Comparative Study of Complete and Incomplete Freund's Adjuvants for Immunization of a Drug Immunogen Using Enzyme Immunoassay Methods

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Highly sensitive and accurate enzyme immunoassays (EIAs), a sandwich EIA for mouse immunoglobulin G (IgG) and an enzyme linked immunosorbent assay for mouse antibody specific to viomycin (VM), were developed. Accuracy and specificity of the assay results were confirmed before their application. The changes of total IgG and antibody specific to VM in mice, immunized with a VM-immunogen with or without two types of Freund's adjuvants under various conditions, were assessed by means of the newly developed EIA methods. Both methods were very useful tools to follow the immunization processes of mice, and complete and incomplete Freund's adjuvant were found to have similar adjuvant activities for production of antibody specific to VM, judging from the amounts of anti-VM antibody formed. It seems to be important that too many booster injections should be avoided in the immunization of mice with a hapten immunogen.

Keywords enzyme immunoassay; ELISA; antibody response analysis; specific antibody; Freund's adjuvant; anti-hapten antibody

Specific antiserum has been a key reagent for various immunological studies, and various immunization schedules of antigens have been proposed.[1-7] Since the titer of the specific antiserum depends largely upon the immunizing schedule of the antigen, such as immunogen dose, kind of adjuvant used, intervals between booster injections and so on,[8] we have been attempting to establish optimal conditions for immunizing animals.

An adjuvant may be described as a substance that, when mixed with an antigen prior to injection, enhances antibody production. A widely used adjuvant is Freund's adjuvant,[9,11] a mixture of an emulsifier such as Arlacel A in mineral oil with (Freund's complete adjuvant, FCA) or without (Freund's incomplete adjuvant, FICA) mycobacteria.

In the preceding paper we reported an analysis of the antibody response of rabbits immunized with four immunogens using a combination of four enzyme immunoassay (EIA) methods.[8] Although the EIA methods were effective for quantitative analyses of immunization processes of rabbits, accurate analyses to compare the adjuvant activities of FCA and FICA *in vivo* requires a group of experimental animals and we chose mice, since an inbred strain is easy to obtain for mice but not for rabbits.

Two highly sensitive and accurate analytical methods to study the dose-response relationship of mouse specific antibody were developed: a sandwich EIA for mouse IgG and an enzyme-linked immunosorbent assay (ELISA) for mouse antibody specific to viomycin (VM). We also report the results of a comparative study on the activities of complete and incomplete Freund's adjuvants using a VM immunogen as a common antigen and the newly developed EIA methods.

Materials and Methods

Reagents Cyanogen bromide-activated Sepharose 4B and DEAE-Sephacel were bought from Pharmacia Fine Chemicals (Uppsala), β-D-galactosidase (GAL) from Boehringer Mannheim, (F.R.G.), mouse IgG, bovine serum albumin (BSA) and pig serum albumin (PSA) from Miles Lab. (Kankakee). Freund's complete and incomplete adjuvants (FCA and FICA) were purchased from Nakarai Chemicals (Kyoto), *N*-(γ-maleimidobutyryloxy)succinimide (GMBS),[12] and Amino-Dylark balls (diameter 6 mm)[13] from Sekisui Chemicals (Osaka). VM-MBS-BSA conjugate was prepared by the reported method.[14] Other chemicals used in this work were of reagent grade.

Immunizations 1. The Preparation of Goat Anti-mouse Immunoglobulin G (IgG): A female goat was immunized by intramuscular injections with 1 ml of a sline solution of mouse IgG (1 mg) emulsified with the same volume of Freund's complete adjuvant. Three booster injections were given at biweekly intervals with half the dose of the first except that Freund's adjuvant of the incomplete type was used instead of the complete type. The goat was bled and the antiserum was kept at −30 °C until use.

