102.2
16	M	96.4	94.6	94.7	100.0	98.9	99.9
16	F	101.4	101.9	101.2	100.1	99.4	102.8

a—*d*) See the footnote of Table I.

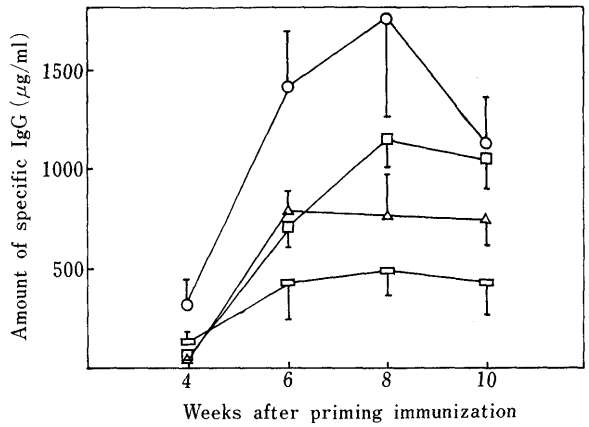

Fig. 3. The Influence of the Number of Booster Injections on the Production of Antibody Specific to VM

Eight-week-old male BALB/c mice were injected with 10 μg of antigen emulsified with FCA, followed by biweekly booster(s) 1 (circles), 2 (squares), 3 (triangles) or 4 (rectangles) time(s) using the same amount of antigen emulsified with FICA for every booster. The specific IgG level in the serum samples collected 4, 6, 8 and 10 weeks after the primary injection are displayed as the means ± S.E.

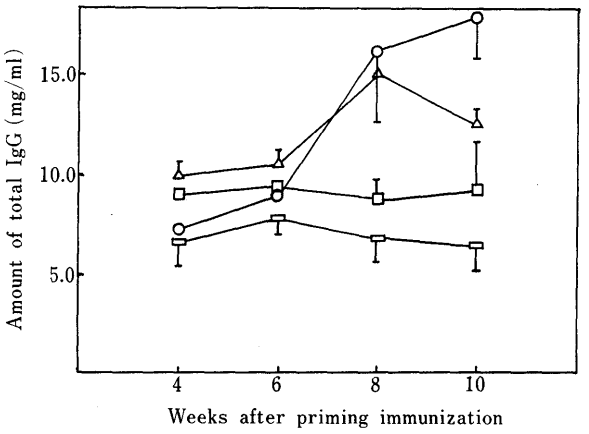

Fig. 4. The Influence of Number of Booster Injections on the Production of Total IgG

The total IgG level in the same serum samples of the mice described in the legend to Fig. 3 were measured. The results are presented in the same way as in Fig. 3.

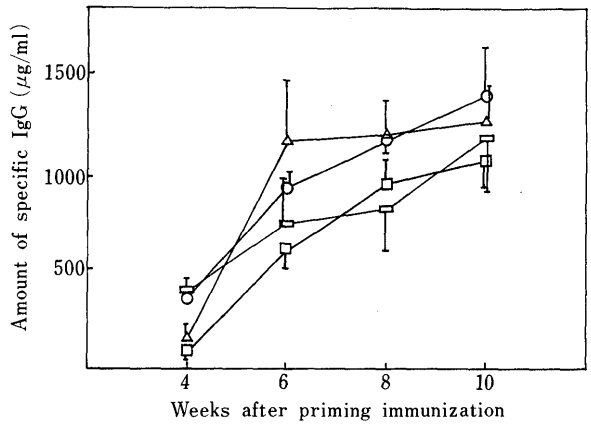

Fig. 5. The Influence of the Interval between Priming and Booster Injection on the Production of IgG Specific to VM

Four groups of male BALB/c mice aged 8 weeks received a priming immunization of 10 μg of antigen emulsified with FCA, followed by booster immunization with the same amount of antigen emulsified with FICA at different periods of time, namely, 2 (circles), 4 (squares), 6 (triangles) and 8 (rectangles) weeks later. The specific IgG level in the serum samples collected 4, 6, 8 and 10 weeks after the priming injection are displayed as the means ± S.E.

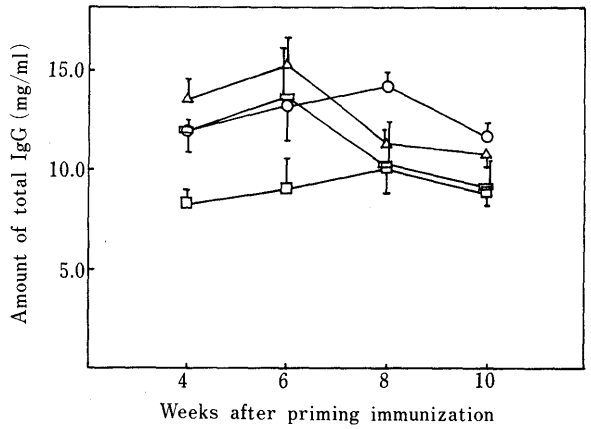

Fig. 6. The Influence of the Interval between Priming and Booster Injection on the Production of Total IgG

The total IgG level in the same samples described in the legend to Fig. 5 were measured. The results are presented in the same way as in Fig. 5.

