Studies on the Optimal Immunization Schedule of Experimental Animals. V.[1] The Effects of the Route of Injection, the Content of *Mycobacteria* in Freund's Adjuvant and the Emulsifying Antigen

Jian-Guo HU,[a] Tesuo YOKOYAMA,[b] and Tsunehiro KITAGAWA*,[a]

Faculty of Pharmaceutical Sciences,[a] and Department of Materials Science and Engineering, Faculty of Engineering,[b] Nagasaki University, Bunkyo-machi 1–14, Nagasaki 852, Japan. *Received January 22, 1990*

The effects of several conditions for the immunization of mice was studied using an aliquot of a viomycin (VM) protein conjugate as the common primary or booster antigen. Responses of the mice were assessed by measuring mouse serum levels of total immunoglobulin G (IgG) and anti-VM antibody respondes using the newly improved two assay methods. The choice of route was found to be a very important factor in immunization and intraperitoneal injection was the most optimal among the four routes studied. The effect of the concentration of *Mycobacteria* in Freund's complete adjuvant (FCA) was also studied, and it was found that a diluted FCA was more effective than a commercial FCA. The effect of the controlled release of the antigen was studied and three important phenomena were observed: The mice immunized by the mini-osmotic pump-aided controlled release of the antigen responded with similar small amounts of both total IgG and anti-VM antibody regardless of the presence or absence of FCA in the antigen; emulsifiing the antigen with FCA was a very important condition for the effective elicitation of the specific antibody; a mixture of antigen and FCA without emulsifying produced little specific antibody and a large amount of total IgG. The more effectively immunizated mice responded with a larger decrease in body weight soon after the primary injection.

Keywords optimal immunization; injection route; *Mycobacteria* content; Freund's adjuvant; mini-osmotic pump; antigen emulsion; total IgG; specific antibody; controlled release

Most claims for a particular method to immunize animals are based on anecdotes which can not be reproduced in the hands of others.[1,2] However, there are certain specific factors which can be related to the success of an immunization procedure, such as the route of immunization,[3-8] the use of an adjuvant[4,9-11] and so on. Since our knowledge on the effect of each factor is meager, we undertook a series of studies to establish the optimal conditions for immunization of experimental animals with a drug immunogen.

Two immunoassay methods, a sandwich enzyme immunoassay (EIA) for mouse immunoglobulin G (IgG)[12] and an enzyme-linked immunosorbent assay (ELISA)[12] for mouse antibody specific to viomycin (VM), were applied to evaluate the effects of adjuvant activities in relation to the optimal immunogen dose,[12,13] as well as the optimal age and sex,[1] using VM as the common immunogen and inbred mice for the experimental animals.

Since both assay methods were effective in evaluating the immune response of mice, improvement of both assay methods by changing the solid-phase antigen support from Amino-Dylark ball to microtiter plate was studied. Comparison of the effects of four routes for the immunization of mice were studied using both the improved methods. In the present paper, we also report the effects of *Mycobacteria* contents in Freund's complete adjuvant (FCA) and of emulsifying an immunogen on the immune response of mice under the conditions studied.

Materials and Methods

Reagents Mini-osmotic pumps model 2002 were bought from Alzet Co., U.S.A. Bovine serum albumin (BSA) and pig serum albumin (PSA) were from Miles Lab., Kankakee, Il. Bovine milk casein, FCA and Freund's incomplete adjuvant (FICA) were purchased from Nakarai Chemicals, Kyoto, Japan. Microtiter plate (96 wells) was from Nunc Co., Denmark, and VM was from Taito Pfizer Co., Tokyo. VM immunogens, VM-(*m*-maleimidobenzydoxy)succinimide–BSA (VM–MBS–BSA) conjugate[14] and VM-(γ-maleimidobutyryloxy)succinimide–PSA (VM–GMBS–PSA) conjugate[15] were prepared according to the cited methods. β-D-galactosidase-labeled goat anti-mouse IgG was prepared according to the cited method[12] and its amount was expressed as the International Unit (U) of galactosidase activity measured according to the method reported.[14] Other chemicals used in this work were of reagent grade.

