# Analyses of Specific and Total Antibody Responses of Rabbits to Four Kinds of Immunogens

Tsunehiro KITAGAWA,* Hideaki TANIMORI, Miwako SHIBATA, Kensei YOSHIDA and Jian-Guo HU

*Faculty of Pharmaceutical Sciences, Nagasaki University, Bunkyo-machi, 1–14, Nagasaki, Nagasaki 852, Japan.* *Received October 11, 1988*

**As a basic study to investigate suitable conditions to immunize rabbits with drug-immunogens, two highly sensitive and accurae enzyme immunoassays (EIAs) for specific antibody to viomycin (VM) and blasticidin S (BLS) were developed using the corresponding standard antibody, the solid-phase antigens, and enzyme-labeled goat anti-rabbit immunoglobulin G (IgG) antibody as immunological reagents. The accuracy of the assay results with these newly developed EIAs was demonstrated. The new EIAs as well as two previously developed EIAs, EIA for antibody specific to neocarzinostatin (NCS) and a sandwich EIA for rabbit IgG, were applied for analyses of the changes in contents of total and specific antibodies in rabbit antisera samples collected during immunizations with four antigens. Total IgG levels increased from 7.0—9.9 mg/ml to 30—50 mg/ml in all rabbits immunized under the same immunizing schedule, despite the use of four kinds of antigens. The highest level of specific antibodies, anti-BLS, anti-VM and anti-NCS, was 0.5 mg/ml in each case.**

**Keywords** enzyme immunoassay; specific antibody response; rabbit antibody; anti-drug antibody; viomycin; blasticidin S; neocarzinostatin

Antibodies specific to a drug are key reagents for analytical, biochemical, pharmacologic, and other studies of drugs. The most common experimental animal used for laboratory preparation of an antiserum specific to a drug has been the rabbit. The procedure, however, is not easy. One of the main difficulties lies in the preparation of an adequate drug immunogen; this is usually done by covalent coupling of drug molecules to a carrier protein, since most drugs are low-molecular haptens which are not antigenic by themselves.[1,2] Another problem is adequate selection of the immunizing schedule of rabbits with hapten immunogens. It is well known that use of too large an immunogen dose at the first immunization leads to immunologic tolerance,[3,4] and also the specific antibody response caused by the booster injection is greater than that induced by the first sensitization.[5,6] A number of immunization schedules have been recommended by various investigators.[7–9] Because of the genetic inhomogeneity of rabbits, precise investigations, however, have seldom been performed on the immune response of rabbits, and our knowledge of the optimal conditions to immunize rabbits with drug immunogens is meager in regard to the antigen dose for the primary injection, the times and intervals of the booster injections, the kind and amount of adjuvant to be used, and so on.

As basic research to study suitable conditions to immunize rabbits with drug immunogens, experiments have designed to develop enzyme immunoassays for three drug-specific antibodies. We also report application of the assays to follow the changes in content of antibody together with those of total immunoglobulin G (IgG) in four series of anti-drug sera samples collected from rabbits.

## Materials and Methods

**Reagents** Sepharose 4B was bought from Pharmacia Fine Chemicals, Uppsala, Amino-Dylark balls (6 mm diameter) from Sekisui Chemical Ind., Osaka, and *N*-(γ-maleimidobutyryloxy)succinimide (GMBS) from Dojin Chemicals, Kumamoto. Bovine and pig serum albumins (BSA and PSA) were purchased from Sigma Chemicals, Saint Louis, Missouri and Miles Lab., Elkhart, respectively. Antibiotics, viomycin (VM) from Taito Pfizer Co., Tokyo, neocarzinostatin (NCS) from Yamanouchi Pharmaceutical Ind., Tokyo and blasticidin S (BLS) from Kaken Pharmaceutical Ind., Tokyo, were commercial products. β-D-Galactosidase (GAL)-labeled goat anti-rabbit IgG antibody,[10] GAL-labeled VM (GAL–VM),[11] GAL-labeled NCS,[12] GAL-labeled BLS,[13] two specific antisera named anti-VM–MBS–BSA,[11] anti-VM–Suc–BSA[14] and, two hapten protein conjugates BLS–MBS–BSA[13] and BLS–GMBS–PSA[15] were prepared by published methods. Two standard antibodies, anti-blasticidin S[15] and anti-NCS[16] used, were purified according to the cited methods. Other chemicals were of reagent grade.

**Media** Buffer A: 0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, and 0.1% $NaN_3$, Buffer B: phosphate–ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.4 (0.03 M sodium phosphate dibasic–1 mM EDTA disodium salt containing 0.1% BSA).