2. The Preparation of Mouse Anti-VM IgG: DDY and ICR female mice were immunized by intraperitoneal injections with 20 μl of a saline solution of 20 μg of VM–MBS–BSA emulsified in 0.2 ml of Freund's complete adjuvant. Booster injections were given at biweekly intervals 7—10 times with half the dose of the first. Ascites was collected and centrifuged at 0 °C for 20 min at 12000 × *g* and supernatant was stored at −30 °C.

3. For the Study of Immunoresponse: Male BALB/C mice (8 weeks of age, weighing 22—27 g) were divided into six groups, each of which contained 3 mice. Three groups of mice were immunized by intraperitoneal injections with the VM immunogen, VM–MBS–BSA, using FCA or FICA and the other three groups of mice were immunized with the antigen, FCA or FICA alone (see below, and Table VI).

The mice were bled before and two weeks after the initial immunization and then every week from the third week. The serum samples were diluted with buffer B. A 1000-fold diluted serum was used for assay of mouse anti-VM specific IgG, and a 10000-fold diluted serum was used for assay of total mouse IgG.

Isolation of the Specific IgG Crude goat anti-mouse IgG fraction collected from 30 ml of goat anti-mouse IgG serum by the ammonium sulfate fraction method was further purified by affinity chromatography on a 1.4 × 10 cm column of mouse IgG-coupled Sepharose 4B as follows: the goat IgG fraction was loaded on the affinity column previously swollen with 0.02 M Tris–HCl buffered saline (pH 8.3). Non-specific IgG was eluted from the column with the same buffer, and the antibody absorbed on the column was then eluted with 0.3 M KCl–0.008 M HCl solution (pH 2.3). The goat anti-mouse IgG fraction was neutralized with 1 M K_2CO_3.

The IgG fraction of mouse anti-VM IgG ascites was collected by means of the ammonium sulfate fraction method, and the IgG fraction was then purified by means of affinity chromatography using a VM–MBS–PSA-coupled cyanogen bromide-activated Sepharose 4B column (1.4 × 12 cm), in the same way as described above.

Enzyme Labeling of Goat Anti-mouse IgG Antibody Goat anti-mouse IgG (14 nmol) was dissolved in 1 ml of 0.05 M phosphate buffer, pH 7.0, and was incubated with 0.1 ml of tetrahydrofuran solution of GMBS (35 nmol) at 30 °C for 30 min. One milliliter of GAL (9 nmol) dissolved in 0.1 M phosphate buffer, pH 7.0, was added to the mixture with vortex mixing and the mixture was incubated at 30 °C for 2 h. The mixture was loaded on a 1.8 × 40 cm column of DEAE-Sephacel. The column was stepwise eluted with 0.05 M phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$ and 0.1 M NaCl, and then with the same buffer containing 0.4 M NaCl. The enzyme and immune activities of each fraction were assayed by

the methods described below.

Measurement of Enzyme Activity A 5 μl aliquot of diluted enzyme solution was incubated at 30 °C for 5 min with 200 μl of 0.1 mM 7-β-D-galactopyranosyloxy-4-methylcoumarin solution in buffer A. The reaction was terminated by adding 2 ml of 0.2 M glycine–NaOH buffer, pH 10.3, and the 7-hydroxy-4-methylcoumarin liberated was measured by spectrofluorometry with excitation and emission wavelengths of 365 and 448 nm, respectively. The amount of enzyme or its conjugate is expressed in units (U) of the enzyme activity, defined as the amount that hydrolyzes 1 μmol of the substrate per min.

Preparation of Goat Anti-mouse IgG-Loaded Amino-Dylark Balls The Amino-Dylark balls were immersed in 1% glutaraldehyde for 1 h, followed by washing with 0.01 M phosphate-buffered saline, pH 7.0 (PBS). Each ball was then immersed in 1 ml of 0.01 M PBS containing 2 μg of goat anti-mouse IgG at room temperature for 30 min with shaking and then at 4 °C for 2 h. After successive washing with buffer B, goat anti-mouse IgG-loaded Amino-Dylark balls were stored in buffer B until used.

