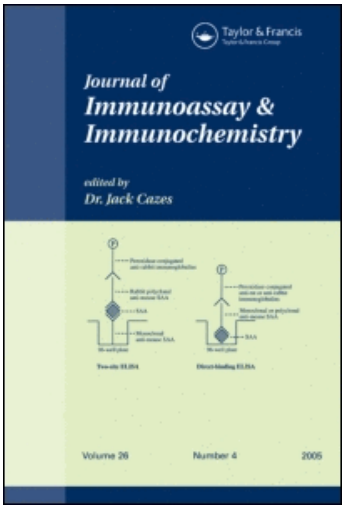

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

The Specificity and Affinity of Polyclonal Antibodies Raised in Rabbits Against a Hapten Conjugated to Rabbit Serum Albumin

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To cite this Article Schöneberg, T. , Erhard, M. H. , Kellner, J. , Kuhn, S. O. and Lösch, U.(1995) 'The Specificity and Affinity of Polyclonal Antibodies Raised in Rabbits Against a Hapten Conjugated to Rabbit Serum Albumin', Journal of Immunoassay and Immunochemistry, 16: 1, 17 — 35
To link to this Article: DOI: 10.1080/15321819508013547
URL: http://dx.doi.org/10.1080/15321819508013547

JOURNAL OF IMMUNOASSAY, 16(1), 17-35 (1995)

THE SPECIFICITY AND AFFINITY OF POLYCLONAL ANTIBODIES RAISED IN RABBITS AGAINST A HAPTEN CONJUGATED TO RABBIT SERUM ALBUMIN

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ABSTRACT

In this study, rabbit antisera to hapten-rabbit serum albumin conjugates were investigated regarding antibody titer, affinity, specificity, and affinity distribution. Methyl phosphonic acid p-aminophenyl 1,2,2-trimethylpropyldiester (MATP) served as model hapten. Four MATP-rabbit serum albumin conjugates with various hapten densities (with and without spacer) were synthesized and used for immunization of rabbits. Antisera were collected over a 130 day-period and characterized with different ELISA methods.

We found that immunogens with rabbit serum albumin gave antisera with lower titers, but similar affinity as compared to polyclonal or monoclonal antibodies obtained with non-rabbit protein as carrier protein. Immunogens with a low hapten density led to higher final titers without affecting antibody affinity or specificity. Immunogens containing a bridging group resulted in higher antibody affinity with a changed specificity. The pattern of antibody affinity distribution differed considerably among individual rabbits and showed a non-Gaussian subpopulation distribution.

(KEY WORDS: hapten, antibody affinity, affinity distribution, ELISA, organophosphorus compound)

INTRODUCTION

The profound knowledge about antibody-hapten-interactions (1-3) and the genetic basis of fine specificity (4-7) contrasts with the limited understanding of antibody generation. Because of their defined epitopes and the possibility to modify their chemical structures, haptens are very useful tools to investigate basic mechanisms of the immune response.

We have used the organophosphorus compound, methyl phosphonic acid p-aminophenyl 1,2,2-trimethylpropyldiester (MATP), as model hapten. In previous studies, MATP was linked to carriers derived from species different from that used to raise the antibody (8-10). Moreover, the effects of various adjuvants on the immune response to these immunogens were previously investigated (11, 12). Only a few systematic studies have been carried out on the influence of hapten density on the immune response (13). Virtually, no information is available at present regarding the effects of using carrier proteins derived from the same species as the immune host. To address this question, we have studied the effects of hapten density and incorporation of a bridging group on antibody titer, affinity and

affinity distribution using various MATP-rabbit serum albumin conjugates for rabbit immunization. Besides an ELISA for estimation of titer values, competitive inhibition enzyme immunoassay (CIEIA) techniques were used for the determination of IC_{50} values of various hapten derivates and measurement of antibody affinity distributions. For comparison, similar studies were carried out with polyclonal and monoclonal antibodies obtained by immunization with a carrier protein derived from a species different from the immune host.