**Immunization** All the immunization schedules for the four antigens were as follows. Two female rabbits were injected subcutaneously and intramuscularly with 1.0 ml of an antigen solution (*ca.* 1.0 mg protein) emulsified in an equal volume of complete Freund's adjuvant. Booster injections of half the first dose were given at biweekly intervals except that Freund's adjuvant of the incomplete type was used instead of that of the complete type. The rabbits were bled from the ear veins 2 weeks after each injection. Antisera were stored at −30 °C. Immunogens used were NCS, VM–MBS–BSA, VM–Suc–BSA, and BLS–MBS–BSA.

**Preparation of VM–PSA Conjugate Used as the Affinity Ligand and Solid-Phase Immunogen** Preparation of VM–GMBS–BSA conjugate was performed essentially according to the method of Kitagawa *et al.*[13] in three steps as shown in Fig. 1.

GMBS-Activation of Viomycin (Step 1): A tetrahydrofuran solution of

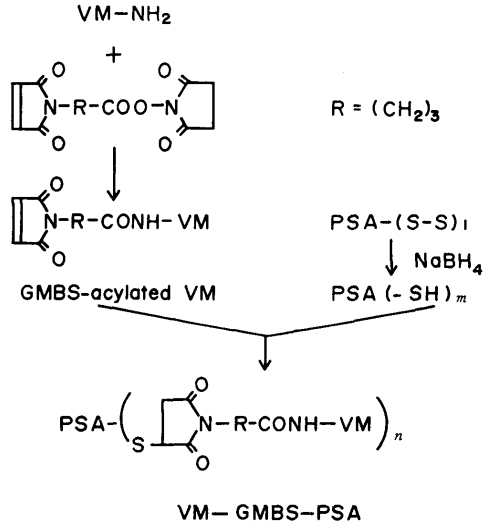


Fig. 1. Scheme for Preparing the VM–GMBS–PSA Conjugate

2.8 mg GMBS (10 μmol/200 μl) was incubated at 30 °C for 30 min with 8.5 mg of VM (10 μmol) dissolved in 1 ml of 0.05 M sodium phosphate buffer. Tetrahydrofuran was removed by flushing with nitrogen for 10 min. Excess GMBS in the aqueous layer was washed with three 5 ml portions of methylene chloride. The aqueous GMBS-acylated VM was immediately used in step 3.

Sodium Borohydride Reduction of Disulfide Bonds in PSA (Step 2): A total of 20 mg of $NaBH_4$ and 0.2 ml of *n*-butanol were alternately added portionwise over 10 min to a solution of 6.6 mg of PSA (0.1 μmol) dissolved in 2 ml of 6 M urea–0.1 M EDTA. After incubation at 25 °C for 20 min excess $NaBH_4$ was decomposed by the additions of 1 ml of 0.1 M sodium phosphate (monobasic) and 0.4 ml of acetone. The solution was immediately used in step 3.

Conjugation of GMBS-Acylated VM to the Reduced PSA (Step 3): The reduced PSA solution (step 2) was incubated at 25 °C for 2 h with an aqueous solution of GMBS-acylated VM (Step 1). The reaction mixture was loaded onto a 25 × 57 cm column of Sephadex G-100, previously swollen with 3 M urea, and was eluted with 3 M urea to isolate the desired conjugate. Fifteen VM molecules were calculated to have been coupled per PSA, judging from the ultraviolet absorption.[17] The solution containing 5 mg of VM–GMBS–PSA conjugate was diluted to 100 ml with 0.05 M Tris–hydrochloric acid-buffered saline, pH 8.5, and then was used to prepare the VM–GMBS–PSA-coupled Sepharose 4B, according to the manual of Pharmacia Fine Chemicals.

**Purification of Rabbit Antibody to VM** The crude immunoglobulin fraction collected from rabbit antiserum to VM (anti-VM–MBS–BSA; 10 ml) by the ammonium sulfate fractionation method was dissolved in 10 ml of 0.02 M Tris-buffered saline, pH 8.3, containing 0.1 M NaCl, and then loaded onto a 1.4 × 10 cm column of VM–GMBS–PSA-coupled Sepharose 4B, previously swollen with the same buffer. Non-specific IgG was eluted from the column with the same buffer. Anti-BSA antibody was then eluted with 200 ml of the same buffer containing 0.1% BSA. BSA was washed with 100 ml of 0.02 M sodium phosphate-buffered saline, pH 7.4. Anti-VM antibody was eluted from the column with 0.3 M KCl–0.008 M hydrochloric acid buffer, pH 2.3. Each fraction was immediately neutralized with 0.1 M glycine–NaOH buffer, pH 10.3, and the optical density at 280 nm was measured. Immune specificities against VM and BSA in 5 μl of each fraction were measured by enzyme immunoassay (EIA) as described below.