Animals Male mice aged 8 weeks of BALB/c weighing 18—24 g were purchased from Otsubo Experimental Animals Lab., Nagasaki, Japan. A group of three mice were used for each experiment.

Buffers Buffer A (60 mM phosphate buffer, pH 7.4, containing 0.01 M ethylenediaminetetraacetic acid (EDTA), 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); buffer C (the same composition as buffer B, except that casein was used instead of BSA); coating buffer (10 mM Tris–hydrochloric acid 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).

Preparation of Modified FCA Diluted FCA: Commercial FCA (abbreviated as ×1 FCA) was diluted with FICA to make two or four times diluted FCA (abbreviated as ×1/2 FCA and ×1/4 FCA, respectively). Concentrated FCA: *Mycobacteria* was separated from commercial FCA by centrifugation at 3000×*g* for 20 min, and the isolated *Mycobacteria* was added to a fresh FCA so as to make double the content of the bacteria of the commercial one (abbreviated as ×2 FCA).

Preparation of Goat Anti-mouse IgG-Loaded Microtiter Plate Each well of microtiter plate was immersed at 37 °C for 30 min with 100 μl of coupling buffer containing 1 μg of goat anti-mouse IgG which was separated from goat anti-mouse IgG according to the method of Hu *et al.*[12] After successive washing with buffer B, goat anti-mouse IgG-loaded microwell plates were blocked by 250 μl of buffer B at room temperature for 1 h.

Sandwich EIA for Mouse IgG Each well of microtiter plate coated with goat anti-mouse IgG was incubated at 28 °C for 1 h with 100 μl of buffer B solution of either standard mouse IgG or serum sample. After three washes 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. Each well was washed three times with 0.3 ml of the washing buffer, and then was filled with 125 μl of the substrate solution and incubated at 37 °C overnight. The enzyme reaction was stopped by adding 75 μl of the stop solution. The enzyme activity bound to the microtiter plate was measured by spectrometry at 414 nm using an ELISA Analyzer.

Preparation of VM–GMBS–PSA-Loaded Microtiter Plate Each well of microtiter plate was coated with 1 μg of VM–GMBS–PSA in 100 μl of the coupling buffer at 37 °C for 30 min, and then washed and blocked by buffer C in the same way described for the preparation of the goat anti-mouse IgG-loaded one.

ELISA for Mouse IgG Specific to VM ELISA for mouse anti-VM antibody was assessed by the same procedure described for sandwich EIA

for mouse IgG except that VM–GMBS–PSA-loaded microtiter plates were used instead of goat anti-mouse IgG-loaded ones.

Intrasplenic Injection The mice were anesthetized by intraperitoneal injection with nembutal at the dosage of 6 μg/g of body weight. A skin incision about 1 cm long was made and the spleen was exteriorized by gently lifting its lower pole. The needle was inserted deeply into the spleen and a solution of 200 μg of VM–MBS–BSA in 20 μl of saline was injected. The needle was pulled out after ensuring that the antigen was distributed into the spleen. After the injection, the incisions of the peritoneal and muscular wells were sutured with 2—3 thread stitches, respectively.

Implantation of Mini-osmotic Pump 185 μl of a solution containing 200 μg of VM–MBS–BSA was filled into a mini-osmotic pump, ensuring that it was free of air bubbles. The mouse was anesthetized with ether and a small midline incision was made in the skin below the rib cage. Another small incision in the abdominal muscle was made directly under the cutaneous incision. The pump, possessing a release rate of 0.48 μl/h, was filled with the antigen solution, the detail of which is described below. The pump was then inserted into the peritoneal cavity. The muscle incision was closed with a suture and then the skin incision was closed with a wound clip.

