Immunogens for the preparation of retinoic acid antibodies

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The generation of antibodies to retinoic acid necessitates the prior synthesis of appropriate immunogens. This paper describes the synthesis and characterization of various retinoic acid immunogens. Immunogens were synthesized by reacting the carboxylic group of retinoic acid with bovine serum albumin, chicken IgG and human IgG; these immunogens are expected to produce antibodies against the ring portion of the retinoid molecule. For the generation of antibodies with specificity towards the retinoid polyene side chain and its carboxyl group, immunogens were prepared with an exposed retinoid polyene side chain and carboxyl group. Retinoic acid was derivatized to methyl-4-oxoretinoate, and carboxymethoxylamine attached via 4-oxo position to the B-ionone ring to yield methyl-4-oxoretinoic acid oxime, m/z 402 for $[M + H]^+$; $E^{1\%}_{1cm}$ 1965 at 365 nm in 45% dioxane; the structure of the oxime was verified by NMR. The hapten was conjugated with bovine serum albumin, chicken IgG, human IgG, or with aminohexyl Sepharose 4B to produce various antigens. The p-hydrazinobenzoic acid derivative of 4-oxoretinoic acid was also used as a hapten. Immunogens were also prepared by conjugating retinoic acid to liposome-protein complexes. The prepared immunogens generated anti-retinoic acid antibodies in mice and rabbits. The best immunogenic response was obtained with 4-oxoretinoic acid oxime-chicken IgG conjugate. Our work provides a systematic approach to synthesis of retinoic acid antigens and describes reproducible techniques for the preparation of retinoic acid haptens and retinoic acid-specific immunogens. The methods can be applied to the generation of immunogens to other retinoids and vitamins.

Keywords: vitamin A; retinoids; retinoic acid-specific antigens; retinoic acid oxime

Introduction

Although it has been evident for some time that vitamin A active molecules, particularly retinoic acid, are potent regulators of cell proliferation and differentiation,[1,2] the exact mechanism of action of vitamin A at the molecular level is still unsolved. The recent demonstration of nuclear receptor(s) for retinoic acid[3-6] has finally confirmed what all in the vitamin A field had long hypothesized: that retinoic acid must be the active form of vitamin A for at least some of its functions, possibly for the majority of vitamin A functions associated with regulation of cellular growth and differentiation. Clearly, a door is now open for a direct approach to the study of retinoid function at molecular level.

Attempts have been made to prepare immunological probes for vitamin A compounds: polyclonal antibodies have been made to retinol and retinoic acid. This work has been pioneered by Wirtz and his associates[7-10]; the antibodies obtained have been used to develop a radioimmunoassay for the assay of serum retinol.[8] The immunoassay, however, does not discriminate between vitamin A compounds with different functional groups, since the immunogen was pre-

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pared by conjugating the retinoid (retinoic acid) molecule through its functional (carboxyl) group to serum albumin. The resulting antibody reacts with all compounds having a B-ionone ring and cannot distinguish the different functional groups on the polyene side chain.

While relatively standard methods can be applied to generate antibodies to protein antigens, the production of antibodies to small molecules is still an experimental area that mandates innovative approaches dependent on the chemical and physical properties of the molecule. In the production of antibodies to retinoic acid or other retinoids, one must overcome several major obstacles: the generation of antibodies to (1) a small molecule, (2) a molecule that is essential for the normal function of the animal, (3) a molecule that is lipid soluble, and (4) a molecule that is extremely sensitive to any chemical and physical manipulation, which makes the generation of chemically well defined derivatives difficult.

New technology is now available for preparation of biological probes to detect small molecules.[11-16] We utilized this technology as well as conventional immunological techniques in combination with our experience in retinoid biochemistry in the preparation of retinoic acid immunogens described in this paper. Several of the immunogens produced high titer polyclonal antibodies to retinoic acid, and in preliminary studies, indicated specificity towards retinoic acid.

Materials and methods

Retinoids, reagents, chemicals

Crystalline all-trans-retinoic acid (RA) was a gift from Hoffmann-LaRoche, Inc., Nutley, NJ. Standards for chromatography, 4-oxoretinoic acid, and 13-cis-ethylretinamide were gifts from Hoffmann-LaRoche, Basel, and National Cancer Institute, respectively. Retinoids were checked for purity and if necessary purified as described earlier.[17] Purification and analysis of retinoids were carried out under amber lights and in N_2 atmosphere. All retinoids were stored under N_2 at $-70°$ C.

Phosphatidyl choline, diacetylphosphate, cholesterol, bovine serum albumin (BSA), methylated BSA (MeBSA), chicken IgG (cIgG), human IgG (hIgG), isobutylchloroformate, N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), carboxylic ester hydrolase (EC 3.1.1.1, Cat. # E.3128, Porcine liver), trypsin (EC 3.4.21.4), protease Type XXVIII (protease K), leucine aminopeptidase (microsomal, EC 3.4.11.2), dimethylsulfoxide (DMSO), and aminohexyl-Sepharose 4B were obtained from Sigma, St. Louis, MO. N-methyl-N-nitroso-p-toluenesulfonamide (Diazald), carboxymethoxylamine, tri-nitrobutylamine, and p-hydrazinobenzoic acid were purchased from Aldrich Chemicals, Milwaukee, WI. Sephadex G 50 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Sep-pak C_{18} cartridges were obtained from Waters, Milford, MA. All other chemicals and solvents were reagent or high pressure liquid chromatography (HPLC) grade.