MATERIALS AND METHODS

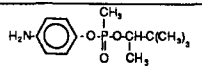
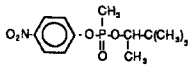
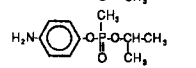
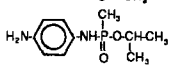
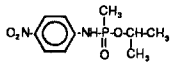
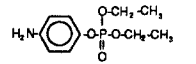
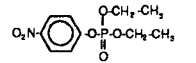
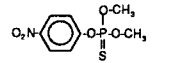
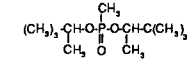
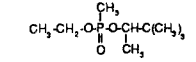
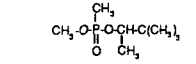
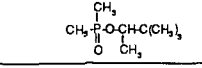
Chemicals

Methyl phosphonic acid, p-aminophenyl 1,2,2-trimethylpropyldiester (MATP, substance I, see table 1) was synthesized by TNO, Rijyswijk (Netherlands). All other organophosphorus compounds (table 1) were gifts from Dr. H. Fischer (Königswusterhausen, FRG; purity >95 %, tested by HPLC). Complete Freund`s adjuvant (FCA) and incomplete Freund`s adjuvant (FIA) were obtained from DIFCO Laboratories, Detroit, USA. Ovalbumin (OvA), rabbit serum albumin (RSA), and o-phenylenediamine were purchased from Sigma, St. Louis, USA. Peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG were obtained from Medac, Hamburg, FRG. All other reagents were from Merck, Darmstadt, FRG.

Syntheses of hapten-carrier conjugates

To provoke an immune response or to coat microtiter plates, MATP was linked to RSA and OvA by means of the azo-reaction (14). A spacer conjugate was synthesized according to Lober et al. (15).

TABLE 1. Specificity of MATP-specific polyclonal and monoclonal antibodies. IC_{50} values were determed for a series of MATP derivates by CIEIA using polyclonal antibodies obtained from rabbit groups A ($MATP_7$-RSA) and D (*succ*$MATP_4$-RSA) *in secondary immune response* (70 and 130 days after priming, respectively) and the monoclonal antibodies F71H10 and F71H9. Data for polyclonal antibodies are presented as means ± S.E. of 15 rabbits, and results obtained with mAbs are mean values (n=2). Differences between mean values were tested for statistical significance by Student's *t* test. The level of significance was chosen at $p<0.05$. Asterisks (*) indicate significant differences between day 70 and day 130 within a group. N.I. = no specific inhibition, IC_{50} value >10 mM

Substance	General formula	Inhibitior concentration IC_{50} [μM]					
		group A		group D		F71H10	F71H9
		70 days	130 days	70 days	130 days		
I MATP		10 ± 3	5.6 ± 1.1*	4.9 ± 1.4	2.1 ± 0.7*	2.5	50
II		4.9 ± 1.6	3.2 ± 0.9	3.5 ± 1.2	3.2 ± 1.3	650	69
III		1047 ± 213	468 ± 73*	355 ± 68	302 ± 51	3600	9300
IV		N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
V		N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
VI		N.I.	N.I.	N.I.	N.I.	9200	N.I.
VII		N.I.	N.I.	N.I.	N.I.	8700	860
VIII		N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
IX		692 ± 162	363 ± 67*	148 ± 31	182 ± 46	61	1400
X		347 ± 42	389 ± 39	83 ± 19	51 ± 23	71	720
XI		457 ± 88	575 ± 101	191 ± 53	166 ± 51	180	700
XII		1023 ± 119	1549 ± 286*	525 ± 132	363 ± 74	99	400

Briefly, 0.2 mmol of MATP-hemisuccinate (O-pinacolyl-O-(N-succinyl-p-aminophenyl)-methylphosphonate; succMATP) and 0.2 mmol tributylamine were dissolved in 2 ml dry dioxane and cooled to 10°C. After addition of 0.2 mmol isobutylchloroformate, the reaction mixture was incubated for 30 min. One ml of reaction mixture was then mixed with 1.2 ml of a RSA solution (27 mM in dioxane/water, 1:1, v/v, pH 8.0). The pH was maintained between 8.0 and 8.5 by addition of 1 M sodium hydroxide for 4 h.

All conjugates were dialyzed exhaustively against phosphate-buffered saline (PBS) and stored at -20°C. Epitope densities were estimated as described by Fenton and Singer (16) or by converting the organically bound phosphorus to inorganic phosphorus with perchloric acid, followed by determination of the phosphorus content according to Chen et al. (17). $MATP_7$-RSA, $MATP_{14}$-RSA, $MATP_{28}$-RSA, $succMATP_4$-RSA, and $MATP_6$-OvA conjugates were obtained. More highly substituted conjugates were insoluble and unsuitable for immunization (15).