Immunization 1. For the study of the effect of the immunization route: four groups of BALB/c mice were immunized at different sites with 10 μg aliquot of a VM immunogen, VM–MBS–BSA, dissolved in 0.1 ml of saline and one booster injection was given 4 weeks later: one group of mice was given an intravenous injection with the immunogen solution and one booster was given in the same way used for the priming four weeks later; two groups of mice were injected with the antigen solution emulsified with 200 μl of FCA, either intraperitoneally or subcutaneously, and each member was given one booster injection four weeks later in a similar way used for the corresponding priming except that FICA was used instead of FCA; the mice in the fourth group were given an intrasplenic injection of the antigen dissolved in 20 μl of saline, detailed procedures of which are given above, and then one booster was given four weeks later in the same way used for the priming.

2. For the study of the effect of emulsion of antigen with FCA: The mice of groups 1 and 2 were given an i.p. injection of 0.2 mg of VM–MBS–BSA conjugate mixed with 0.2 ml of FCA with or without emulsification, respectively. Members of the two other groups were immunized using mini-osmotic pumps; mice of group 3 used the pump containing only 0.2 mg of the antigen in saline, and every member of group 4 was implanted with the pumps containing 0.2 mg of the antigen mixed with FCA without emulsification.

Results

Improved Immunoassays for Mouse IgG and Specific Antibody to VM A highly sensitive sandwich EIA for mouse IgG with a working range between 1 and 1000 ng/well was established using a goat anti-mouse IgG antibody-coated microtiter plate as the solid-phase antibody and Gal-labeled goat anti-mouse IgG antibody as the tracer. A typical dose response curve for mouse IgG is shown in Fig. 1.

Experimental results for accuracy and precision of the assay are summarized in Table I. Good recoveries (93.3—110.3%) were obtained for 5 different dose samples with coefficients varying less than 20.6% for both intra- and inter-assays.

A highly sensitive ELISA for mouse anti-VM antibody was also developed using the same process used for sandwich EIA of mouse IgG, except that the VM–GMBS–PSA conjugate-coated plate was used instead of the antibody-coated plate as the solid phase antigen. A typical dose response curve with a measuring range between 1 and 1000 ng/well is also shown in Fig. 1. Precision tests are summarized in Table II. Good recoveries (96.7—105.0%) were obtained with coefficients of variation of less than 21% for both intra- and inter-assays.

Effect of Immunization Routes The total IgG levels of serum samples of the four groups of mice, immunized in different ways, were measured by a newly developed modified sandwich EIA. The levels of the mice receiving intravenous and intra-splenic injections without using FCA were low (Table III). The levels of the mice which received FCA-aided intraperitoneal and subcutaneous injections were different: the former was high and the later was low.

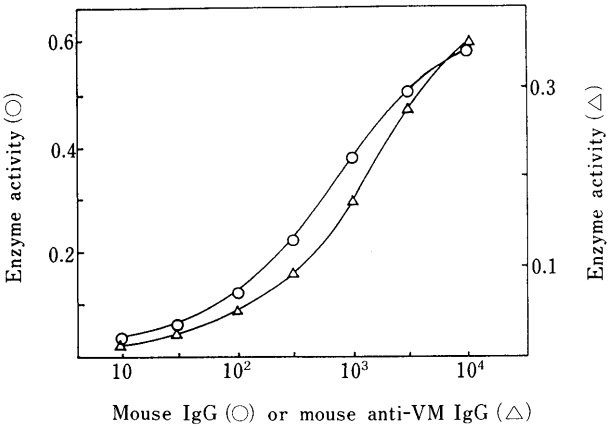

Fig. 1. Typical Dose-Response Curves of Sandwich EIA for Mouse IgG and ELISA for Mouse Anti-VM Antibody

Table I. Quality Control Data for a Sandwich EIA of Mouse IgG

Addition (ng/tube)	Estimated ng/tube[a] Intra-assay[b]	Inter-assay[b]
3	3.1 (110.3)[c] ±0.42[d] (19.0)[e]	2.8 (93.3) ±0.44 (20.3)
10	10.4 (104.0) ±0.40 (3.9)	10.1 (101.0) ±0.28 (3.3)
30	31.0 (103.3) ±1.38 (4.4)	29.5 (99.7) ±1.78 (6.0)
100	97.0 (97.0) ±4.00 (4.1)	96.3 (96.3) ±8.20 (9.5)
300	315.0 (105.0) ±50.3 (16.0)	320.0 (106.7) ±65.3 (20.6)