Preparation of liposomes

Liposomes containing retinoic acid were prepared by the method of Shek et al.[18] Negatively charged multilamellar liposomes were prepared by dissolving phosphatidyl choline, cholesterol, and phosphatidic acid (molar ratios 7:2:1, respectively) and all-trans-retinoic acid, 4.7 μmol, in 5 mL chloroform in a round bottom flask; the solvent was removed by rotating the flask under a stream of N_2 until a uniformly thin layer was formed. A suspension of 2.3 μmol of all-trans-RA (in 100 μL chloroform) and 33 nmol of MeBSA in 7 mL of 0.02 M, pH 7.4 phosphate buffered saline (PBS) was used to disperse the lipid film by sonication for 20 min in ice bath. The yellow RA-MeBSA-liposome complex was allowed to stand overnight at 4° C. It was then centrifuged at 105,000*g* for 30 min to remove retinoic acid not associated with liposomes. Retinoic acid remaining in supernatant was quantitated by UV at 350 nm. Most of the retinoic acid was found to be incorporated into liposomes.

Synthesis of 4-oxoretinoic acid methyl ester

For the conjugation of RA to a large vector via the B-ionone ring of the molecule (so as to leave the side chain and the carboxylic group free), RA was first methylated, then the B-ionone ring oxidized with MnO_2 to generate the 4-oxo-RA methyl derivative according to the method of Barua and Gosh.[19] The compound was purified by HPLC and characterized by comparison with the elution pattern of authentic methyl-4-oxoretinoate on HPLC,[17] by nuclear magnetic resonance (NMR), and by positive ion fast atom bombardment (FAB-MS).

Alternatively, 4-oxoretinoic acid was methylated by adding 0.1 mmol of it to 4 mL of diazomethane dissolved in diethyl ether at RT; complete methylation was achieved in 30 min. After removal of solvent and excess diazomethane, the mixture was analyzed by HPLC and the eluted compounds identified by comparison with elution patterns of authentic standards.[17]

Addition of spacer onto the derivatized retinoic acid: preparation of haptens

Methyl ester of 4-oxo-all-trans-retinoic acid was reacted with carboxymethoxylamine[12] to generate methyl-4-oxoretinoate oxime, a retinoic acid derivative with a relatively short spacer chain that provides a choice carboxyl linkage of the hapten to a carrier molecule. All-trans-methyl-4-oxoretinoate, 0.1 mmol, dissolved in 4 mL pyridine, was reacted with carboxymethoxylamine, 0.3 mmol, with stirring at RT for 24 h. Pyridine was removed by a stream of N_2, and the reaction products dissolved in 4 mL of chloroform. Insoluble side products were removed by centrifugation. Supernatant (chloroform) containing the product, methyl-4-oxoretinoate oxime, was washed with water,

then evaporated by a stream of nitrogen and the product redissolved in dioxane and purified in two solvent systems by HPLC using methanol and water (*Figure 1*). Characterization was by UV spectrum, FAB-MS, and NMR. The $E^{1\%}_{1cm}$ in 45% dioxane was determined to be 1956 at 365 nm. Methyl-4-oxoretinoate was also reacted with p-hydrazinobenzoic acid, another spacer molecule, to generate methyl-4-oxoretinoate carboxyphenyl hydrazone, as described elsewhere.[2]

Conjugation of methyl-4-oxoretinoate oxime to protein: preparation of antigens

The carboxyl-containing hapten, methyl-4-oxoretinoate oxime was conjugated via the free carboxyl group on the oxime to BSA, cIgG, and hIgG. A *modified* N-hydroxysuccinimide ester (NHS) method,[11] was used for all proteins and is outlined for cIgG as follows. To methyl-4-oxoretinoate oxime, 0.1 mmol, dissolved in 2.4 mL dioxane, was added 0.1 mmol of NHS in 0.8 mL dioxane and 0.1 mmol of dicyclohexylcarbodiimide (DCC) in 0.8 mL dioxane and the reaction was allowed to proceed at RT for 30 min. Insoluble side-products were removed by centrifugation. The supernatant was added to 0.001 mmol of cIgG dissolved in 6 mL of water, pre-adjusted with NaOH to pH 8.5. The reaction mixture was stirred at RT for 2 h, then dialyzed overnight against 0.02 M PBS, pH 8, followed by dialysis against 0.02 M PBS, pH 7.4 for 4 h. The antigen was purified by chromatography on a Sephadex G 50 column (22 × 2.5 cm) that had been equilibrated with 0.02 M PBS, pH 7.4; elution was with 0.02 M PBS, pH 7.4. The collected fractions were analyzed by spectrophotometry at 365 nm to quantitate the hapten, using $E^{1\%}_{1cm}$ 1956, determined above. Protein was measured by the Lowry reaction,[20] and the molar ratio of hapten to protein calculated. Similarly, the phenylhydrazone derivative was conjugated to BSA and cIgG by the above procedure. The concentration of the retinoid-phenylhydrazone derivative was determined at 345 nm.

Unblocking of the carboxyl group: enzymatic deesterification

Several methods were tried to unblock the carboxylic group on the polyene side chain of the methyl retinoate molecule. Hydrolysis with nonspecific esterase (Carboxylic ester hydrolase, Sigma, porcine liver, carboxylic ester hydrolase) was selected as the method of choice to remove the methyl group from the methylated carboxyl in the derivatized retinoic acid moiety of haptens. Using methyl-4-oxoretinoate as substrate, conditions were first established that were suitable for the use with retinoid-protein conjugates as substrates. The reaction was optimum (70% deesterification) in 0.1 N sodium bicarbonate, pH 8.1, using 2.5 μM substrate per 1 mL of reaction mixture with 50 μL (30 U) of esterase at 37° C for 6 h. Longer incubation times (16 h) and increased amounts of the esterase resulted in complete deesterification. The esterase was free of