Immunization

Four groups (A-D) of 15 rabbits (2-3 kg body weight) were immunized intradermally with 0.5 mg/kg of the various hapten-carrier conjugates (A = $MATP_7$-RSA; B = $MATP_{14}$-RSA; C = $MATP_{28}$-RSA; D = $succMATP_4$-RSA) emulsified in FCA (1:1, v/v), and boosted subcutaneously 60, 90, and 120 days later with 0.1 mg conjugate/kg emulsified in FIA (1:1, v/v). Animals were bled 10 days after injections (10, 70, 100, and 130 days, respectively, after priming). Sera were stored at -20°C prior to use.

The production of anti-MATP polyclonal or monoclonal antibodies (mAbs) obtained by using non-rabbit protein as carrier protein has been described recently (12, 18, 19). These rabbit antisera and four

of the previously produced mAbs (F71B12, F71D7, F71H9, and F71H10) were also included in this study.

Titer estimation

Microtiter plates (Immunoplate II, Fa. Nunc, Denmark) were coated with 150 µl/well of $MATP_6$-OvA solution (2 µg protein/ml in 0.05 M borate buffer, pH 9.0) and incubated for 12 h at 4°C. The plates were blocked with 0.5 % gelatin in PBS-Tween (200 µl/well) for 12 h at 4°C and washed afterwards three times with PBS-Tween. Antisera (predilution 1:100 in PBS-Tween) were applied in doubling dilution (PBS-Tween; 25 µl/well) for 1 h at 37°C. Plates were washed three times with PBS-Tween. For the next incubation step (1 h, 37°C), peroxidase-conjugated species-specific antibodies (goat anti-rabbit IgG, 1:1000, or goat anti-mouse IgG, 1:500, respectively; 50 µl/well) were added. H_2O_2 and o-phenylenediamine (2.5 mM in 0.1 M phosphate-citrate buffer, pH 5.0; 100 µl/well) served as substrates. The enzymatic reaction was stopped after 10 min with 50 µl/well of a 2 M HCl solution, and the color development was measured bichromatically in the SLT ELISA reader (EAR 400 AT) at 492 and 620 nm. The antibody titer was defined as the dilution of antibody giving an extinction of 0.5.

Fine specificity analysis

The fine specificity of anti-MATP antibodies was determined with a CIEIA according to Buenafe and Rittenberg (3). Microtiter plates were coated and blocked as described above (Titer estimation). Antisera or mAbs were diluted in PBS-Tween to give an extinction

not higher than 1.0. Optimal dilutions of antibodies and antisera were determined in the titer assay (see above). Antibodies (25 µl/well) were incubated with 10-fold dilutions (10^{-3} - 10^{-9} M; 25 µl/well) of the various organophosphorus compounds for 1 h at 37°C. Dry isopropyl alcohol (final concentration: 5 % in PBS-Tween) was used to dissolve the more hydrophobic analogues, and was consequently added to all test samples. Brimfield et al. (1) have shown that the CIEIA can be performed even in 10 % isopropyl alcohol. The incubation step with peroxidase-conjugated species-specific antibodies followed by the enzymatic reaction were carried out as described above (Titer estimation).

Measurement of relative antibody affinity distribution

Antibody affinity distributions were determined by an ELISA technique as described by Nieto et al. (20). Briefly, microtiter plates were coated with 150 µl/well of doubling dilutions of $MATP_6$-OvA in 0.05 mM borate buffer, pH 9.0 (highest concentration = 10 µg protein/ml, e.g. column 2A-H of microtiter plate = 10 µg/ml, column 3A-H = 5 µg/ml, etc.) and incubated for 12 h at 4°C. The plates were blocked with 0.5 % gelatin in PBS-Tween (200 µl/well) for 12 h at 4°C and washed afterwards three times with PBS-Tween. Antisera and mAbs were diluted in PBS-Tween to give an extinction of less than 1.0. Antibody samples (25 µl/well) were mixed with 10-fold dilutions (10^{-3} - 10^{-9} M) of MATP (25 µl/well) for each coating antigen concentration and incubated for 1 h at 37°C. The incubation step with peroxidase-conjugated species-specific antibodies and the enzymatic reaction were carried out as mentioned above (Titer estimation).