Sandwich EIA for Mouse IgG Each Amino-Dylark ball coated with goat anti-mouse IgG was incubated at 30 °C for 2 h with a standard mouse IgG or a sample solution in a final volume of 0.2 ml buffer B. After washing with buffer A, the ball was incubated at 30 °C for 2 h with 500 μU of GAL-labeled goat anti-mouse IgG in 0.2 ml of buffer A. Each ball was washed twice with 1 ml of buffer A and transferred to another test tube to eliminate nonspecific binding. The enzyme activity bound to the ball was measured.

ELISA for Antibody Specific to VM ELISA for mouse anti-VM antibody was done by the same procedure described for sandwich EIA for mouse IgG except that VM–GMBS–PSA–loaded Amino-Dylark balls were used instead of goat anti-mouse IgG-loaded ones.

Results

Sandwich Enzyme Immunoassay of Mouse IgG A highly sensitive sandwich EIA for mouse IgG with the assay range of 1—1000 ng/tube was established using goat anti-mouse IgG antibody-coated Amino-Dylark balls as the solid-phase antigens and GAL-labeled goat anti-mouse IgG antibody as the tracer. A typical dose–response curve with mouse IgG is shown in Fig. 1.

Experimental results for accuracy and precision of ELISA are summarized in Table I. Good recoveries (92—106%) were obtained for 5 samples with intra-assay coefficients of variation of less than 17%. Inter-assay, 13 samples also showed good recoveries (94.6—105.3%) with coefficients of variation of less than 21.8% (Table I).

This assay can be applied to 10^4 to 10^6 fold diluted serum samples with high sensitivity and accuracy: the reliability of the assay values for serum samples was confirmed by the recovery tests of added mouse IgG (10 and 100 ng). The recovery percentages are 92% to 120% as shown in Table II.

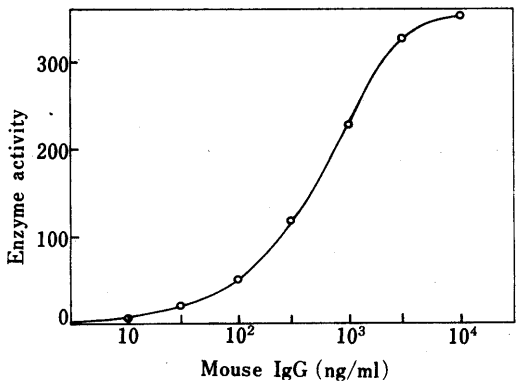

Fig. 1. A Typical Dose–Response Curve of a Sandwich EIA for Mouse IgG

The bound enzyme activity is plotted against logarithmic dose of mouse IgG (10.0—10000 ng/ml).

Specificity of the EIA for Mouse IgG This assay is highly specific to mouse IgG and hardly any crossreactivities were observed for human, horse, rabbit and pig IgGs (Table III).

ELISA for Mouse Antibody Specific to VM A highly sensitive ELISA for mouse anti-VM antibody was de-

Table I. Intra- and Inter-assay Quality Control Data for a Sandwich EIA of Mouse IgG

Added amount (ng/tube)	Estimated ng/tube[a] Intra-assay[b]	Inter-assay[b]
3	2.97 (92.0)[c] ± 0.39[d] (14.0)[e]	3.00 (100.0) ± 0.11 (3.7)
10	10.38 (103.0) ± 0.39 (3.8)	9.90 (99.3) ± 0.81 (8.2)
30	30.70 (102.0) ± 1.32 (4.3)	29.90 (99.8) ± 1.80 (6.0)
100	97.50 (97.5) ± 3.8 (3.9)	94.50 (94.6) ± 9.40 (10.0)
300	320.00 (106.0) ± 55.4 (17.0)	316.0 (105.3) ± 69.0 (21.8)

a) Concentration is given as ng/0.1 ml of sample solution. *b*) Number of experiments was 5. *c*) Number in parentheses, recovery (%). *d*) Mean ± S.D. *e*) Number in parentheses, coefficient of variation (%).