**Measurement of GAL Activity** Five microliters of diluted enzyme solution was incubated at 30 °C for 30 min with 0.15 ml of 0.1 mM 7-β-D-galactopyranosyloxy-4-methylcoumarin in buffer A. The reaction was stopped by the addition of 2 ml of 0.2 M glycine–NaOH buffer, pH 10.3, and the 7-hydroxy-4-methylcoumarin liberated was measured by spectrofluorometry. The GAL-labeled anti-rabbit IgG was expressed in units of GAL activity; 1 U of the enzyme activity was defined as the amount able to hydrolyze 1 μmol of substrate/min.

**Preparation of VM–GMBS–PSA Conjugate-Coated Amino-Dylark Balls** The Amino-Dylark balls were immersed in 1% glutaraldehyde for 1 h with shaking. This was followed by washing with 0.02 M phosphate-buffered saline, pH 7.0 (PBS). The solid supports were immersed in 0.01% VM–GMBS–PSA conjugate in PBS at 4 °C for 2 h. After being washed with PBS and buffer A successively, the Amino-Dylark balls coated with VM–GMBS–PSA were stored in buffer A at 4 °C until use.

Three solid-phase antigens, NCS-, BSA-, or BLS–GMBS–PSA conjugate-coated Amino-Dylark balls, were prepared in a similar manner.

**Immunoassay by Enzyme Linked Immunosorbent Assay (ELISA)** ELISA for specific antibody was performed by a similar method to that used for ELISA of anti-NCS antibody.[16] Amino-Dylark balls coated with the corresponding antigen were incubated at 30 °C for 2 h under mechanical shaking, with standard antibody or sample dissolved in a final volume of 0.2 ml of buffer B. After 2 washes with 1 ml of buffer A, the bound rabbit IgG on the balls was reacted with GAL-labeled goat anti-rabbit IgG (200 μU) dissolved in 0.2 ml of buffer A by incubation at 30 °C for 2 h with shaking. Each ball was washed twice with 1 ml of buffer A and was transferred to a fresh tube to eliminate non-specific enzyme activity bound to the wall of the test tube. The enzyme activity bound to the solid support was assayed.

Sandwich EIA for rabbit IgG[10] and ELISA for anti-NCS antibody[16] were performed according to the published methods.

## Results

**Preparation of the Standard Sample of Rabbit Anti-VM Antibody** Typical elution profiles of the crude IgG fraction of anti-VM–MBS–BSA antiserum from the affinity column of a VM–GMBS–PSA conjugate-coupled Sepharose 4B, assayed in terms of optical density at 280 nm, and immune specificities against VM and BSA, are shown in Fig. 2.

The purified anti-VM antibody contained little anti-BSA antibody, since it hardly bound to BSA-coupled Amino-Dylark balls in buffer C (same constituents as buffer B except that 0.1% BSA was replaced with 0.1% egg white albumin) (Table I).

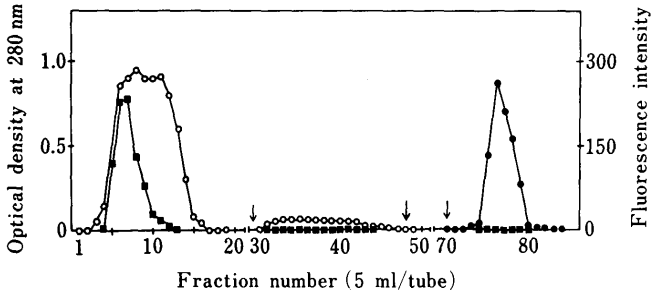


Fig. 2. Elution Profiles of Anti-VM Antibody in the Crude IgG Fraction of Anti-VM–MBS–BSA Serum from a 1.4 × 10 cm Column of VM–GMBS–PSA-Coupled Sepharose 4B

The left arrow indicates the changes of the eluent from 0.02 M Tris–hydrochloric acid buffer (pH 8.3) containing 0.1 M NaCl and 0.01% BSA to 0.05 M sodium PBS containing 0.01% BSA. The middle arrow indicate the change of eluent to PBS. The right arrow indicate the change of eluent to 0.3 M KCl–0.008 M hydrochloric acid (pH 2.3). Open circles, optical density at 280 nm; closed circles, immune specificity of anti-VM antibodies in 5 μl fractions (2.5 ml/tube) determined by ELISA for anti-VM antibody; closed squares, immune specificity of anti-BSA antibody in 5 μl fractions determined by ELISA for anti-BSA antibody.