RESULTS

Titer estimation

In all rabbit groups (A-D), MATP-specific antibodies could be induced with MATP-RSA conjugates (figure 1). Surprisingly, antibody titers obtained with the immunogen with the highest hapten density (group C) were significantly lower then those found in groups A, B, and D in the secondary response. Incorporation of a spacer into the MATP-RSA conjugate (group D) had no significant effect on antibody titers (figure 1). An interassay variability of <7.25 % was determined for the titer value of an anti-MATP standard serum (n=20). In about 30 % of rabbits, low antibody titers (<1:100) were detected against the carrier protein, RSA, in a simple ELISA for RSA-specific antibodies (data not shown).

Fine specificity analysis

The fine specificity of the different anti-MATP antibodies was determined by CIEIA. Table 1 provides a summary of this analysis by giving the IC_{50} values of various MATP derivates. The hapten density, investigated with various MATP-RSA azo-conjugates (groups A, B, and C), similar to the use of a MATP-non-rabbit-protein azo-conjugate for immunization (12), had no significant effect on antibody fine specificity. For this reason, only data obtained with $MATP_7$-RSA (group A) are shown in table 1.

An about 2-fold increase in antibody affinity to MATP (all groups) and to substances III and IX (groups A-C) could be observed between the 70th and 130th day after priming. In general, the antibody affinities of group D (spacer between hapten and carrier) were

