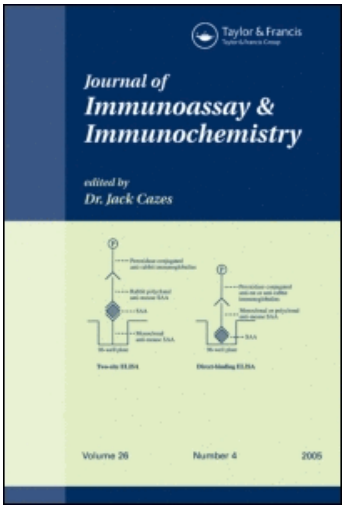

Journal of Immunoassay and Immunochemistry

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

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Jos Raats[a]; Nicole van Bree[b]; Jochem van Woezik[b]; Ger Pruijn[a]
[a] Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands [b] Future Diagnostics B.V., Wijchen, The Netherlands

Online publication date: 05 December 2003

To cite this Article Raats, Jos , van Bree, Nicole , van Woezik, Jochem and Pruijn, Ger(2003) 'Generating Recombinant Anti-idiotypic Antibodies for the Detection of Haptens in Solution', Journal of Immunoassay and Immunochemistry, 24: 2, 115 — 146
To link to this Article: DOI: 10.1081/IAS-120020081
URL: http://dx.doi.org/10.1081/IAS-120020081

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JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 24, No. 2, pp. 115–146, 2003

Generating Recombinant Anti-idiotypic Antibodies for the Detection of Haptens in Solution

Jos Raats,[1,*] Nicole van Bree,[2] Jochem van Woezik,[2] and Ger Pruijn[1]

[1]Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands
[2]Future Diagnostics B.V., Wijchen, The Netherlands

ABSTRACT

A new method is described for generating recombinant human and chicken antibody fragments for accurate quantification of haptens in solution. The chemistry of labelling small molecules has always been a problem in the development of immunoassays. Here, we describe a specific panning procedure that enables the selection of recombinant anti-idiotypic phage antibodies that bind to hapten binding

*Correspondence: Jos Raats, Department of Biochemistry, University of Nijmegen, P.O. Box 9101, NL-6500 HB, Nijmegen, The Netherlands; E-mail: j.raats@ncmls.kun.nl.

DOI: 10.1081/IAS-120020081 1532-1819 (Print); 1532-4230 (Online)

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molecules (e.g., antibodies) in the absence of the hapten, but are displaced in a highly specific and concentration dependent manner, in the presence of the hapten. The major advantage of such a detection system is that there is no need to label the hapten or to covalently attach it to a solid phase. In this study we demonstrate, using cortisol and aldosterone as model haptens, that the recombinant antibody phage display technology offers great possibilities to generate recombinant anti-idiotypic antibodies. Furthermore, we show that such antibodies can be used successfully to design highly sensitive immunoassays for the quantification of small molecules.

Key Words: Phage display; Haptens; Cortisol; Aldosterone; Competition assay.

INTRODUCTION

Cortisol is the major glucocorticoid produced in the adrenal cortex under control of the hypothalamic-pituitary-adrenal axis. Cortisol is bound to proteins, mainly to cortisol binding globulin (CBG) with some binding to albumin. Cortisol affects the metabolism of protein, fat, and carbohydrates, the maintenance of muscle and myocardial integrity, and the suppression of inflammatory and allergic activities. Abnormal changes in cortisol levels occur due to hypothalamic, pituitary, or adrenal malfunction. If undiagnosed and untreated, disorders such as Cushing's Syndrome (hypercortisolemia) and Addison's disease (primary adrenal insufficiency) can lead to severe metabolic imbalance and can be life threatening.[1–3]

The interaction of aldosterone, renin, and angiotensin is important in the regulation of extracellular fluid volume, blood pressure, and the balance of sodium and potassium. A change in one of these variables leads to changes in the others. Both increased or decreased production of aldosterone by the adrenal glands may occur. The result of increased production of aldosterone is retention of water and sodium, an increase in extracellular volume, and a decrease in serum potassium. Decreased production of aldosterone may be seen due to enzyme blocks in aldosterone synthesis, as in congenital adrenal hyperplasia, destructive lesions of both adrenal glands, as in primary adrenal insufficiency with mineralocorticoid deficiency associated with glucocorticoid deficiency, or deficient production of renin by the kidney leading to secondary hypoaldosteronism.[4–6]

It is obvious that accurate determination of the amount of haptens such as cortisol and aldosterone, present in the body fluids

of individuals provides vital information for clinical and nutritional diagnosis.