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. Quality Control Data for ELISA of Anti-VM Antibody

Addition (ng/tube)	Estimated ng/tube[a] Intra-assay[b]	Inter-assay[b]
3	2.9 (96.7)[c] ±0.11[d] (3.5)[e]	3.1 (103.3) ±0.30 (12.6)
10	10.1 (101.0) ±0.42 (4.3)	9.9 (99.0) ±0.80 (8.1)
30	29.7 (99.0) ±0.60 (2.1)	30.3 (101.0) ±2.50 (8.9)
100	99.0 (99.0) ±3.50 (3.5)	98.4 (98.4) ±12.3 (11.1)
300	310.0 (103.3) ±35.6 (11.0)	315.0 (105.0) ±58.9 (21.0)

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 III. Effect of Immunization Route on Immune Response of Mice

Route[c]	Total IgG[a]		Specific IgG[b]		
	4 weeks	6 weeks	4 weeks	6 weeks	8 weeks
i.p.	9.6±1.0	13.5±1.1	119±46	773±123	1389±460
s.c.	7.5±1.2	4.4±0.4	34±11	192±34	252±56
i.v.	4.6±0.3	3.3±0.1	3±0	16±3.5	95±7.8
i.sple.	4.2±0.4	3.7±0.1	<3	<3	<3

a) Amount of total IgG in the sera collected 4 and 6 weeks after the priming were expressed as mean±S.E., mg/ml. *b*) Amount of anti-VM antibody in the sera collected 4, 6 and 8 weeks after the priming were expressed as mean±S.E., μg/ml. *c*) Immunization route is abbreviated as: i.p., intraperitonel; s.c., subcutaneous; i.v., intravenous; i.sple., intra-splenic.

Among them, the mice in the third group showed the highest level, 13.5 mg/ml, but, with intravenous and intrasplenic immunizations the mice responded with only 3.3—3.7 mg/ml of IgG 6 weeks after priming.

The contents of antibody specific to VM in the serum samples collected 4, 6 and 8 weeks after priming are also summarized in Table III. The mice in the third group responded with the highest content of the specific antibody at about 1.4 mg/ml of serum. On the contrary, the mice which received intra-splenic injections produced little specific IgG. With a subcutaneous injection the level of the mice was about 0.25 mg/ml of anti-VM antibody, and with an intravenous one the response was only 95 μg/ml.

The Effect of the Content of *Mycobacteria* in FCA Four groups of mice were given primary injection of 10 μg aliquot of VM–MBS–BSA but using 0.2 ml of four kinds of modified FCA, differing in the contents of *Mycobacteria*. The levels of total IgG which responded in the serum samples collected 4 and 6 weeks after the priming are shown in Fig. 2. The higher the concentration of *Mycobacteria* used as an adjuvant, the larger the amount of total IgG which responded: the concentration of the samples of the ×2 FCA group was 26.8 mg/ml, about two times as high as other groups 6 weeks later. Those of the ×1 and ×1/2 FCA groups were 13.5 and 12.2 mg/ml, respectively, and that of ×1/4 FCA group was only 11.5 mg/ml.

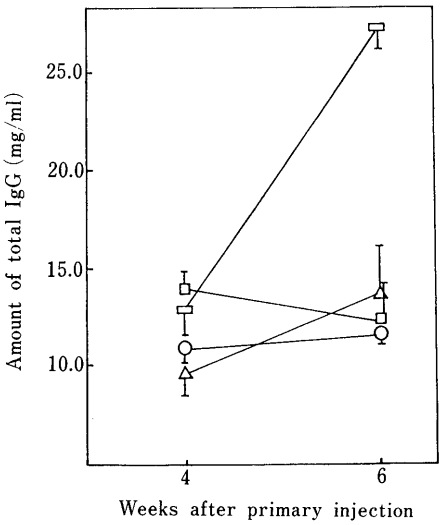

Fig. 2. The Influence of *Mycobacteria* Concentration in FCA on the Production of Total IgG

Four groups of male BALB/c mice received primary injection of 10 μg antigen emulsified with ×2 (rectangles), ×1 (triangles), ×1/2 (squares) and ×1/4 (circles) FCA, respectively, and received booster injections of the same amount of antigen emulsified with FICA. The data is expressed as mean±S.D.