TABLE I. Binding Activities of Three Different Doses of Anti-VM Antibody to VM–GMBS–PSA- or BSA-Coupled Amino-Dylark Balls Determined by Using GAL-Labeled Goat Anti-rabbit IgG as an Indicator (Amounts of Anti-VM Antibody Were Determined by Sandwich EIA for Rabbit IgG)

| Antibody | Solid-phase antigen | 1 ng/tube | 10 ng/tube | 100 ng/tube |
|---|---|---|---|---|
| Anti-VM | VM–GMBS–PSA | 58[a] ±4 | 285 ±6 | 723 ±8 |
| | BSA | 2.6±0.7 | 3.2±0.9 | 4.6±0.6 |

*a*) One μU of the bound enzyme activity gave a value of 600 measured under the same conditions.

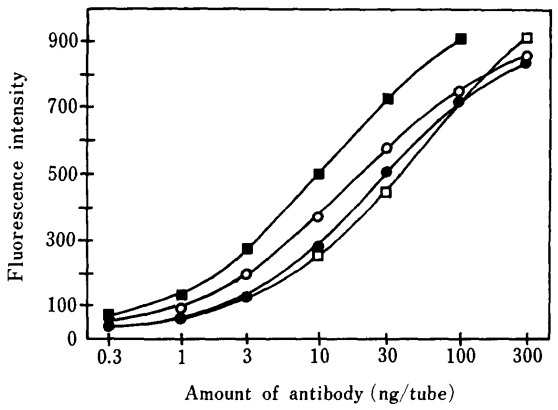


Fig. 3. Dose–Response Curves of Three ELISAs for Rabbit Anti-VM (Open Circles), Anti-BLS (Closed Circles) and Anti-NCS Antibodies (Open Squares)

Anti-VM antibody was also measured by the use of sandwich EIA for rabbit IgG (open squares). The bound enzyme activity is plotted against logarithmic dose of the corresponding specific antibody (0.3—300 ng/tube).

When the antiserum to VM containing 46 mg of total IgG was subjected to the affinity chromatography, 45.8 mg of non-specific IgG and 0.165 mg of anti-VM antibody were recovered as determined by sandwich EIA for rabbit IgG.

**EIAs** Every EIA for rabbit specific antibody, anti-VM, anti-BLS, or anti-NCS, was performed by an ELISA type procedure using a standard antibody and the corresponding solid-phase antigen as the immunological reagents, and GAL-labeled goat anti-rabbit IgG as a tracer. The amount of every standard antibody was determined by a sandwich EIA for rabbit IgG. Three higly sensitive and accurate ELISAs for the three specific antibodies were developed. The ranges of measurement of all three ELISAs are the same, from 0.3 to 300 ng/tube, as shown in Fig. 3, which also includes the dose–response curve of anti-VM antibody measured by sandwich EIA for rabbit IgG.

**Accuracy of the ELISAs** Experimental results concerning accuracy and precision tests of the ELISA of anti-VM antibody are summarized in Table II. Good recoveries (96 to 114%) for five samples were obtained with coefficients of variation of less than 12% for all the intra- or inter-assay results. Similar satisfactory results were obtained in accuracy and precision tests of the ELISAs of anti-BLS and anti-NCS, with 95 to 116% recoveries and less than 18% coefficient of variation (data not shown).

The accuracy of the assay results for sera samples have also been confirmed by the recovery tests. Assay recoveries of 5 ng of anti-BLS antibody added to diluted solutions of anti-BLS antiserum are shown in Table III. Good recoveries were obtained.

**Comparison of Assay Methods** A comparison of two different methods, the ELISA method and sandwich EIA of rabbit IgG, to quantify rabbit anti-VM antibody is shown in Fig. 4. There was a good correlation between the results obtained by the two methods for the antibody. Similar good correlations were obtained for the comparisons in the cases of anti-BLS and anti-NCS antibodies (data not shown).

**Changes in Content of Total IgG and Specific Antibodies in Rabbits during Immunizations 1) Immunization with BLS–MBS–BSA Conjugate** The chenges in content of total IgG as well as the specific antibody in a series of anti-BLS antisera samples collected during seven months are shown in Fig. 5. Titers for anti-BLS activity of these sera

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

| Added amount (ng/tube) | Estimated ng/tube Intra-assay[a] | Inter-assay[a] |
|---|---|---|
| 3 | 2.98 (99.3)[b] ± 0.06[c] (2.0)[d] | 2.95 (98.3) ± 0.17 (5.8) |
| 10 | 10.2 (102.0) ± 0.9 (3.0) | 9.96 (99.6) ± 0.6 (6.0) |
| 30 | 29.7 (99.0) ± 0.9 (3.0) | 29.2 (97.3) ± 2.3 (7.9) |
| 100 | 98.6 (98.6) ± 5.6 (5.7) | 103.6 (103.6) ± 7.6 (7.3) |
| 300 | 290.0 (96.7) ±10.8 (3.7) | 290.6 (96.9) ±32.6 (11.2) |

*a*) Number of experiments was 5. *b*) Number in parentheses, recovery (%). *c*) Mean ± S.D. *d*) Number in parentheses, coefficient of variation (%).