Table II. Recoveries of 10 or 100 ng of Mouse IgG Added to Three Diluted Solutions of Normal Mouse Serum Measured by a Sandwich EIA for Mouse IgG

	Content of anti-VM antibody in diluted anti-VM serum solutions (ng/tube)		
Dilution fold	10^6	10^5	10^4
Mouse IgG level (A)	1.8 ± 0.3	12.0 ± 0.9	113.0 ± 5.3
(with 10 ng addition) (B)	12.5 ± 1.3	22.0 ± 0.8	125.0 ± 5.3
Recovery (%)[a]	107.0	100.0	120.0
(with 100 ng addition) (C)	110.0 ± 4.1	105.0 ± 2.2	205.0 ± 7.2
Recovery (%)	108.2	93.0	92.0

a) Recovery percentages were calculated by applying the following equation:

$$(B \text{ or } C - A)/(\text{added amount}) \times 100\%.$$

Table III. Cross-Reactivity of Anti-mouse IgG Serum Against Various IgGs Determined by a Sandwich EIA for Mouse IgG

Sample amount (μg)	Percentage of cross-reactivity Human IgG	Horse IgG	Rabbit IgG	Pig IgG
1	ND	ND	ND	ND
10	0.0008[a]	ND	ND	0.002
100	0.024	ND	0.0003	0.0012

ND, not detected. *a*) The percentage value was calculated from the determined value by using the dose–response curve of mouse IgG.

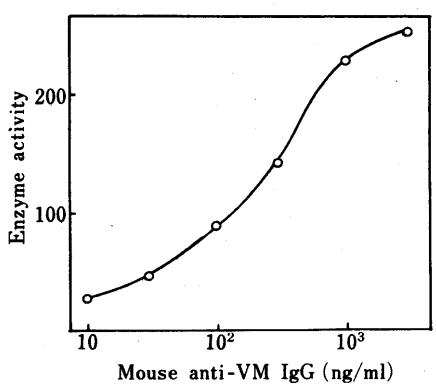

Fig. 2. A Typical Dose–Response Curve of the ELISA for Mouse Antibody Specific to VM

TABLE IV. Intra- and Inter-assay Quality Control Data for ELISA of Anti-VM Antibody

Added amount (ng/tube)	Estimated ng/tube[a] Intra-assay[b]	Inter-assay[b]
3	2.97 (99.0)[c] ± 0.09[d] (3.0)[e]	2.95 (98.3) ± 0.24 (8.4)
10	9.95 (99.5) ± 0.32 (3.2)	9.92 (99.2) ± 0.82 (8.2)
30	29.50 (98.5) ± 0.67 (2.3)	29.50 (98.4) ± 2.86 (9.7)
100	98.10 (98.1) ± 3.70 (3.8)	100.50 (100.5) ± 10.7 (10.5)
300	310.0 (104.0) ± 40.5 (12.9)	312.6 (104.2) ± 56.6 (18.1)

a) Concentration is given as ng/0.1 ml of sample solution. *b*) Number of experiments was 10. *c*) Number in parentheses, recovery (%). *d*) Mean ± S.D. *e*) Number in parentheses, coefficient of variation (%).

TABLE V. Precision Data for Assay of Mouse Anti-VM Antibody in Serum Sample

Dilution fold of serum sample	Content of anti-VM antibody in diluted anti-VM serum solution			
	Found (ng/tube)	Added (ng)	Found (ng/tube)	Recovery[a] (%)
10^3	44 ± 6.1	10	54 ± 1.3	100.0
		30	75 ± 3.8	103.3
		100	150 ± 5.9	106.0

a) Recovery percentage of 10, 30 or 100 ng of mouse antibody specific to VM, measured by an ELISA for mouse anti-VM antibody.