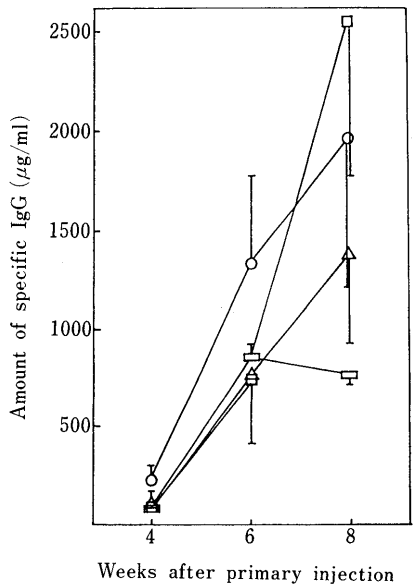

Fig. 3. The Influence of *Mycobacteria* Concentration in FCA on the Production of IgG Specific to Viomycin

Legend is the same as that of Fig. 2.

On the contrary, reverse results were obtained for the contents of antibody specific to VM (Fig. 3). The highest level in the samples collected 8 weeks after priming was 2.5 mg/ml measured for the ×1/2 FCA group. Meanwhile the ×2 FCA group responded with the lowest specific antibody, which contained only 0.76 mg/ml, less than one third of that of the ×1/2 FCA group. The anti-VM level of the ×2 FCA group decreased 6 weeks later, while those of other groups increased for two more weeks.

Effect of the Controlled Release of Antigen Four groups of mice were used to study the effect of the controlled release of the antigen (Table IV). The mice which received a FCA-aided i.p. injection responded with a high level of total IgG, regardless of whether or not the antigen was emulsified

TABLE IV. Effect of Controlled Release of Antigen on Immune Response of Mice

Group	Immunization	Total IgG[a]		Specific IgG[b]	
		3 weeks	4 weeks	3 weeks	4 weeks
1	FCA+Ag emul.	19.2±5.1	15.8±3.0	170±49	246±61
2	FCA+Ag mix.	24.0±0.5	16.0±2.0	<3	<3
3	Ag pump	8.3±0.3	6.4±1.2	15±4	22±5.5
4	FCA+Ag mix. pump	7.8±0.3	7.6±2.4	15±3	28±8.3

a) Amount of total IgG in the sera collected 3 and 4 weeks after the priming were expressed as mean±S.E., mg/ml. *b*) Amount of anti-VM antibody in the sera collected 3 and 4 weeks after the priming were expressed as mean±S.E., μg/ml. Abbreviation: Ag, antigen; emul., emulsion; mix., mixture without emulsifying; pump, in mini-osmotic pump.

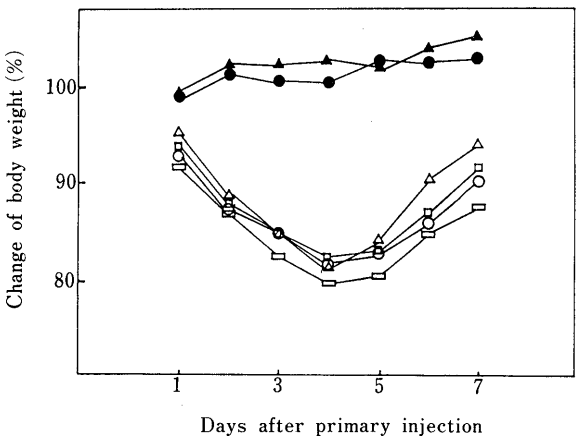

Fig. 4. Changes of Body Weight after Primary Injection in Different Route

The data show the changes of body weight after mice received s.c. (close circles), i.v. (close triangles) injection, or the i.p. injection of emulsion of ×1/4 FCA (open triangles). ×1/2 FCA (open squares), ×1 FCA (open circles) and ×2 FCA (open rectangles). Data are expressed as the mean value of three mice.

with FCA. The mice implanted with a mini-osmotic pump produced a low level of total IgG, regardless of whether or not the antigen was mixed with FCA.