TABLE III. Recovery of 5 ng of Anti-BLS Antibody Added to Three Diluted Solutions of Anti-BLS–MBS–BSA Antiserum Measured by ELISA for Anti-BLS Antibody

| | Content of anti-BLS antibody in diluted anti-BLS serum solutions (ng/tube) | | |
|---|---|---|---|
| Dilution (fold) | $3\times10^3$ | $3\times10^4$ | $1\times10^5$ |
| Anti-BLS antibody (a) | 16.8 ±0.67 | 1.82±0.11 | 0.95±0.04 |
| (With 5 ng addition, b) | 22.08±0.68 | 6.86±0.31 | 5.30±0.07 |
| b−a | 5.26 | 5.04 | 4.71 |
| Recovery (%) | 105.2 | 100.8 | 94.2 |
| Number of assays | 5 | 5 | 5 |

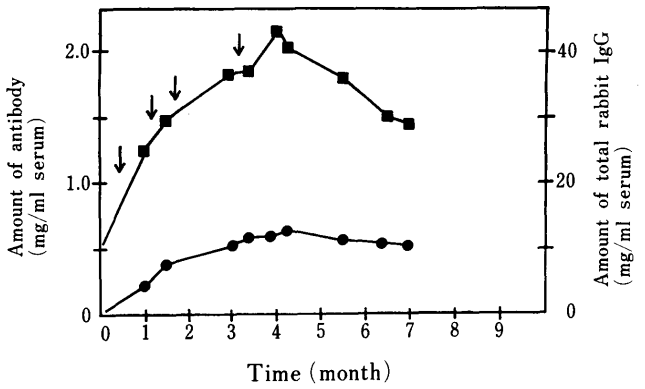


Fig. 5. Changes in Rabbit Serum Levels of Total IgG (Closed Squares: Right Ordinate) and Anti-BLS Antibody (Closed Circles: Left Ordinate) during Nine Months after Starting from the First Immunization

The sera samples were collected from the rabbit immunized with BLS–MBS–BSA conjugate and arrows indicate the points when booster injections were given.

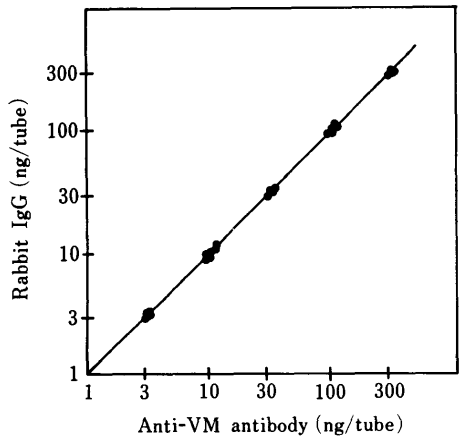


Fig. 4. Correlation of Values for Anti-VM Antibody Determined by the ELISA Method and by the Sandwich EIA for Rabbit IgG

$Y=0.999X\pm0.002$. $r=0.999$. $n=45$.

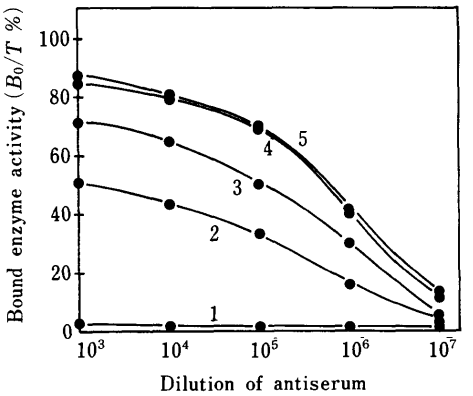


Fig. 6. Binding Activities of Anti-BLS Antiserum Samples to GAL-Labeled BLS Determined by a Double Antibody Method

Sample 1 is the serum before immunization and samples 2, 3, 4 and 5 are the sera collected at 2, 4, 6 and 8 weeks, respectively.

TABLE IV. Contents of Total IgG and Anti-VM Antibody in Rabbit Anti-VM-MBS-BSA and Anti-VM-Suc-BSA Antisera Samples Collected at Different Days, Measured by a Sandwich EIA for Rabbit IgG and an ELISA for Rabbit Anti-VM Antibody[a]