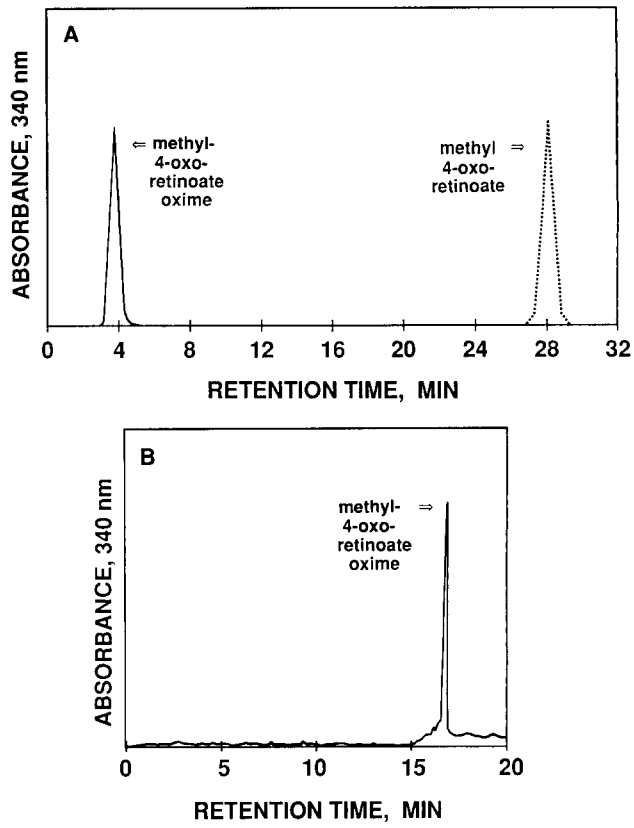


Figure 1 Purification of methyl-4-oxoretinoate oxime. (A) Separation of methyl-4-oxoretinoate oxime from methyl-4-oxoretinoate by reversed phase HPLC; solvents methanol:water, 75:25 for 15 min, followed by methanol:H_2O, 85:15. Elution profile of reaction mixture at the beginning of synthesis of oxime, (- - -); elution profile at the completion of synthesis of oxime, (———). Complete conversion to the oxime occurred after 16 h of reaction. (B) HPLC profile of methyl-4-oxoretinoate oxime in methanol:water, 60:40, 12 min, followed by methanol:water, 85:15. The oxime eluted at 17 min.

proteinase activity as assessed by incubation of the enzyme with BSA. Deesterification was quantitated by separating the retinoids in the reaction mixture by HPLC and by quantitating methyl-4-oxoretinoate and retinoic acid before and after the reaction by HPLC-UV using the integrated peak area method.[17] HPLC was achieved on an ODS-3 reversed phase column described in *Figure 2*. The above deesterification procedure was used to remove the methyl group from the hapten after preparation of the antigens.

Deesterification of methyl-4-oxoretinoate oxime cIgG conjugate

Methyl-4-oxoretinoate oxime-cIgG conjugate (molar ratio retinoic acid/cIgG, 25:1), 16 mg (100 nmol) was incubated with 100 μL (60 U) of carboxylic ester hydrolase in 4 mL of 0.1 M sodium bicarbonate, pH 8.1, in water bath at 37° C for 16 h. To assess the completeness of deesterification, the 4-oxoretinoate acid oxime-cIgG conjugate was subjected to proteolytic digestion as follows. After deesterification, 0.16 mg of trypsin in 0.1 mL was added and the mixture incubated for 18 h, following which 0.16 mg of protease K

in 0.1 mL was added and incubation continued for an additional 24 h. Final digestion was by incubation with 2 U of amino-peptidase for 8 h. The digest will contain amino acids and enzyme proteins, as well as retinoid-lysine adducts, anticipated, because in the carbodiimide method the conjugates are formed via lysyl residue of the protein.[11]

The protein digest was loaded onto a Sep-pak C_{18} column, which had been prewashed with 5 mL of methanol and equilibrated with water. Amino acids and salts were eluted with 10 mL of distilled water. Lysine adducts with the retinoid moiety as well as enzyme proteins were eluted with 5 mL of 85% methanol, the eluate concentrated by evaporation under vacuum, dissolved in methanol, and analyzed by FAB-MS spectrometry.

Preparation of Sepharose 4B-linked retinoic acid immunogens

Ten mg (25 μmol) of methyl-4-oxoretinoate oxime, dissolved in 0.6 mL of dioxane was mixed with 5.15 mg (25 μmol) of DCC in 0.2 mL of dioxane and 2.88 mg (25 μmol) of NHS in 0.2 mL H_2O. The mixture was added to 1 mL of AH-Sepharose 4B gel, pH 8.5 and allowed to react at RT overnight. The gel was extracted with 45% dioxane and the amount of unreacted methyl-4-oxoretinoate oxime determined. The gel was then washed with H_2O. The amount of retinoic acid per mL of gel was calculated from the difference in UV absorption at 365 nm of the total methyl-4-oxoretinoate oxime added and the amount unreacted, using $E^{1\%}_{1cm}$ 1956 in 45% dioxane. The above procedure was also used to conjugate the phenylhydrazone derivative to Sepharose 4B.

Conjugation of retinoic acid to protein

All-trans-retinoic acid was conjugated via its carboxyl group to BSA and cIgG by the modified NHS method[21] as described above. The molar ratios of retinoic acid to protein were determined as described for other protein antigens above, except that retinoic acid absorbance was measured at 348 nm.

Selection of immunogens for immunization

Immunogens to be used for antibody generation were selected on the basis of their solubility and high retinoic acid hapten-to-protein carrier or nonprotein carrier ratios. Based on these criteria the following immunogens were selected and used as follows: (1) 4-oxoretinoic acid oxime-cIgG was used for the immunization of mice and the corresponding BSA conjugate was used for enzyme linked immunosorbent assay (ELISA) solid plate; (2) all-trans-retinoyl-BSA was used for immunization of rabbits, and retinoyl cIgG conjugate was used for ELISA solid plate; (3) 4-oxoretinoic acid oxime-AH-Sepharose 4B conjugate; and (4) retinoic acid-MeBSA-liposome complex. Four-oxoretinoic acid phenylhydrazone-cIgG and 4-oxoretinoic acid phenylhydrazone-AH-Sepharose 4B conjugates were also used to immunize mice, but in preliminary testing were found to be very weak immunogens and were not used in subsequent studies.