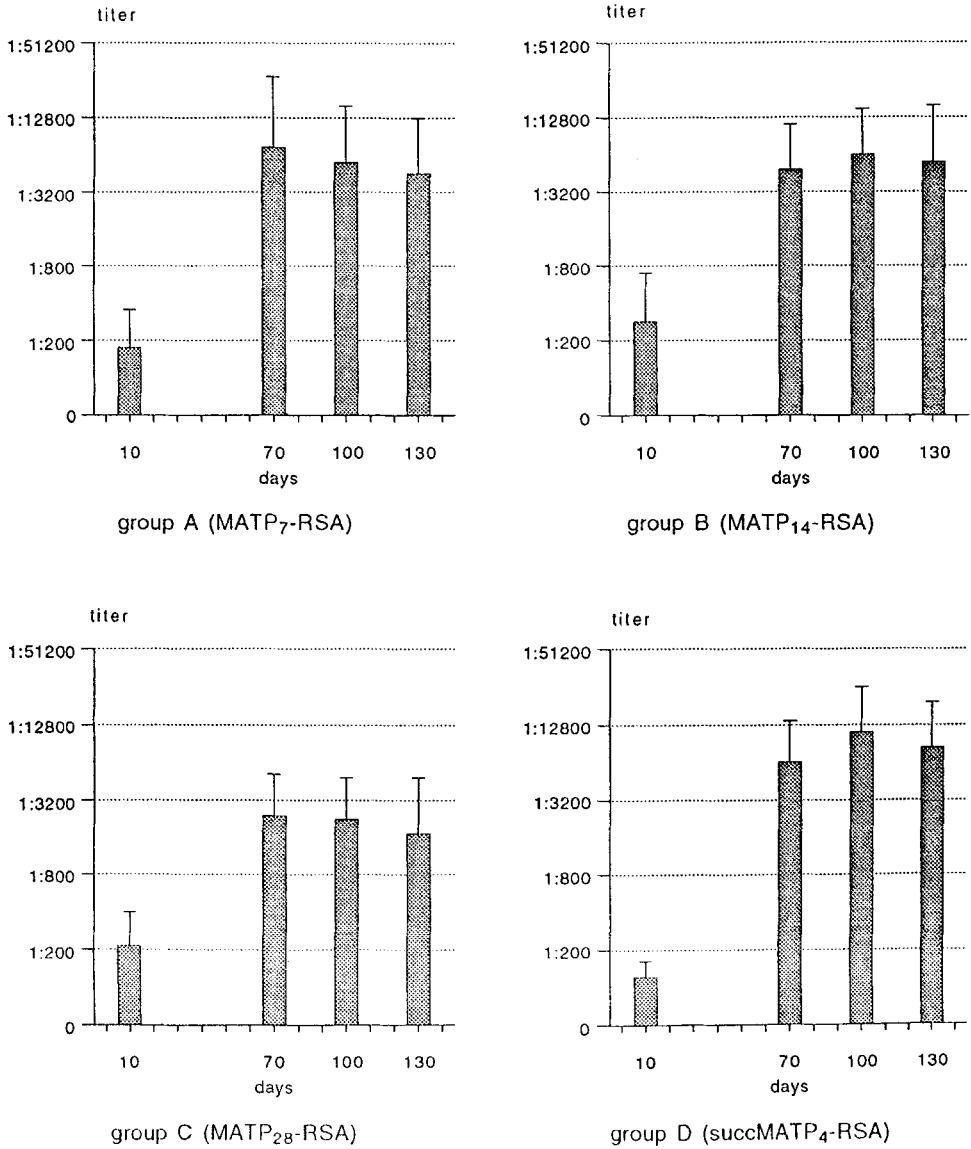


FIGURE 1. Development of antibody titers in rabbit groups A, B, C and D. Blood samples were collected 10 days after each injection over a 130 day-period (n=15). The antibody titer is defined as the dilution of antibody giving an extinction of 0.5.

significantly higher (up to 7.6 times in case of substance X, 130 days after priming) than those obtained in all other groups. In all cases, antisera failed to recognize amido derivates (IV and V), p-amino-paraoxon (VI), paraoxon (VII), and methylparathion (VIII). Moreover, small differences in antibody affinities to derivates without phenyl moiety were found in all groups (substance X>IX=XI>XII).

To compare fine specificities of polyclonal and monoclonal antibodies, four mAbs (F71B12, F71D7, F71H9, and F71H10) were also included in these experiments (18). IC_{50} values obtained with two of these mAbs (F71H10 and F71H9) are shown in table 1. MAbs F71D7 and F71B12 gave results virtually identical to those obtained with F71H10 (data not shown). The mAbs F71H10, F71D7, and F71B12 bound to MATP with similarly high affinity as the polyclonal antibodies. In contrast to the polyclonal antibodies, these three mAbs discriminated significantly between MATP and substance II displaying an about 200-fold lower affinity to substance II. Like group D antisera, these mAbs displayed relatively high affinities to derivates without phenyl moiety. The mAb F71H9 showed a different affinity pattern binding to most derivates with low affinities. In contrast to the other mAbs, it did not discriminate between MATP and substance II. Interestingly, substance VII was able to bind to this mAb with relatively high affinity.

An interassay variability of <10 % and an intraassay variability of <2.1 % was observed for IC_{50} determinations of MATP using an anti-MATP standard serum (n=21).

Measurement of relative antibody affinity distribution

Antibody affinity distribution patterns were determined by a simple and reliable ELISA technique as described by Nieto et al. (20). To ex-

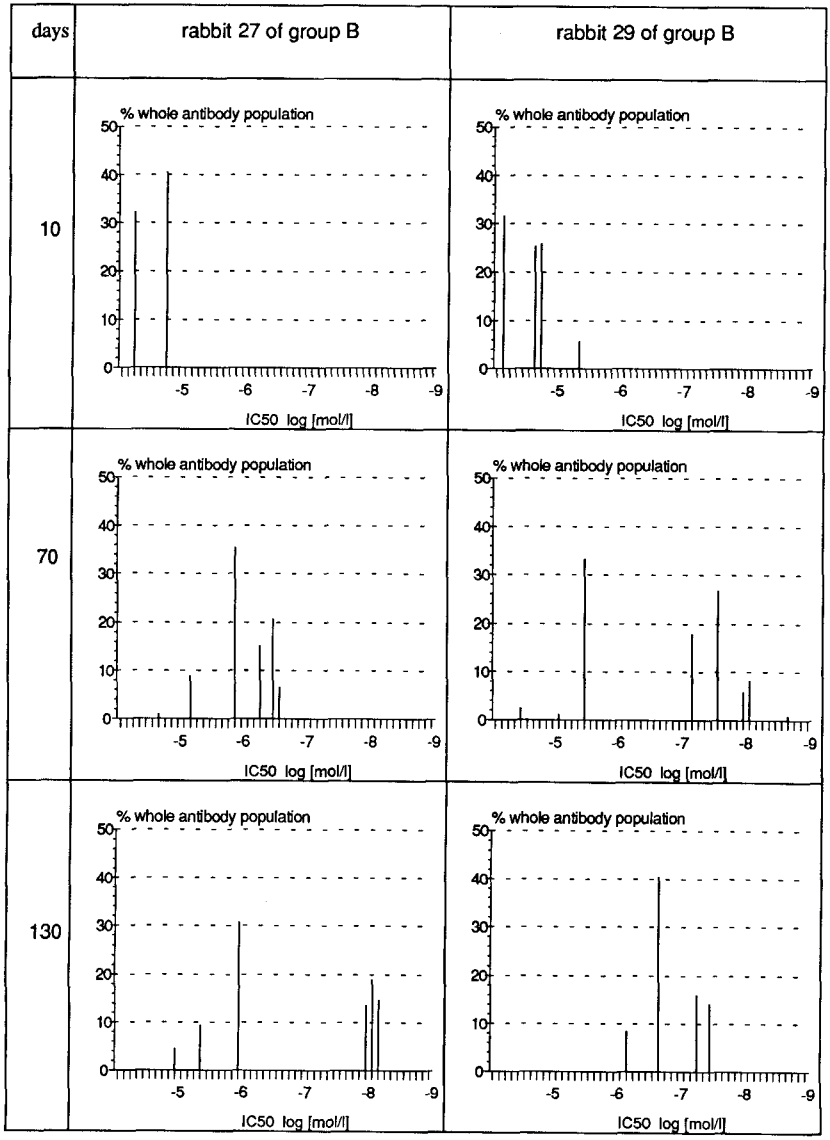
amine the sensitivity of this assay, affinity distribution patterns were studied with single mAbs and mixtures of mAbs with different affinities. These preliminary investigations showed that differences in IC_{50} values of less than 20 % were within experimental error and IC_{50} values differing in >20 % were indicative of two different affinity subpopulations.