| Sample | Content of total IgG | | | | Content of anti-VM antibody | | | |
|---|---|---|---|---|---|---|---|---|
| | Anti-VM-MBS-BSA | | Anti-VM-Suc-BSA | | Anti-VM-MBS-BSA | | Anti-VM-Suc-BSA | |
| Collected date[b] | 780527 | 780627 | 770809 | 770816 | 780527 | 780627 | 770809 | 770816 |
| Measured (ng, a) | 5.1±0.31 | 4.4±0.10 | 2.8±0.17 | 2.4±0.15 | 2.4±0.2 | 5.1±0.04 | 0.5±0.04 | 0.6±0.06 |
| (Added with 3 ng, b) | 8.4±0.41 | 7.3±0.15 | 5.7±0.21 | 5.3±0.42 | 5.4±0.4 | 8.2±0.6 | 3.4±0.2 | 3.5±0.3 |
| b−a | 3.3 | 2.9 | 2.9 | 2.9 | 3.0 | 3.1 | 2.9 | 2.9 |
| Recovery (%) | 110.0 | 96.7 | 96.7 | 96.7 | 100 | 103 | 97 | 95 |
| Number of assays | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Serum level (mg/ml) | 50.7±3.3 | 44.2±1.2 | 27.8±1.7 | 24.0±2.8 | 0.24±0.03 | 0.51±0.04 | 0.046±0.01 | 0.061±0.02 |

*a*) Every serum sample was diluted $10^6$- and $10^4$-fold and 0.1 ml aliquots of the diluted solutions were used for measurements of total IgG and anti-VM antibody, respectively. *b*) For example, 780527 means May 27, 1978.

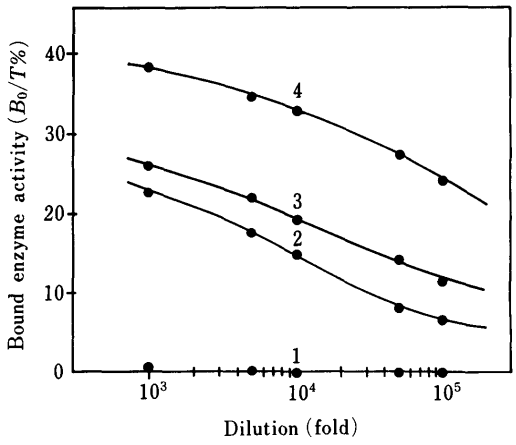


Fig. 7. Binding Activities of Anti-NCS Antiserum Samples to GAL-Labeled NCS

Sample 1 is the serum before immunization and samples 2 and 3 are the sera collected at 4 and 6 weeks, respectively. Sample 4 is the serum collected at 8 months.

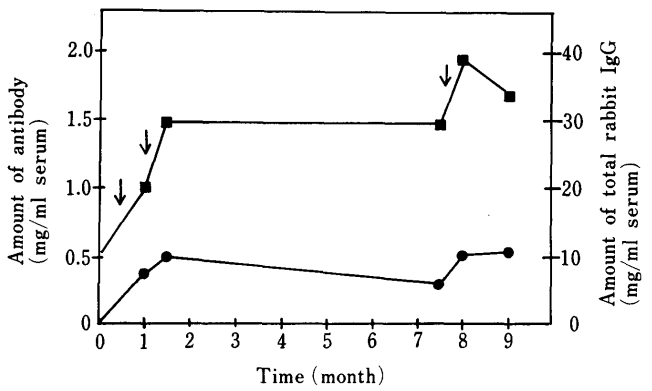


Fig. 8. Changes in Rabbit Serum Levels of Total IgG (Closed Squares: Right Ordinate) and Anti-NCS Antibody (Closed Circles: Left Ordinate) during Nine Months

The arrows indicate the points when booster injections were given.

samples are shown in Fig. 6. The titer was increased by the three booster injection but the fourth booster did not increase the titer. Total IgG levels were increased by the booster injections from 9.2 to 35 mg/ml after the third booster and the fourth booster further increased the value to 40 mg/ml. The IgG level then gradually decreased to 30 mg/ml during the next four months.

**2) Immunization with NCS** The titer of antibody specific to NCS was increased very rapidly by the two booster injections and a $10^5$-fold diluted solution of the antiserum sample collected after the second booster showed sufficient binding to use for EIA of NCS (Fig. 7). The third booster injection was given after six months. The titer of the final antiserum obtained 2 weeks after the booster was higher than that of the serum obtained before the third booster. Total IgG level increased from 7.2 to 30 mg/ml after two booster injections and the level was maintained until seven and half months. After the final booster the level increased to 40 mg/ml, and then decreased to 33 mg/ml at 1 month after the final booster. The anti-NCS antibody levels increased to 0.5 mg/ml at one and half months after the primary and two booster injections and decreased to 0.3 mg/ml during the next six months; the final booster increased the level to 0.5 mg/ml which was maintained during the next 1 month as shown in Fig. 8.

**3) Immunizations with Two VM Immunogens** Contents of total IgG and the antibody specific to VM were also measured with use of the two kinds of specific antisera samples of higher titer obtained from rabbits immunized with VM-MBS-BSA and VM-Suc-BSA conjugates. Two samples of each kind of anti-VM antiserum were used, and the results obtained are summarized in Table IV.