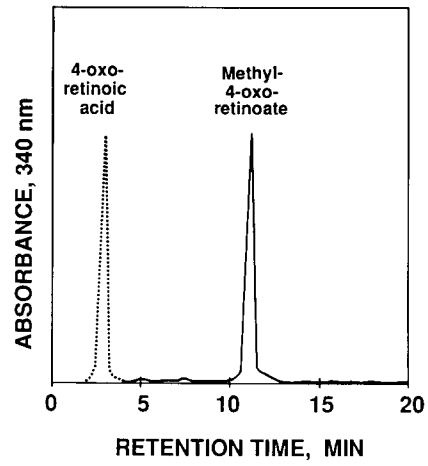


Figure 2 Separation of methyl-4-oxoretinoate from 4-oxoretinoic acid by reversed phase HPLC. Solvents methanol:water, 75:25 for 5 min, followed by methanol:water, 85:15. Elution of standard 4-oxoretinoic acid, (. . . .); methyl-4-oxoretinoate, (———).

Animals

Female BALB/c and C57BL/6 inbred mice, 8–10 wk of age, and female New Zealand white rabbits, weighing approximately 2 kg, were purchased from Charles River, Portage, MI, and housed in Michigan State University Laboratory Animal Research Committee approved animal quarters at 21° C, 40% humidity, and equipped with automatically controlled 12-h light-dark cycle. The animals were maintained in accordance with NIH guidelines and were under veterinary care. Mice were fed Rodent Blox (Wayne Pet Food Division, Continental Grain Co., Chicago, IL); rabbits were fed high fiber Rabbit Chow (Ralston Purina Mills, Inc., Richmond, IN).

Immunization

Ten BALB/c or C57BL/6 mice were used for immunization with each immunogen. Four-oxoretinoic acid oxime-cIgG (1.14 nmol) or 4-oxoretinoic acid phenylhydrazone-cIgG (1.22 nmol) were injected intraperitoneally, 200 μg of protein conjugate/injection, in 0.2 mL of PBS: Freund's complete adjuvant, 1:1. Retinoic acid-MeBSA-liposome complex was injected introperitoneally in 0.5 mL of emulsion containing PBS: Freund's complete adjuvant, 1:1 and containing 0.27 μmol of retinoic acid in the hapten. The mice were boosted three times at 4-wk intervals using the above immunogen prepared with Freund's incomplete adjuvant.

Four-oxoretinoic acid oxime-AH-Sepharose 4B beads were implanted in spleen as described by Spitz et al.[22] Briefly, mice were anesthetized with ether, the spleens exposed, and the antigen in an emulsion inoculated into the spleen by a needle. The immunogen emulsion was prepared as follows: one vial of RIBI

adjuvant (monophosphoryl Lipid A and Trehalose Dimycolate) was immersed in hot water for 10 min; to it were added 2 mL of saline and 1 mL of 4-oxoretinoic acid oxime-AH Sepharose 4B conjugate, 9.3 μmol/mL gel, and the mixture was vortexed for 3 min. For each intrasplenic inoculation, 0.05 ml of the above immunogen preparation was used. A total of three inoculations were performed at 2-wk intervals.

Three rabbits were immunized each with 2 mg (30 nmol) of retinoyl-BSA conjugates in 2 mL of PBS: Freund's complete adjuvant, 1:1; the immunogen was injected around the lymph nodes of front and back feet, in the neck area, and behind the ears. The rabbits were boosted three times at 4-wk intervals using the same conjugates, but they were prepared with Freund's incomplete adjuvant.

Preparation of sera

Mice. One week after the last immunization, 60 μL of blood were obtained from the tail vein of each mouse and mixed immediately with 1.5 mL of saline to obtain a 1:50 serum dilution; the diluted sera were used for antiserum titration.

Rabbits. One week after the last immunization, blood from the rabbits was collected from the ear vein. The antiserum was precipitated with 50% ammonium sulfate, the precipitate dissolved in a small amount of PBS, dialysed overnight against PBS, the dialysate adjusted to original volume and used for determination of antiserum titer.

Antiserum titration by indirect enzyme linked immunosorbent assay (ELISA)

Indirect ELISA was devised to determine titers of retinoic acid antisera. Four-oxoretinoic acid oxime-BSA conjugate was used as the solid phase to coat the well; it was dissolved in 0.1 M sodium bicarbonate buffer, pH 9.6, to contain 0.5 μg (7 pmol)/100 μL; 100 μL of the solution was added to each well in a 96-well microtiter plate (Immulon II Removawells, Dynatech Laboratories, Alexandria, VA) and incubated overnight at 4° C. The coated wells were washed 4 times by filling each well with 300 uL of 0.02 M PBS, pH 7.4, containing 0.05%, v/v Tween 20. To block unbound solid phase sites and to minimize nonspecific binding, 300 uL of 1% gelatin in PBS were added to each well and wells incubated for 45 min at 37° C. The wells were washed 4 times with PBS-Tween and duplicate aliquots of antiserum serially diluted in 100 μL PBS were added to the wells and incubated for 1 h at 37° C. Duplicate wells of diluted normal serum were used as controls. Unbound antibody was removed by washing 4 times with PBS-Tween. Goat anti-mouse polyvalent IgG perixodase conjugate, 100 μL, diluted 1:350 in 1% ovalbumin-PBS was then added to each well; after an incubation for 30 min at 30° C, the plate was washed 6 times with PBS-Tween. Bound peroxidase was determined with 100 μL of ABTS substrate.[21] Absorbance was read to 405 nm; endpoint titer for each serum was arbitrarily designated as the maximum dilution which gives twice or greater than twice the absorbance of the non-immune control serum.