770 μg/ml, respectively, and with four boosters the value was only 490 μg/ml. The mice given one booster produced more than about 3.5 times as much specific IgG as the mice given four boosters.

Serum levels of total IgG of the same mice were also determined at four, six, eight and ten weeks after the primary immunization. The level of the mice given one booster was 17.8 mg/ml, while that of mice given four boosters was only 6.4 mg/ml, about one third of the former value.

Influence of the Interval between Priming and Booster Injection on the Production of Total and Specific IgGs The same primary injections were given to all four groups of male BALB/c mice aged 8 weeks with 10 μg of VM-MBS-BSA emulsified with 200 μl of FCA. Each group of mice was given one booster at different intervals, namely two, four, six or eight weeks later, in the same way and with the same amount of the immunogen as those used for the first injection, except that FICA was used instead of FCA.

Figures 5 and 6 shows the specific and total IgG levels in the sera collected four, six, eight and ten weeks after priming injection. Small differences, most of which are within the range of assay variations, were observed in the curves showing the serum levels of both the specific antibody (Fig. 5) and total IgG (Fig. 6) of the four groups of mice. It suggested that the interval between priming and booster had little influence on the production of total IgG and the specific antibody.

Discussion

It is well known that there are many factors which can affect the success of an immunization procedure, such as the nature and dose of the immunogen, the use of adjuvant, the animal species, the route of immunization, and so on. Since our knowledge of the relation between an individual factor and the immune response of an experimental animal is meager, we undertook a series of studies to analyze the relations of these factors using a hapten protein conjugate, VM-MBS-BSA as the common immunogen, and inbred BALB/c mice as experimental animals.[1,22)]

In a previous paper, we reported that two new analytical methods, a sandwich EIA for mouse IgG and ELISA for

mouse anti-VM antibody, are very useful tools to follow the immunization process in mice.[22] By means of these methods, the optimal immunogen dose and the optimal combination of the two kinds of adjuvants, FCA and FICA, were studied.[1] Since the immunogen dose and the kind of adjuvant showed marked effects on the immune response of mice,[1] the optimal conditions for immunization of mice, in terms of selecting both the immunogen dose and the kind of adjuvant, were applied to the present studies to find the optimal age and sex of mice, as well as to assess the effect of the number of booster injections of the immunogen.

The effects of age and sex of mice on the humoral immune response of mouse were first investigated in the present study. A set of five groups of male and female mice of various ages were immunized with VM-MBS-BSA and their immune responses were evaluated. It was found that the mice aged from 4 to 12 weeks are suitable for the immunological experiment. The mice aged eight weeks seemed to have the maximal immune response, judging from the produced amounts of both total and specific IgGs. It was also found that the sex of the mice had no significant effect on the immune response.

In a previous paper,[1] we reported that both the immunogen dose and the kind of adjuvant used for the primary and booster injections were extremely important for the immune response of mouse.

As an extension of that study, other conditions for booster injection were studied using the optimal immunogen dose and adjuvant established previously, by changing either the interval between the priming and booster injection, or the number of boosters. It was found that a change in the interval had no marked influence on immune response. On the contrary, the number of booster injections strongly affected the immune response in terms of both total and specific IgGs; the greater the numbers of boosters given, the smaller the amounts of total and specific IgGs elicited in mice. This may be ascribed to the phenomenon of immune tolerance.

Concerning the relationship between body weight and immune response of animals, it was observed that the animals showing high titers of specific antibodies sometimes decreased in weight during the immunization processes.[1] Since the conditions for immunization of mice have a large effect on the decrease in weight of mice,[1] the optimal conditions for immunization were again used for this study. Judging from the data in Table I, all the mice decreased in weight soon after priming despite differences in their sex and age; the female mice decreased in weight less than the corresponding male mice. The eight-week old mice, which showed the highest immune response, also showed the largest decrease in weight; compared to older mice, the weights of younger mice showed smaller decreases and also faster recoveries. Only young mice recovered in weight to over 100% within a week. No marked decreases, however, were observed for these mice after their booster injections (Table II).

Other important conditions for the immunization of experimental animals are under investigation.

Acknowledgment This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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