The amounts of specific IgG contained in mouse serum are also shown in Table IV. The mice injected intraperitoneally with the antigen emulsified with FCA produced a high level of specific IgG of 246 μg/ml within a month. Mice which received the antigen mixed, but not emulsified, with FCA responded with a large amount of total IgG but produced little specific IgG. Two groups of mice implanted with mini-osmotic pumps which contained either a simple saline solution of the antigen or a mixture of the saline solution of the antigen and FCA, not emulsified, produced almost the same amount of specific IgG of 22—28 μg/ml. It was found that the presence of FCA hardly affected the production of IgG in the mini-osmotic pump implant immunizations.

Change of Body Weight after Primary Injection in Different Routes The percentage change of body weight after primary injection using different routes are shown in Fig. 4, defining the body weight before primary injection as 100%. The mice which received an intraperitoneal injection tended to decrease in body weight till the fourth day, and then recovered. It was observed that the higher the concentration of *Mycobacteria* in FCA used, the larger was the decrease in the body weight. Both mice which received intravenous and subcutaneous injections responded with small amounts of the specific antibody. Their body weight decreased slightly on the first day and then recovered and gradually increased.

Discussion

Two analytical methods, a sandwich EIA for mouse IgG and ELISA for mouse anti-VM antibody had been developed.[12] Before immunization the inbread mice BALS/c contained no specific IgG and only averaged 0.8 mg/ml of total IgG.[12] The changes of both the contents had been applied to follow the immune response of mice.[1,12,13] Improvement of both methods, involving the change of the antigen support from Amino-Dylark balls to microtiter plates, was first studied to simplify their assay processes. Handling of the improved methods for measuring a number of samples was easier than handling the original methods by using an ELISA analyzer.

Various routes for immunization have been studied.[3-8,16-22] The efficiency of stimulation of the immune response may be related to the site of inoculation.[3,4] The most widely used route has been the subcutaneous injection. The intraperitoneal injection has rarely been used for animals.[2]

We compared the effect of four injection routes, two of which were performed with the presence of FCA (i.p. and s.c.), and the other two without the aid of FCA. It was found, however, that both the serum levels of total IgG and anti-VM antibody (Table III) were the highest for the mice receiving i.p. injection among the tested mice. Only one-fourth the amount of anti-VM antibody responded in the mice receiving s.c. injection compared to those receiving the i.p. injection. The non FCA-aided routes of immunization seemed very weak in immune response.

An appropriate use of FCA which contain *Mycobacteria* was a very important condition for the primary immunization as reported in the previous papers.[12,13] Thus, the effect of the concentration of the bacteria were compared. Modified FCAs which contain a two-fold diluted series of the bacteria were prepared. It was found that when the higher concentrated *Mycobacteria* was used, the more total IgG responded. While the response of the antibody specific to VM was different: the higher the concentration used, the lower the level of anti-VM IgG responded under the conditions studied. The reason for this is not clear, though it could be related to the tolerance resulting from an overdose of *Mycobacteria*. The differences in immune response of mice by these conditions, however, was not drastic compared to that caused by the selection of the route nor by the effect of antigen dose.[1,13]

Various effective mechanisms of a water-in-oil type emulsion of antigen with an adjuvant have been proposed.[9-11,23] It is generally recognized that one of the main roles of FCA is the control release of antigen over a long period of time.[10] Mini-osmotic pumps, which release the antigen slowly during one month, were used to study the effects of the controlled release of the immunogen. The mice which received i.p. injections of the immunogen either mixed or emulsified with FCA, gave significant differences on the anti-VM antibody response. It was interesting that their total IgG responses were similar (Table IV). All mice receiving the pump-aided immunizations produced similar levels of total IgG and anti-VM antibody, regardless of the presence of FCA (Table IV). On the other hand, the same mixture of the immunogen and FCA responded with little anti-VM antibody wighout using the pump. These results also support that an antigen must be exposed to an animal bit by bit.