## Discussion

We have developed a new method which has been used to prepare more than 30 kinds of drug immunogens.[17-19] During the course of the studies, we found that the titers of antisera vary depending upon the immunizing schedule of rabbits, such as immunogen dose, interval between booster injections and so on. We were able to establish a tentative standard immunizing schedule for rabbits, which was successfully applied to more than 20 kinds of immunogens.

Despite the progress in recent immunoassay procedures,[17,20-23] titer of effective maximal dilution of antiserum has been used as the standard index of specific antibodies,[24-29] since assays for specific antibodies have not been easy to perform. A semiquantitative dose-response relationship between immunogen dose and the subsequent antibody formation in mice was established in connection with the study of immunologic tolerance,[30] but this has not been done in rabbits. One of the main reasons for this could be that preparation of a standard antibody for ELISA is not easy, owing to the contamination by denatured antibodies formed during purification of the unstable antibody.

As a basic study to find suitable conditions for immunization of rabbits with drug immunogens, we developed

three ELISA methods to quantify specific antibodies elicited in rabbits. Every ELISA requires the standard antibody, an adequate solid-phase antigen, and GAL-labeled anti-rabbit IgG antibody as immunological reagents.

Recently we reported a method to purify two specific antibodies.[15] The method was modified and applied to obtain three kinds of specific antibodies from rabbit antiserum, specific to BLS, VM, or NCS. Drug–GMBS–PSA conjugates were prepared and used as both solid-phase antigens in ELISAs and affinity ligands to purify standard specific antibodies. PSA, the least cross-reactive protein against anti-BSA antibody among human, pig, horse, rabbit, goat, and mouse albumins (unpublished data), was chosen as the carrier protein, since the three kinds of anti-hapten antisera used in this study contain antibody specific to BSA, the common carrier protein of these drug–immunogens. The method for preparing drug–PSA conjugates was essentially the same as that of the VM-immunogen VM–MBS–BSA[11,17] except that GMBS and PSA were replaced with *N*-(*m*-maleimidobenzoyloxy)succinimide (MBS), and BSA, respectively. GMBS differs from MBS in the substitution of *n*-butyric acid for benzoic acid, to avoid cross-reaction of anti-hapten antibodies against MBS, because MBS was used as the cross-linking part of the hapten immunogens.

The purity of the standard anti-VM antibody was established by proving little contamination with anti-BSA antibody (Table I) or inactivated antibodies by repeated chromatography on the same affinity column used for the first purification. Non-specific IgG was not detected by a highly sensitive sandwich EIA for rabbit IgG (assay limit less than 1 ng/ml[10]; data not shown). The purity of other specific antibodies was confirmed in the same way (data not shown). The amount of every standard antibody was determined by means of the accurate sandwich EIA for rabbit IgG.[10]

Three highly specific and sensitive ELISAs against anti-VM, anti-BLS, and anti-NCS antibodies were developed using the corresponding solid-phase antigen and a standard antibody, and a GAL-labeled anti-rabbit antibody. The accuracy of each ELISA was first confirmed (Table II), and the specificity of each ELISA was then proved by confirming that little cross-reactivity occurred with normal rabbit IgG.

The process of immunization of a rabbit was followed in terms of the titer of specific antibody, detected by measuring the binding of the antibody to the GAL-labeled antigen using the double antibody method, and further booster injection was stopped when the maximum titer was obtained.

The changes in levels of total IgG were then measured, since little is known about such changes. It was found that total IgG increased during the immunization process of all rabbits to more than 20 mg/ml, even though four kinds of immunogens were employed.

It was interesting that the maximal levels of specific antibody to a hapten, BLS or VM, were similar (0.5 mg/ml) for anti-BLS–MBS–BSA and anti-VM–MBS–BSA. Both immunogens, BLS–MBS–BSA and VM–MBS–BSA, were prepared similarly by applying the Kitagawa method. The highest content of the antibody specific to VM in anti-VM–Suc–BSA serum was 0.061 mg/ml. Although no obvious difference was observed between anti-VM–MBS–BSA[11] and anti-VM–Suc–BSA[14] in terms of their titers, assay sensitivities and measurable ranges[11] of EIAs for VM using them as antibodies, as well as their affinity constants to VM (unpublished data), the difference in preparation methods of the VM immunogens could be responsible for the different contents. NCS is not a hapten, but the highest level of anti-NCS antibody was also 0.5 mg/ml after immunization using the same schedule as for both BLS–MBS–BSA and VM–MBS–BSA immunogens.