To enable the design of diagnostic assays that can accurately determine the amount of haptens in solution, we devised a specific panning procedure that enables the selection of recombinant anti-idiotypic phage antibodies that bind to hapten-binding molecules (e.g., antibodies) in the absence of the hapten, but, in the presence of the hapten, are displaced in a highly specific and concentration dependent manner. In this study we demonstrate that the recombinant antibody phage display technology offers great possibilities to generate recombinant antibodies with such characteristics.

The chemistry of labelling small molecules has always been a problem in the development of immunoassays. The major advantage of the assay system that we designed, using the recombinant antibody technology described in this article, is that there is no need to label the hapten or attach it to a solid phase. We demonstrate here that the selected antibodies can be used successfully in highly sensitive immunoassays to quantify small molecules in solution.

EXPERIMENTAL

Bacterial Strain

The *E. coli* strain TG1 was used as the bacterial host for the preparation of phagemids, as host for the bacteriophage M13, and for the production of soluble monomeric and dimeric scFv fragments.

Antibodies and Haptens Used in This Study

Antibodies

1. Anti-cortisol-mab (Fitzgerald Industries International Inc., Concord, MA, USA, art#10-C30 lot#493, clone M94144).
2. Anti-aldosterone-mab antibodies (kindly provided by De Lauzon, Université R. Descartes, Paris, France),
3. Anti-testosterone-mab1 (Fitzgerald Industries International Inc., testosterone-3 CMO BSA, clone: M211244),
4. Anti-testosterone-mab2 (BIODESIGN International, Saco, Maine, USA, testosterone-3-CMO-BSA, clone: 7004),

5. Anti-insulin-mab (DAKO Denmark A/S, Glostrup, Denmark, clone HUI-018).

Steroids

1. Cortisol (Steraloids Inc., Newport, Rhode Island, USA, Art.# Q3880 lot# G725), stock solution 1 mg/mL in dimethylformamide (DMF).
2. Aldosterone (Steraloids Inc., Art# Q2000, batch H158), stock solution 0.1 mg/mL in ethanol.
3. Testosterone (SIGMA-ALDRICH CHEMIE Gmbh, Steinheim, Germany, Art# T-1500 lot# 106H0323), stock solution 0.1 mg/mL in ethanol.

Introduction of VSV, His, and Fos Tags in scFv Expression Vectors

Using oligo VSV-NotI and oligo pHENBACK, the 11 amino acid vesicular stomatitis virus derived VSV-G tag (YTDIEMNRLGK)[7,8] sequence was amplified by PCR. This fragment was digested with *Not*I and *Bam*HI and ligated in a *Not*I and *Bam*HI digested pHENIX vector,[9] resulting in pHENIXVSV.

For introduction of VSV- and His-tags in the pUC119MycHis6Sfi/Not vector,[10] we used a PCR amplification with oligos LMB3 and VSVHis-EcoRI together with the plasmid pHENIXVSV. The resulting PCR fragment was digested with *Not*I and *Eco*RI restriction enzymes and ligated in pUC119MycHis6Sfi/Not digested with *Not*I and *Eco*RI. This resulted in the vector pUC119VSVHis6Sfi/Not.

The Fos dimerization domain was introduced as a *Not*I fragment in a scFv fragment containing pUC119VSVHis6Sfi/Not vector as described previously.[11] Subsequently, the Fos fragment was amplified by PCR using oligos LMB3 and Fos and a pUC119FosVSVHis6Sfi/Not vector containing the Fos insert. In a parallel PCR reaction a VSVHis6 fragment was amplified from the same vector using oligos VSVBglII and M13Forward. In a subsequent overlap PCR reaction, products from the first PCR reactions (Fos, and VSVHis6) were amplified together with oligos LMB3 and M13Forward, digested with *Not*I and *Eco*RI and ligated in a *Not*I and *Eco*RI digested pUC119MycHis6Sfi/Not vector. The resulting vector is indicated as pUFosVH6. All oligos used in this study are listed in Table 1.