The 50% ammonium sulfate fraction of serum from rabbits immunized with the retinoyl-BSA conjugate was serially diluted and incubated over a microtiter plate coated with retinoyl-cIgG as the solid phase; total bound antibodies were subsequently detected with goat anti-rabbit IgG peroxidase conjugate as described above.

HPLC and spectrometric analysis

Reversed phase HPLC was accomplished on a C_{18} reversed-phase column (Whatman ODS-3 10/25, 25 × 0.46 cm) with a precolumn (7 × 0.46 cm) containing Whatman Co:Pell ODS pellicular support. Step gradient elution of retinoid standards (100 ng or 250–330 pmol/2 μL) and biological samples (in 100–200 μL) was accomplished with mixtures of water and methanol (v/v) containing 0.01 M ammonium acetate and mixtures of $CHCl_3$ and methanol. UV absorbance was monitored with a Waters model 400 detector (Millipore Waters, Milford, MA). The HPLC-UV system is equipped with a reporting integrator (CHP-3390A, Hewlett-Packard, Avondale, PA). Ultraviolet spectra were recorded with a Beckman model 35 spectrophotometer equipped with a Sargent model SR recorder. Positive ion fast atom bombardment mass spectrometry (FAB-MS) was performed in a JEOL HX-110 mass spectrometer using dithioerythreitol:dithiothreitol, 1:5 or m-nitrobenzyl alcohol as the matrix. Proton nuclear magnetic resonance (NMR) spectra were determined in a Varian VXR 500 spectrophotometer operating at 300 MH_Z, using $CDCl_3$ as solvent with tetramethylsilane as an internal reference.

Other procedures

All above operations were carried out in a laboratory equipped with amber fluorescent lights.

The prepared retinoic acid immunogens can be stored frozen in 0.02 M PBS, pH 7.4 at −70° C for at least 18 months.

Results

Methyl-4-oxoretinoate and its oxime

The synthesized methyl-4-oxoretinoate was separated from 4-oxoretinoic acid by HPLC; a mixture of methanol:H_2O (75:25) eluted 4-oxoretinoic acid at 2.5 min, while the ester was eluted at 11.5 min with methanol:H_2O (85:15) methyl-4-oxoretinoate (*Figure 1*). *Figure 3* shows the UV spectrum of methyl-4-oxoretinoate oxime. The oxime has an absorption maximum at 365 nm and a minimum at 286 nm in 45% dioxane. $E^{1\%}_{1cm}$ in 45% dioxane was determined to be 1965 at 365 nm. FAB-MS spectrum of methyl-4-oxoretinoate oxime (*Figure 4B*) shows the expected m/z 402 for $[M + H]^+$. FAB-MS spectrum of methyl-

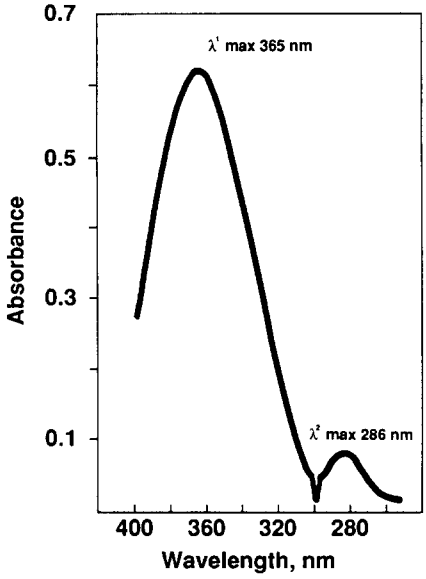


Figure 3 UV spectrum of methyl-4-oxoretinoate oxime.

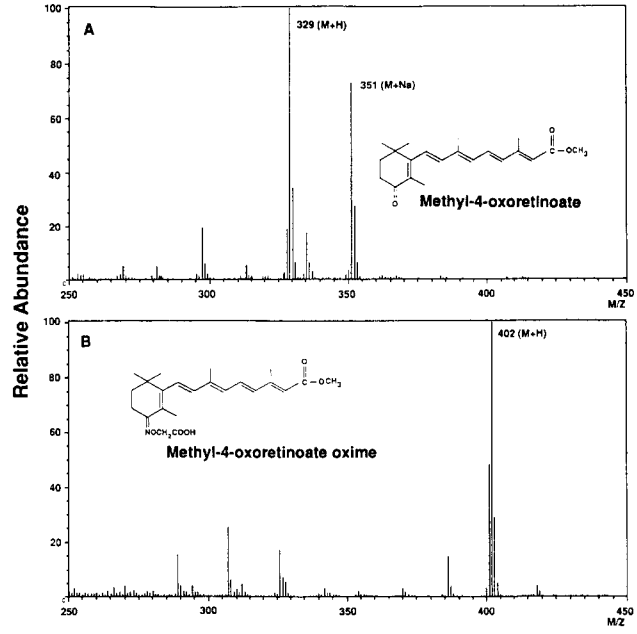


Figure 4 Mass spectra of (A), methyl-4-oxoretinoate and (B), methyl-4-oxoretinoate oxime. FAB-MS spectra were determined in dithioerythreitol:dithiothreitol, 1:5.