Two examples of antibody affinity distribution development are shown in table 2. The relatively high antibody titers after priming in group B rabbits allowed the estimation of affinity distribution already after the primary immune response (day 10). In general, a shift from low to high affinity antibody subpopulations was found between priming (day 10) and the first booster injection (day 70). Further booster injections resulted in highly variable antibody affinity distributions. Depending on the individual rabbit examined, either an increase, a decrease, or no change in high affinity antibody populations was observed in all groups (data not shown). This pattern was not significantly affected by different hapten densities, spacer interposition, or the use of a non-rabbit carrier protein. In general (groups A-D), the average number of antibody affinity subpopulations decreased from 3.1 (70 days) to 2.3 subpopulations (130 days after priming) during the secondary immune response.

DISCUSSION

Organophosphorus compound-protein conjugates linked via azo-coupling are frequently used hapten-carrier conjugates to investigate basic immunological mechanisms. Beside the adjuvants and animal species (11, 12), the properties of the hapten-carrier conjugate also influence titer development as well as affinity and specificity of an antiserum. Hapten-carrier conjugates can differ in their

TABLE 2. Antibody affinity distribution pattern during primary and secondary immune response. Columns with differences of IC_{50} values >20 % indicate different antibody affinity subpopulations. Two typical examples are shown.



carrier protein, hapten density, coupling procedure and spacer interposition. Usually, hapten-azo-conjugates with a carrier protein derived from a species different from the immune host are employed for immunization. To the best of our knowledge, this is the first study investigating the influence of hapten density and spacer interposition on antibody titer and affinity using hapten-RSA conjugates for immunization of a large number of rabbits.

Our data suggest that the source of the carrier protein primarily affects the antibody amount generated. Investigations similar to those described here using a non-rabbit protein as carrier led to considerably higher titers (12) but not to significant changes in antibody affinity. Consistent with a previous study (21), we found that a carrier protein derived from the same species as the immune host is immunologically not inert. Chemical and structural changes of the carrier protein due to coupling procedures or emulsification in FCA and the local application combined with an inflammation may be responsible for the immune reaction to species-identical proteins.

We found that hapten density plays an important role in inducing high antibody titers. In contrast to a report by Laralde and Janof (13), a direct correlation between hapten density and antibody titer could not be clearly estimated in the primary immune response. However, the less highly substituted $MATP_7$-RSA or $MATP_{14}$-RSA conjugates lead to significantly higher antibody titers in the secondary response, as compared with the $MATP_{28}$-RSA conjugate. Moreover, we found that the interposition of a spacer between hapten and carrier did not significantly influence titer development.