It was also interesting that the same mixture of the immunogen and FCA without emulsification but immunized by different routes, direct i.p. injection and by using a mini pump, responded with different amounts of total IgG in mice (Table IV). It may suggest that a rapid exposure of an aliquot of *Mycobacteria* in FCA could be important for starting the immune response.

Change of the body weight of mice after immunization was found to be related to their immune response.[1,13] The result presented in this paper showed a similar phenomenon. Although the mice aged 8 weeks increased in weight naturally to 108% in a week,[13] the mice which responded more intensely decreased their body weight. The i.v. and s.c. injection seemed to cause a gentle immune response in the mice, and their body weights hardly decreased.

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.

References

1) Part IV: J.-G. Hu, T. Yokoyama, and T. Kitagawa, *Chem. Pharm. Bull.*, **38**, 448 (1990).
2) T. Chart, "An Introduction to Radioimmunoassay and Related Techniques," 3rd, Ed., Elsevier, Amsterdam, New York, Oxford, 1988, pp. 93—102.
3) R. G. Webster, *Immunology*, **14**, 29 (1968).
4) Y. Katsura, *Jpn. J. Microbiol.*, **16**, 223 (1972).
5) J. W. Moorhead, *J. Immunol.*, **119**, 315 (1977).
6) R. J. Johnson, G. R. Pasternach, and H. S. Shin, *J. Immunol.*, **118**,

489 (1977).
7) W. Gerhard, Y. Iwasaki, and H. Koprowski, *J. Immunol.*, **120**, 1256 (1978).
8) B. A. L. Hurn and S. M. Chantler, in "Methods in Enzymology," Vol. 70, ed. by H. V. Vunakis and J. J. Langone, Academic Press Inc., New York, 1980, pp. 112—113.
9) A. E. Stuart and G. M. Cooper, *J. Pathol. Bacteriol.*, **83**, 245 (1962).
10) A. G. Johnson, S. Gains, and M. Landy, *J. Exp. Med.*, **103**, 225 (1956).
11) B. A. Askonas and J. H. Humphrey, *Biochem. J.*, **60**: X (1955).
12) J.-G. Hu, H. Tanimori, M. Shibata, T. Yokoyama, and T. Kitagawa, *Chem. Pharm. Bull.*, **37**, 1316 (1989).
13) J.-G. Hu, A. Ide, T. Yokoyama, and T. Kitagawa, *Chem. Pharm. Bull.*, **37**, 3042 (1989).
14) T. Kitagawa, H. Tanimori, K. Yoshida, H. Asada, T. Miura, and K. Fujiwara, *Chem. Pharm. Bull.*, **30**, 2487 (1982).
15) T. Kitagawa, H. Tanimori, M. Shibata, K. Yoshida, and J.-G. Hu, *Chem. Pharm. Bull.*, **37**, 1013 (1989).
16) M. F. Kagnoff, *J. Immunol.*, **120**, 395 (1978).
17) P. S. Morahan, M. C. Breinig, and M. B. McGeorge, *J. Immunol.*, **119**, 2030 (1977).
18) S. Leskowitz and B. H. Waksman, *J. Immunol.*, **84**, 58 (1960).
19) G. W. Boyd and W. S. Peart, *Lancet*, II, 129 (1968).
20) B. O. Nilsson, K. O. Gronvik, and P. C. Svalander, *Upsala J. Med. Sci.*, **88**, 151 (1983).
21) M. Spitz, L. Spitz, R. Thorpe, and E. Eugui, *J. Immunol. Methods*, **70**, 39 (1984).
22) B. O. Nilsson, P. C. Svalander, and A. Larsson, *J. Immunol. Methods*, **99**, 67 (1987).
23) M. M. Dale, *J. Exp. Pathol.*, **42**, 297 (1961).