4-oxoretinoate shows the expected m/z 329 for $[M + H]^+$ (*Figure 4A*). NMR of methyl-4-oxoretinoate is shown in *Figure 5A* and is consistent with published data.[23] NMR of methyl-4-oxoretinoic acid oxime: ^{1}H-NMR ($CDCl_3$) δ 1.07 (S,6H), 1.60 (t,J = 6.70 Hz, 2H), 1.86 (S,3H), 2.00 (S,3H), 2.34 (S,3H), 2.67 (T,J = 6.70 Hz, 2H), 3.70 (S,3H), 4.65 (S,2H), 5.78 (S,1H), 6.25 (m, 4H), 6.97 (dd, J = 11.44 Hz, 15.08 Hz, 1H). The position of the oxime group in methyl-4-oxoretinoate oxime was verified.

Retinoic acid immunogens

The structures of the immunogens produced by conjugating retinoic acid haptens to various vectors are shown in *Table 2*. The wavelength maxima of the haptens and the molecular ratios of retinoid-to-protein, or to other carriers in the immunogens, are shown in *Table 1*.The highest amount of retinoic acid hapten was

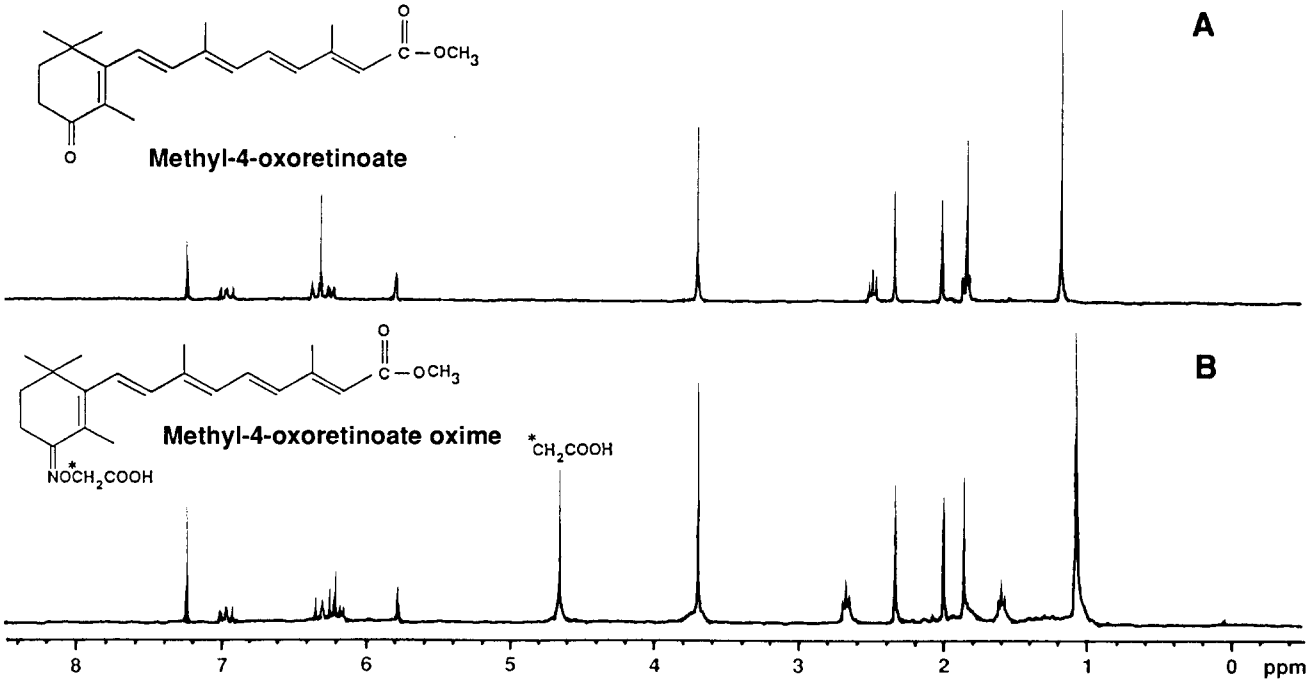


Figure 5 Proton NMR spectrum of (A) methyl-4-oxoretinoate and (B) methyl-4-oxoretinoate oxime. Both compounds were determined at 300 MHz in $CDCl_3$. Asterisk (*) indicates the presence of $-CH_2COO^-$ group.

Table 1 Ratios of retinoids to various antigenic moieties in immunogens

Retinoid conjugate	Wavelength (maximum of hapten, nm[a])	Molar ratios of retinoid/protein
4-Oxoretinoic acid oxime-BSA	365	19.2
4-Oxoretinoic acid oxime-cIgG	365	42.0
4-Oxoretinoic acid oxime-hIgG	365	38.3
All-trans-retinoyl-BSA	346–348	11.6
All-trans-retinoyl-cIgG	346–348	27.6
All-trans-retinoyl-hIgG	346–348	37.8
4-Oxoretinoic acid-phenylhydrazone-BSA	345	10.8
4-Oxoretinoic acid-phenylhydrazone-cIgG	345	32.4
Retinoic acid-MeBSA-liposome complex		200 (0.17 μmol/mg lipid)
4-Oxoretinoic acid oxime-Sepharose 4B		9.3 μmol/mL gel
4-Oxoretinoic acid-phenylhydrazone-Sepharose 4B		6.0 μmol/mL gel

bound to cIgG. Oxime- and phenylhydrazone derivatives of retinoic acid bound to Sepharose 4B in a ratio of 9.3 and 6.0 μmol per mL of Sepharose beads, respectively. Purification of retinoic acid-protein linked immunogens by chromatography on Sephadex G 50 is illustrated in *Figure 6*. The immunogens eluted is a broad peak with 30–55 mL of PBS buffer, 0.02 M, pH 7.4; unreacted methyl-4-oxoretinoate oxime eluted with 80-100 mL of the buffer.