CIEIA studies with different MATP derivates were carried out to characterize antibody affinities. It has been demonstrated that IC_{50} values determined by CIEIA are a good measure of average affinities (6, 22, 23). In accordance with a previous study (2), we could

demonstrate that the phenyl moiety as well as the aliphatic side chain are strong epitope regions of MATP. Considerable structural changes such as those present in substances IV-VIII led to a loss of antibody affinity. However, even minor changes in the MATP hapten such as the modification of the number of methyl groups in the pinacolyl side chain or the use of stereo isomers have been shown to influence the antibody-hapten-interactions (1). In addition, moderate changes in fine specificity were found when a spacer was used for hapten-carrier coupling. Similar findings have also been obtained by Vallejo et al. (24).

The monoclonal antibodies F71D7, F71H10, and F71B12 showed the same high affinity to MATP as the polyclonal antibodies but considerably lower affinities to derivates II and III. Interestingly, mAb F71H10 did not only show high affinity to MATP but also to compounds with lack a phenyl moiety (substances IX-XII).

Pauling et al. (25) assumed an unimodal, in fact, normal distribution of individual antibody affinities of an antiserum. Sips (26) made allowance for experimental findings by implementation of a heterogeneity index in the Scatchard-plot. Today, simple CIEIA techniques for determining antibody affinity distribution (20) and computer programs fitting data from antigen-antibody binding studies are available (27, 28). The method used in this study determines antibody affinity distribution by measuring the number and IC_{50} values of subpopulations and the relative abundance of each antibody subpopulation in an antiserum without any prefractionation procedures. As expected, we found a shift from low to high affinity antibody subpopulations from primary to secondary immune response. However, subsequent booster injections resulted in individually highly variable antibody affinity distributions which did not correlate with hapten density, spacer interposition, or the choice of the carrier

protein. Variabilities on the genomic level or somatic mutations could be responsible for this heterogeneity of individual immune responses (4, 6, 29).

In conclusion, the present study provides strong evidence that the carrier protein has a strong effect on the generated antibody amount but that the affinity and specificity pattern is determined by the chemical structure of the hapten. Besides providing basic information about the immune response, the determination of antibody affinity distribution pattern should become an useful tool to establish the optimum moment for obtaining high affinity antisera or for extirpation of the spleen for the preparation of highly specifically mAbs.

ACKNOWLEDGMENTS

The authors are grateful to Dr. P. Rausch (Munster, FRG), Prof. H. Bleyer (Greifswald, FRG), and Dr. J. Wess (Bethesda, USA) for their support and helpful discussions.

REFERENCES

1. Brimfield, A.A., Hunter, Jr., K.W., Lenz, D.E., Benschop, H.P., Van Dijk, C. and de Jong, L.P.A. Structural and stereochemical specificity of mouse monoclonal antibodies to the organophosphorus cholinesterase inhibitor soman. Mol. Pharmacol. 1985; 28: 32-9.

2. Lenz, D.E., Brimfield, A.A., Hunter, Jr., K.W., et al. Studies using a monoclonal antibody against soman. Fundam. Appl. Toxicol. (1984); 4: 156-64.

3. Buenafe, A.C. and Rittenberg, M.B. Combining site specificity of monoclonal antibodies to the organophosphate hapten soman. Mol. Immunol. 1987; 24: 401-7.

4. Allen, D., Cumano, A., Simon, Th., Sablitzky, F. and Rajewsky, K. Modulation of antibody binding affinity by somatic mutation. Int. J. Cancer: Supplement 1988; 3:, 1-8.

5. Griffiths, G.M., Berck, C., Kaartnem, M. and Milstein, C. The maturation of immune response to 2-phenyloxaxolone. Nature 1984; 312: 271-5.

6. Buenafe, A.C., Makowski, F.F. and Rittenberg, M.B. Molecular analysis and fine specificity of antibodies against an organophosphorus hapten. J. Immunol. 1989; 143: 539-45.

7. Bruderer, U., Stenzel-Poore, M.P., Bächinger, H.P., Fellman, J.H. and Rittenberg, M.B. Antibody combining site heterogeneity within the response to phosphocholine-keyhole limpet hemocyanin. Mol. Immunol. 1989; 26: 63-71.

8. Hunter, Jr., K.W., Lenz, D.E., Brimfield, A.A. and Naylor, J.A. Quantification of the organophosphorus nerve agent soman by competitive inhibition enzyme immunoassay using monoclonal antibody. FEBS Letters 1982; 149: 147-51.