TABLE VI. Grouping and Immunization Schedules of Mice Used to Test Adjuvant Activities of FCA and FICA with VM–MBS–BSA Conjugate as the Antigen (AG)

Group[a]	Injection days and kind of adjuvant used				
	1st d	17th d	30th d	36th d	43rd d
1	FCA[b]	FCA[c]	FCA	FCA	FCA
2	FICA[b]	FICA[c]	FICA	FICA	FICA
3	AG[d]	AG	AG	AG	AG
4	AG+FICA	AG+FICA	AG+FICA	AG+FICA	AG+FICA
5	AG+FCA	AG+FCA	AG+FCA	AG+FCA	AG+FCA
6	AG+FCA	N	N	AG+FCA	N

a) Three mice were used for each group. *b*) A 0.18 ml aliquot of FCA or FICA was emulsified with 0.02 ml of saline solution of the antigen (0.4 mg) and then injected. *c*) The same components as in the first injection[b] except that 0.2 mg of antigen was used. *d*) A saline solution of antigen alone (0.4 mg/0.02 ml) was used for the first injection and 0.2 mg/0.02 ml solutions were used for booster injections. N, not injected.

veloped using VM–GMBS–PSA conjugate-coated Amino-Dylark balls as the solid-phase antigen, a purified anti-VM as the standard antibody, and GAL-labeled goat anti-mouse IgG antibody as the tracer. The dose of the standard antibody was determined by a sandwich EIA for mouse IgG. A typical dose–response curve for ELISA of mouse anti-VM antibody with the measuring range of 1 to 1000 ng/tube is shown in Fig. 2.

Precision tests for the ELISA are summarized in Table IV. Good recoveries (98—104%) were obtained, with coefficients of variation of less than 18% for both intra- and inter-assays.

The recovery test was performed by adding various amounts of mouse anti-VM IgG to diluted mouse anti-VM antiserum, and good recoveries of between 100% and 106% were obtained (Table V).

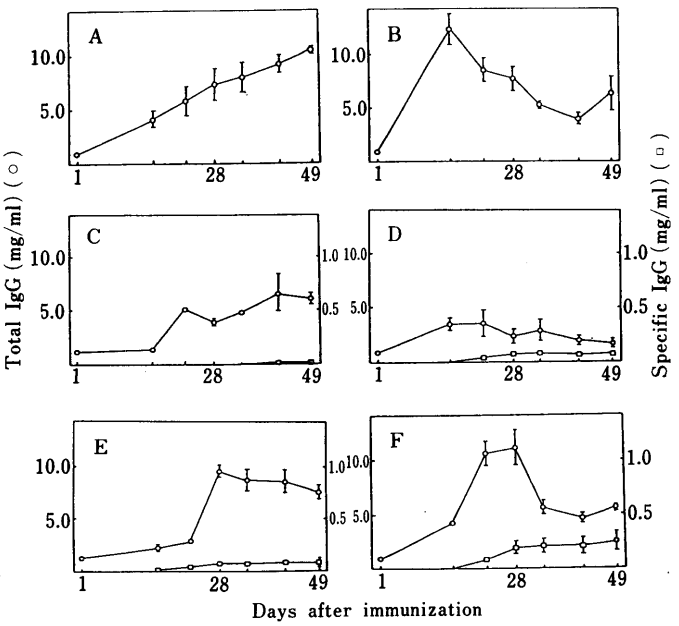

Fig. 3. Changes in Serum IgG Levels of Total IgG (Open Circles, Left Ordinate) and Anti-VM IgG (Open Squares, Right Ordinate) of the Mice of Group 1 (3A), Group 2 (3B), Group 3 (3C), Group 4 (3D), Group 5 (3E), and Group 6 (3F)

Immunization Schedule Immunization schedules of six groups of mice designed to compare the adjuvant activities of FCA and FICA are summarized in Table VI.