Characterization of demethylated 4-oxoretinoic acid oxime-cIgG conjugate

The demethylation of methyl-4-oxoretinoate oxime-cIgG conjugate was verified by FAB-MS characterization of the retinoid oxime-lysine adducts isolated from the proteolytic digest of the immunogen (*Figure 7*). In the region of FAB-MS spectrum where the lysine adducts of the methylated and demethylated retinoid oxime would be anticipated, the only significant peak observed represents the free (unmethylated) retinoic acid derivative (i.e., the 4-oxoretinoic acid oxime-lysine adduct, m/z 516 for $[M + H]^+$. No peak was observed at m/z 530 where the methylated adduct would be found. An additional peak at m/z 538 was seen in the spectrum for the sodium adduct $[M + Na]^+$ of the retinoic acid oxime-lysine conjugate.

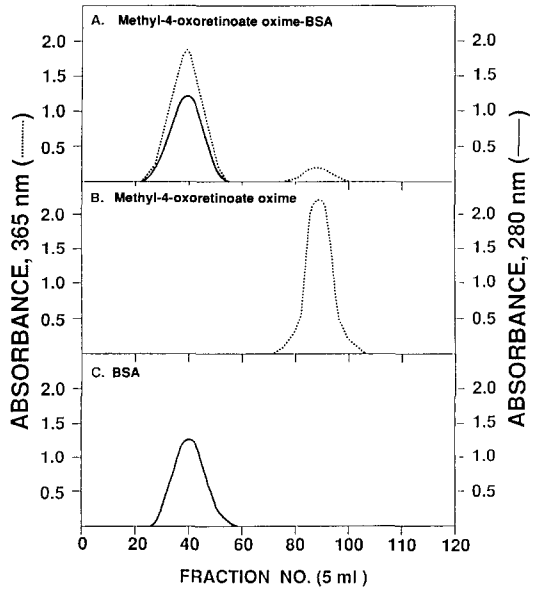


Figure 6 Chromatography of retinoic acid hapten-protein conjugates on Sephadex G 50. (A) methyl-4-oxoretinoate oxime-BSA conjugate; (B) methyl-4-oxoretinoate oxime; (C) BSA.

Production of antibodies against retinoic acid

The immunogenic response of the retinoic acid immunogens in mice and rabbits is shown in *Table 2*. With 4-oxoretinoic acid oxime-cIgG as the immunogen, six out of 10 mice produced anti-retinoic acid antibodies, while no anti-retinoic acid antibodies were generated using 4-oxoretinoic acid phenylhydrazone-cIgG conjugate. When 4-oxoretinoic acid oxime was conjugated to AH-Sepharose 4B beads and implanted into the spleens of mice, 4 out of 10 mice produced anti-retinoic acid antibodies. The immunization of mice with retinoic acid-MeBSA-liposome complex generated anti-retinoic acid antibodies in only one of 10 mice. BALB/c and C57BL/6 mice produced antibodies of similar titers. BALB/c mice were subsequently selected for the production of antibodies because of their suitability for possible monoclonal antibody production.

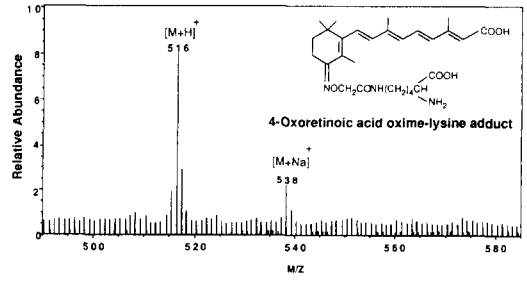
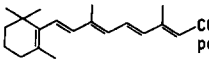
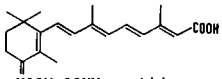
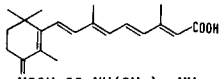
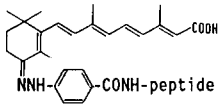


Figure 7 Mass spectrum of 4-oxoretinoic acid oxime-lysine conjugate. FAB-MS spectra were determined with m-nitrobenzyl alcohol as the matrix. Peak m/z 516 for $[M + H]^+$ represents the free retinoic acid oxime-lysine adduct; peak m/z 538 $[M + Na]^+$ represents its sodium adduct.

Table 2 Production of anti-retinoic acid antibodies in mice and rabbits

IMMUNOGEN	No. of animals producing antibodies / No. of animals immunized	Antibody titer by ELISA
 Retinoyl-BSA	2/3 rabbits	6400-25600
 4-oxoretinoic acid-oxime-O-methylene carboxy cIgG	6/10 mice BALB/C or C57BL/6	6400-12800
 4-oxoretinoic acid-oxime-O-methylene carboxy-aminohexyl Sepharose 4B	4/10 mice BALB/C or C57BL/6	1600-3200
 4-oxoretinoic acid phenyl-hydrazone-p-carboxy cIgG	0/10 mice BALB/C or C57BL/6	---
 Retinoic acid-MeBSA-liposome-complex	1/10 mice BALB/C or C57BL/6	800

Retinoyl-BSA conjugate was used to immunize rabbits against retinoic acid; two out of three rabbits responded with the production of anti-retinoid antibodies.

Antibody titers by ELISA

Indirect ELISA titers of retinoic acid antisera from mice immunized with 4-oxoretinoic acid oxime-cIgG ranged from 1:6400 to 1:12800; in contrast, the antibody titers from sera of mice immunized by intrasplenic inoculation of retinoic acid oxime-Sepharose 4B beads were much lower (*Table 2*). The highest antibody titer was obtained in sera from rabbits immunized with retinoyl-BSA.