Phage Display Libraries

Synthetic Phage Display Library

For initial testing of our screening and selection procedures, a synthetic scFv library made by Nissim[12] was used.

Immunization of Chicken and Generation of Chicken Antibody Phage Display Libraries

Four Red/Black Cornish Cross chickens were immunized with anti-cortisol-mab (2 animals) and anti-aldosterone-mab (2 animals), using 200 μg antigen in PBS, pH 7.4, emulsified in Freunds Complete Adjuvant (CFA, Difco Laboratories, Detroit, MI, USA) per chicken. Three subsequent boosts were given at two-week intervals using 100 μg antigen in Incomplete Freunds Adjuvant (IFA) per chicken. After each boost, the immune response was analyzed by ELISA. After 6 weeks, the chickens were euthanized and bone marrow, spleen, and peripheral blood were collected.

Construction of Recombinant Chicken Antibody Libraries

mRNA extraction (using B-lymphocytes from bone marrow, spleen, and peripheral blood), cDNA synthesis, polymerase chain reaction (PCR) amplification of the variable antibody regions, and subsequent assembly of the single chain variable fragments by overlap PCR was performed as described previously.[13] cDNAs generated from chickens immunized with the same antigen were pooled and amplified by PCR. The scFv fragments were digested with Sfi-I and cloned into the display vector pComb3H.[14–17] Ligations were transformed into electro-competent TG1 cells. Libraries were grown o/n on large $2 \times$ TY agar plates, harvested and frozen as dense glycerol stocks at -80°C. From each immunization two separate libraries were generated. One containing a short linker sequence GQSSRSS between the Vl and Vh regions for diabody formation, and one containing a long linker sequence GQSSRSSGGGGSSGGGGS between the Vl and Vh regions to favor monomeric scFv. Short and long linker libraries were pooled for selections. All oligos used in this article are listed in Table 1.

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Table 1. Oligonucleotides used for PCR amplifications.

VSVHis-EcoRI	5′CGGAATTCTTATTAATGGTGATGATGGTGCGGCCCCTTTCCTCTATGTCTG3′
VSV-NotI	5′AAGGAAGAATGCGGCCGCCATATACAGACATAGAGATGAACCGGCCGCATAGACTGT-CCGCATAGACTGTTGAAAGTTGTTTA′3
VSV-BglII	5′GGTGGTTGCAGATCTTATACAGACATAGAGATGAAC′3
Fos	5′ATAAGATCTGCAACCACCGTGTGC′3
FdSeq1	5′GAATTTTCTGTATGAGG3′
pHENBACK	5′GTTGAGGCAGGTCAGACGATT3′
LMB3	5′TCACACAGGAAACAGCTATGAC3′
M13forward	5′CGCCAGGGTTTTCCCAGTCACGAC3′
CSCVK sense	5′GTGGCCCAGGCGGCCCTGACTCAGCCGTCCTCGGTGTC3′
CKJ0-B antisense	5′GGAAGATCTAGAGGACTGACCTAGGACGGTCAGG3′
CSVH0-F sense	5′GGTCAGTCCTCTAGATCTTCCGCCGTGACGTTGGACGAG3′
CSCVHo-FL sense	5′GGTCAGTCCTCTAGATCTTCCGGCGGTGGTGGCAGCTCCGGTGGCTGACGTTGGACG-CTGACGTTGGACGAG3′
CSCG-B antisense	5′CTGGCCGGCCTGGCCACTAGTGGAGGAGACGATGACTTCGGTCC3′
CSC-F sense	5′GAGGAGGAGGAGGAGGTGGCCCAGGCGGCCCTGACTCAG3′
CSC-B antisense	5′GAGGAGGAGGAGGAGGAGCTGGCCGGCCTGGCCACTAGTGGAGG3′
PCRCs	5′GGCCCAGGCGGCCCTGACTCAG3′
PCRCa	5′CTGGCCGGCCTGGCCACTAGTG3′