Changes of Total IgG Levels in Mouse Sera Immunized with Adjuvant Alone Changes in serum total IgG levels of mice immunized with FCA or FICA during seven weeks were first studied and the results are showed in Figs. 3A and 3B. When the mice received FCA injections alone, serum total IgG levels increased gradually and steadily from 0.8 mg/ml to the maximum of 10.5 mg/ml at the 49th d. With FICA injections, the total IgG content increased quickly in the first two weeks, reaching the maximum of 12.2 mg/ml and then decreasing to 3.9 mg/ml.

Changes of Mouse Serum Levels of the Specific and Total IgGs after Immunization with VM–MBS–BSA Conjugate without the Use of Adjuvant Changes in the levels of total IgG and anti-VM antibody in antiserum samples of the mice immunized with VM–MBS–BSA solution alone are shown in Fig. 3C. The total IgG increased gradually from 0.8 mg/ml to the maximum value of 6.6 mg/ml at the 49th d. Contents of antibody specific to VM, determined by the ELISA for anti-VM antibody, were less than 0.01 mg/ml.

Changes in Contents of Total and Anti-VM IgGs after Immunization with VM–MBS–BSA Conjugate Emulsified with Adjuvants The changes in total and specific IgGs of the mice immunized with the VM immunogen emulsified with FICA are shown in Fig. 3D. As in the mice immunized with FICA alone, total IgG level increased to the maximum (3.5 mg/ml) within two weeks and then gradually decreased to 1.7 mg/ml. The maximum content of the specific IgG (0.07 mg/ml) appeared at the 28th d, two weeks later than that of the total IgG, and the level was maintained until the seventh week. The FICA-aided immunization gives less total IgG and more specific IgG than those obtained by non-adjuvant immunization.

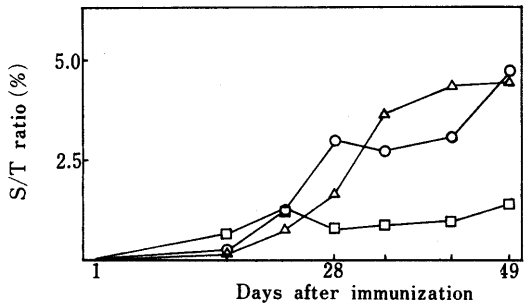

Fig. 4. Changes in Ratio of Mouse Sera Levels of anti-VM per Total IgG Induced by Immunization of VM-Immunogen under Various Conditions

Open squares, open circles and open triangles represent groups 4, 5 and 6.

Figure 3E shows the changes of total and specific IgGs of the mice immunized with VM–MBS–BSA and FCA. The total IgG increased gradually in the first three weeks and then rapidly increased until the 4th week. The maximum content of total IgG was 9.62 mg/ml at the 28 th d and decreased gradually thereafter. The content of anti-VM antibody increased slowly to 0.08 mg/ml without showing a peak.

The FCA-aided immunization yielded the highest total IgG (Fig. 3E), while the FICA-aided immunization gave less total IgG than the non-adjuvant aided one (Fig. 3D). The contents of specific IgG produced by FCA- and FICA-aided immunizations are similar, though the total IgG level of the former is 4.4 times higher than that of the latter.

Effect of Injection Interval with Use of FCA The changes of total and anti-VM IgG levels of mice immunized with VM–MBS–BSA using FCA at different intervals of injection were studied. The mice of group 5 received 4 booster injections (Fig. 3E); the group 6 members received only one booster (Fig. 3F). The total IgG levels of group 6 was lower than that of group 5 after 30 d, but the anti-VM antibody level was 2.5 times higher than that of group 5 at the 49 th d.

Ratio of Specific IgG to Tatal IgG The time courses of the ratio of specific IgG to total IgG produced under different immunizing schedules are summarized in Fig. 4. All the groups showed an increase of the ratio. The ratio of group 4 is almost the same as that of group 6, but higher than that of group 5.