Discussion

The focus of our work is directed towards the preparation of retinoid immunogens specific for retinoic acid, the most active form of vitamin A in somatic functions. Vitamin A and its metabolites are not naturally antigenic. In order to generate antibodies to retinoids, it is necessary to prepare appropriate haptens for binding to antigenic moieties. We utilized 4-oxoretinoic acid to prepare carboxyl-containing haptens by attaching a spacer to the ring portion of the retinoic acid molecule in carbon-4 position, while simultaneously protecting the carboxyl on the retinoic acid side chain. Subsequently, using the activated NHS ester as coupling agent and conditions optimized for retinoids, we were able to generate retinoic acid immunogens with an exposed carboxyl group on the retinoic acid side chain. Analysis of the conjugates showed the molar ratios of retinoic acid to protein to be in the range of 10.8 to 42.0, cIgG giving the highest and BSA giving the lowest retinoic acid-to-protein ratios with either the oxime or phenylhydrazone as the spacer molecules. The 4-oxoretinoic acid-cIgG conjugate produced a strong immunogenic response. This conjugate promises to be a useful retinoic acid immunogen, since, in preliminary testing the antiserum showed considerable specificity towards retinoic acid: competitive indirect ELISA with polyclonal antibodies resulted in 75% inhibition with all-trans-retinoic acid, 3.3 μmol/mL; with a monoclonal antibody 86% inhibition was obtained with 0.1 μg (0.33 μmol) of retinoic acid/mL; cross reactivity with retinol in the polyclonal antibody was 23%, in the monoclonal antibody, 8%, with respect to retinoic acid (full details to be published separately). The good immunogenic properties of the retinoic acid oxime-cIgG conjugate can be attributed mainly to its solubility and its high retinoic acid-to-protein ratio. Solubility was also a significant factor in the development of ELISA methods in which the retinoic acid oxime-BSA conjugate was used as the solid phase antigen.

Conrad and Wirtz using the mixed anhydride method[7] conjugated retinoic acid directly through its carboxyl group to BSA and produced polyclonal antibodies to vitamin A compounds.[9] However, this method has been reported to generate immunoreactive mixed anhydride-conjugated immunogens that result in mixed anhydride specific cross-reactions.[24] To avoid these problems, we adapted the activated NHS ester method for the conjugation of retinoic acid through its carboxyl group to several proteins, and obtained retinoic acid-to-protein ratios in the range of 11.6 to 37.8, comparable to those obtained with the mixed anhydride method.[7] Although this immunogen produced high titer polyclonal antibodies in rabbits, preliminary testing indicated that the antiserum reacted to the same extent with all vitamin A compounds. These results confirm the work of Wirtz and his associates.

Immunogens linked to solid matrix and implanted into spleen have been advocated for use with small amounts of immunogens[22] and have been reported to be highly immunogenic against BSA.[25] Sepharose has been successfully used for a solid-phase enzyme linked assay for riboflavin.[26] More important for us, however, was the likelihood that upon implantation at

the site of antibody-generating cells, there would be an extended stimulus for antibody production which would result in an enhanced immunogenic response.[25] We coupled methyl-4-oxoretinoate oxime and phenylhydrazone haptens to Sepharose 4B beads and implanted the beads into the spleens of mice. However, this method did not result in a greater immunogenic response compared to conventional immunization methods. It is possible that the retinoic acid moiety of the complex is subject to non-specific enzymatic degradation by the spleen cells and, thus, over a period of time the immunogen was altered and lost specificity.

An obstacle to the preparation and delivery of retinoic acid immunogens to cells is the hydrophobic character of these molecules. Liposomes are known to be useful carriers of lipid soluble substances for delivery of antigens.[18,27-30] We incorporated retinoic acid into liposomes sensitized with antigenic moieties such as methylated BSA and rabbit IgG and verified the incorporation of retinoic acid into liposomes by ultracentrifugation of liposomes and the removal of free retinoic acid. It has been suggested that acidic substances such as retinoic acid form ionic bonds with methylated BSA, and that the immunogenicity of this complex is further enhanced by lecithin in the liposome carrier.[28] We anticipated that the carrier would solubilize retinoic acid and also provide a near-physiological membrane vector for the interaction of the antigen with the surface of immune cells. However, the retinoic acid-MeBSA-liposome complex was a poor immunogen. It is possible that the carboxyl group of retinoic acid became tightly bound to the carrier protein or was not exposed and was not available as an immunogenic determinant, or that upon disintegration of the liposome complex the released retinoic acid was rapidly metabolized and eliminated.

We anticipated problems in the enzymatic removal of the methyl blocking group from the retinoid moiety of the immunogen. However, as verified by mass spectrometry, a complete deesterification was achieved with prolonged incubation times in the presence of sufficient amounts of enzyme.

The novel feature of our work is the synthesis of retinoid immunogens with the carboxylic group on the retinoid polyene side chain exposed, so that they can give a very specific antigenic response to retinoic acid. We have demonstrated that high titer anti-retinoic acid sera can be produced by the protein linked immunogens reported in this paper. Our studies suggest that the combined features of exposed carboxylic group on the polyene side chain, oxime as the spacer molecule and chicken IgG as the protein vector, can produce retinoid immunogens with a potential to generate antibodies with a high specificity towards retinoic acid. The methods described in our paper will open the field for the preparation of retinoid-specific antibodies to synthetic therapeutic and natural retinoids with different functional groups on the polyene side chain. The methods will also be useful for the preparation of antibodies against other lipid soluble vitamins.

Nomenclature

RA	retinoic acid
HPLC	high-pressure liquid chromatography
NMR	nuclear magnetic resonance
FAB-MS	positive ion fast atom bombardment mass spectrometry
ELISA	enzyme-linked immunosorbent assay
BSA	bovine serum albumin
MeBSA	methylated BSA
cIgG	chicken immunoglobulin G
hIgG	human immunoglobulin G
PBS	phosphate-buffered saline
NHS	N-hydroxysuccinimide ester
DCC	dicyclohexylcarbo-diimide
DMSO	dimethylsulfoxide

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