9. Schmidt, P., Kühlmann, R. and Lösch, U. A competitive inhibition enzyme immunoassay for detection and quantification of organophosphorus compounds. Z. Naturforsch. 1988; 43c: 167-72.

10. Erhard, M.H., Jüngling, A., Schöneberg, T., Szinicz, L. and Lösch, U. A homogeneous immunological detection system for soman using the in vitro protection of acetylcholinesterase by monoclonal antibody. Arch. Toxicol. 1993; 64: 580-5.

11. Kellner, J., Erhard, M.H., Schranner, I. and Lösch, U. The influence of various adjuvants on antibody synthesis following immunization with a hapten. Biol. Chem. Hoppe-Seyler 1992; 373: 51-5.

12. Erhard, M.H., Kellner, J., Kühlmann, R. and Lösch, U. Influence of various adjuvants on synthesis of specific antibodies of chicken,

sheep and rabbit following immunization with an hapten. J. Vet. Med. A 1991; 38: 21-7.

13. Larralde, C. and Janof, P. The effects of the DNP:HSA molar ratio on the quantity and the affinity of rat anti-DNP antibodies in the primary response. Immunochemistry 1972; 9: 1209-19.

14. Suttnar, J., Hrkal, Z. and Vodrazka, Z. Affinity chromatography of serum haemopexin. J. Chromatogr. 1977; 131: 453-7.

15. Lober, M., Krantz, S. and Herrmann, I. Erzeugung und Charakterisierung von Antiseren gegen O,O-Diäthyl-o-(p-nitrophenyl)-phosphat (Paraoxon). Acta Biol. Med. Germ. 1982; 41: 487-96.

16. Fenton, J.W. and Singer, S.J. Affinity labeling of antibodies to the p-azophenyltrimethylammonium hapten, and a comparison of affinity-labeled antibodies of two different specificities. Biochemistry 1971; 10: 1429-37.

17. Chen, Jr., P.S., Toribara, T.Y. and Warner, H. Microdetermination of phosphorus. Anal. Chem. 1956; 28: 1756-8.

18. Erhard, M.H., Schmidt, P., Kühlmann, R. and Lösch, U. Development of an ELISA for detection of an organophosphorus compound using monoclonal antibodies. Arch. Toxicol. 1989; 63: 462-8.

19. Erhard, M.H., Kühlmann, R., Szinicz, L. and Lösch, U. Detection of the organophosphorus nerve agent soman by an ELISA using monoclonal antibodies. Arch. Toxicol. 1990; 64: 580-5.

20. Nieto, A., Gaya, A., Jansa, M., Moreno, C. and Vives, J. Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. Mol. Immunol. 1984; 21: 537-43.

21. Atassi, M.Z. Antigen structures of proteins. Eur. J. Biochem. 1984; 145: 1-20.

22. Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L. and Goldberg, M.E. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods 1985; 77: 305-19.

23. Rath, S., Stanley, C.M. and Steward, M.W. An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity heterogeneity. J. Immunol. Methods 1988; 106: 245-9.

24. Vallejo, R.P., Bogus, E.R. and Mumma, R.O. Effects of hapten structure and binding groups on antisera specificity in parathion immunoassay development. J. Agric. Food Chem. 1982; 30: 572-80.

25. Pauling, L., Pressmann, D. and Gromberg, A.L. The theoretical properties of simple substance. A quantitative theory of inhibition by haptens of precipitation of heterogeneous antisera with antigens and comparison with experimental results for polyhaptenic simple substances and for azoproteins. J. Am. Chem. Soc. 1944; 66: 784-92.

26. Sips, R. On the structure of a catalyst surface. J. Chem. Phys. 1948; 16: 490-5.

27. Flanaga, M.T., Tattam, F.G. and Green, N.M. The characterization of heterogeneous antibody hapten interaction using nonlinear regression analyse on fluorescence quenching curves. Mol. Immunol. 1978; 15: 261-7.

28. Sciutto E., Garat, B., Ortega, E. and Larralde, C. Antibody heterogeneity: Theoretical and experimental evaluation of a simple procedure to describe differing affinities in hapten binding reactions. Mol. Immunol. 1987; 24: 577-85.

29. Sharon, J. Structural correlates of high antibody affinity: Three engineered amino acid substitutions can increase the affinity of an anti-p-azophenylarsonat antibody 200-fold. Proc. Natl. Acad. Sci. USA 1990; 87: 4814-7.