Discussion

Two new analytical method, a sandwich EIA for mouse IgG and an ELISA for mouse anti-VM antibody were developed. A female goat was immunized with mouse IgG isolated from mouse ascites, and anti-mouse IgG was purified from the goat antiserum by modifying the published method.[15] The purified antibody was converted to two immunological reagents, the GAL-labeled antibody and the solid-phase-fixed antibody. A highly sensitive sandwich EIA for mouse IgG was developed using both reagents. Ascites containing anti-VM antibody was elicited in mice immunized with VM–MBS–BSA conjugate, and highly purified mouse anti-VM antibody was obtained from the ascites using a VM–GMBS–PSA-coupled affinity column.[16] A highly accurate ELISA for anti-VM antibody was developed using the purified antibody as the standard sample, VM–GMBS–PSA-linked Amino-Dylark balls as the solid-phase antigen, and the GAL-labeled goat anti-mouse IgG as the second antibody. The accuracy of these methods was confirmed by the good recoveries and small CV values at 5 point determinations (Table III) in intra- and inter-assay experiments with both assay methods. The specificity of the ELISA was excellent and no cross-reactivity was observed with normal mouse IgG.

Six groups of mice were used to compare the adjuvant activities of FCA and FICA. Three groups, 1 to 3, of mice were used to examine the effect of FCA, FICA or VM-immunogen injection. Mice of group 4 were immunized with FCA-aided VM-immunogen and those of group 5 with FICA-aided VM-immunogen. Biweekly booster injections were given to all members of groups 1 to 5, while the mice of group 6 were immunized with FCA-aided VM-immunogen with only one booster injection. The changes in anti-VM and total IgG levels in the six series of antisera samples collected during 7 weeks were followed by the use of the two newly developed EIA methods.

Injection of VM-immunogen alone gave an extremely low anti-VM formation response, but increased the total IgG level from 0.8 to 6.6 mg/ml during seven weeks. It was concluded that an effective adjuvant is necessary for immunization with VM-immunogen. Dose–response relationships of anti-VM antibody in FCA- and FICA-aided immunizations were quite similar (Figs. 5 and 6), while the total IgG response showed a large difference depending on the adjuvant used. The total IgG level of the mice immunized with FCA-aided antigen showed a peak of 10 mg/ml at the third week, while that with FICA-aided immunizations reached a maximum value of 3.5 mg/ml at the second week, about one-third of that of the former. At the 49 th d after the initial immunization, the ratio of the specific IgG to the total IgG of the former was only 1.42%, whereas it was 4.28% for the latter, almost 3 times higher than the former. Judging from anti-VM and total IgG levels, as well as the ratios of produced anti-VM/total IgG (Fig. 8), it was concluded that FICA was a better adjuvant than FCA to immunize mice with a hapten immunogen under the conditions used, since FCA-aided immunization produced a larger amount of undesired antibodies than FICA-aided immunization.

An interesting observation was that anti-VM antibody levels in mice receiving only one booster injection were higher than those in the mice given four biweekly booster injections. Four booster injections further increased the level of total IgG but not that of the specific IgG.

In conclusion, two new analytical methods developed for the present study are very useful tools to follow the immunization process in mice. By means of these methods, the adjuvant activities of FCA and FICA were compared quantitatively, and it was found that the effects of FCA- and FICA-aided immunizations were similar in terms of levels of anti-VM antibody formed. In addition, though we have set a tentative standard immunization schedule for rabbits, in which several biweekly booster injections were given and which was successfully applied to more than 20 kinds of hapten immunogens,[8] the present study suggest that our standard schedule may not be optimum, since biweekly booster injections could be too frequent.

In the present study, we applied 400 μg of VM immu-

nogen for the first injection and 200 μg for each booster injection per mouse. The effect of booster injections could be influenced by immunogen dose, and a further study to elucidate the relationship between immunogen dose and times of booster injection, as well as the effects of other important immunizing conditions is in progress.

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