Although female Japanese white rabbits were randomly used as experimental animals, and despite the use of different kinds of antigens, the present study shows that increases in the levels of total IgG and specific antibodies were similar for all rabbits immunized under the same immunizing schedule, in regard to the immunogen dose, intervals between primary and booster injections and also kind and amount of adjuvant. It was also suggested that selection of the method for preparation of a hapten immunogen could be very important in preparing anti-drug serum, and ELISA methods are useful tools to establish suitable conditions to administer drug immunogens. Further studies are inprogress.

**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) K. Landsteiner, "The Specificity of Serological Reactions," Harvard Univ. Press, Cambridge, Mass., 1945.
2) V. P. Butler Jr., *Pharmacol. Rev.*, **29**, 103 (1978).
3) N. A. Mitchison, *Immunology*, **15**, 531 (1968).
4) G. W. Siskind, "Fundamental Immunology," ed. by W. E. Paul, Raven Press, New York, 1984, pp. 537—558.
5) J. Klein, "Immunology the Science of Self-Nonself Discrimination," A Wiley Interscience Publication, New York, 1982, pp. 518—519.
6) H. N. Eisen, *Cancer Res.*, **26**, 2005 (1966).
7) R. Bercks, R. Koening and G. Querfurth, "Principles and Techniques in Plant Virology," ed. by C. I. Kado, and H. O. Agrawal, Van Nostrand-Reinhold, Princeton, New Jersey, 1972, pp. 466—490.
8) M. F. Clark and M. Bar-Joseph, *Methods Virol.*, **7**, 51 (1972).
9) S. L. Barclay and A. M. Smith, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 4336 (1986).
10) H. Tanimori, F. Ishikawa and T. Kitagawa, *J. Immunol. Methods*, **62**, 123 (1983).
11) T. Kitagawa, H. Tanimori, K. Yoshida, H. Asada, T. Miura and K. Fujiwara, *Chem. Pharm. Bull.*, **30**, 2487 (1982).
12) H. Tanimori, T. Kitagawa, T. Tsunoda and R. Tsuchiya, *J. Pharmacobio-Dyn.*, **4**, 812 (1981).
13) T. Kitagawa, T. Kawasaki and H. Munechika, *J. Biochem.* (Tokyo), **92**, 585 (1982).
14) T. Kitagawa, T. Fujitake, H. Taniyama and T. Aikawa, *J. Biochem.* (Tokyo), **83**, 1493 (1978).
15) H. Tanimori, K. Yoshida, H. Motomura, K. Kitada, S. Yagisawa and T. Kitagawa, *Chem. Pharm. Bull.*, **35**, 2062 (1987).
16) T. Kitagawa, H. Tanimori and K. Yoshida, *J. Immunol. Methods*, **72**, 297 (1984).
17) E. Engvall, "Methods in Enzymology," 70, ed. by H. V. Vunakis and J. J. Langone, Academic Press, New York, 1980, pp. 419—439.
18) T. Kitagawa, "Methods of Enzymatic Analysis," Vol. 12, ed. by H. V. Bergmyer, VCH Publishers, Weinheim, 1986, pp. 200—215.
19) K. Fujiwara and T. Kitagawa, "Advances in Immunity and Cancer Therapy," Vol. 2, ed. by P. K. Ray, Springer-Verlag, New York, 1986, pp. 251—292.
20) T. Kitagawa, T. Kanamaru, H. Kato, S. Yano and Y. Asanuma, "Enzyme Labelled Immunoassay of Hormones and Drugs," ed. by S. B. Pal, Wlater de Gruyter and Co., Berlin, 1978, pp. 59—66.

21) C. N. Hales and J. S. Woodhead, "Methods in Enzymology," 70, ed. by H. V. Vunakis and J. J. Langone, Academic Press, New York, 1980, pp. 334—355.
22) E. Engvall and P. Perlmann, *Immunochemistry*, **8**, 871 (1971).
23) J. J. Langone and H. V. Vunakis, "Methods in Enzymology," 73, Academic Press, New York, 1982.
24) K. Droessler, D. Parker and J. L. Turk, *Int. J. Immunopharmacol.*, **5**, 323 (1983).
25) L. M. Parker and A. D. Steinberg, *J. Immunol.*, **110**, 742 (1973).
26) T. Kohno, S. Hashida and E. Ishikawa, *J. Biochem.* (Tokyo), **98**, 379 (1985).
27) M. Suter, *J. Immunol. Methods*, **53**, 103 (1982).
28) K. Droessler, D. Parker and J. L. Truk, *Int. Arch. Allergy Appl. Immunol.*, **76**, 37 (1985).
29) M. Takahashi, S. A. Fuller and J. G. R. Hurrell, *IRCS Med. Sci.*, **13**, 559 (1986).
30) K. L. McCoy, L. Kendrick and T. M. Chused, *J. Immunol.*, **136**, 1217 (1986).