Guided Selection of Recombinant Anti-idiotypic Antibodies

To select recombinant antibodies that can compete for binding of the hapten to the target antigen, several selection methods were assessed. Test selections were performed using the anti-cortisol-mab as a target antigen for selections with the synthetic "Nissim"-library.[12]

Test-Selection Protocols

Method A, No Pre-absorption

Nunc Maxisorb tubes (4 mL) (NUNC A/S, Kamstrup, Roskilde, Denmark) were coated with 1 μg/mL antigen in 1 mL phosphate-buffered saline (PBS), pH 7.4, o/n at 4°C. Tubes were washed 5 times with PBS pH 7.4, and blocked with 4 mL 4% Marvel (non fat dry milk), PBS pH 7.4, and 0.05% Tween-20 (MPBST) for 2 h at room temperature (RT). Blocking solution was removed and 2 mL phage stock ($>10^{12}$ phages) in 4% Marvel (non fat dry milk), PBS pH 7.4 (MPBS) was added and rotated for 30 min at RT. After an additional 90 min incubation without rotation at RT, phage solution was removed and 4 mL of washing solution PBS pH 7.4, 0.05% Tween-20 (PBST) was added. Each wash was incubated for 5 min and mixed vigorously. Washing was performed 5 times in the first, 10 times in the second, and 20 times in the third and subsequent rounds of selection. After washing, phages were eluted by addition of 1 mL 200 ng/mL cortisol in PBS and rotation for 30 min at RT. The eluted phages were added to a fresh TG1 bacterial culture for infection, phage amplification and titration.

Method B, Pre-absorption Against Control IgG1

Selection was performed essentially as described for method A. However, before the actual selection a pre-absorption was performed using a non-related mouse antibody with the same isotype (IgG1) as the anti-cortisol-mab used for the actual selection. Two milliliters of the pre-absorption antibody was coated at 1 μg/mL in PBS, pH 7.4, o/n at 4°C in Nunc Maxisorb tubes. Tubes were blocked using 4% MPBST. Pre-absorption was performed using 2 mL of phage stock in 2 mL 4% MPBS containing 1 μg/mL soluble IgG1. After 2 h incubation at RT the phage solution was transferred to the selection tube coated with anti-cortisol-mab and the selection was performed as outlined in method A.

Method C, Pre-absorption Against Hapten Specific Antibody

Selection was performed essentially as described in Sec. b. Instead of using an unrelated IgG of identical isotype, the anti-cortisol-mab itself, saturated with cortisol, was used in the pre-absorption step. 1 mL 1 μg/mL anti-cortisol-mab was coated in PBS, pH 7.4 o/n at 4°C. Subsequently, 3 washes with PBS were performed and the tubes were blocked using 4% MPBST for 2 h. After three washes with PBS, 1 μg/mL cortisol in PBS was added. After 30 min incubation at RT, tubes were washed 10 times with PBST and 10 times with PBS. Subsequently, 2 mL of phage stock in 2 mL of MPBS was added and incubated for 2 h at RT. After this pre-absorption step, the phage solution was dialyzed o/n against PBS pH 7.4 to remove any traces of cortisol, and subjected to the selection protocol described in method A.

Selection protocols described in methods A through C were performed using either VCS M13 helper phage (A and B) or a trypsin-sensitive M13K07 helper phage (A, B, and C).[18]

Selection Protocol Used for Chicken Libraries

Selections using the chicken libraries were performed according to the protocol described in method C using the wildtype M13VCS helper phage, except that the selections were carried out in microtiter wells (Nunc 96 well Maxisorb plates). For each selection 2 to 4 wells (300 μL per well) were used. Pre-absorptions were carried out in 4 mL Nunc Maxisorb tubes.

Growth of Phage Libraries

After each selection round, eluted phages were added to a fresh TG1 bacterial culture (2 mL bacterial culture for 200 μL eluted phages) for infection, phage amplification, and titration. Cells were incubated at RT in 2 × TY medium containing 2% glucose (2 × TYG) for 15 min, after which the culture volume was increased to 10 mL with pre-warmed 2 × TYG. The output phage titre was determined by plating 0.1, 1, and 10 μL on 2 × TYG ampicillin plates. The cultures were then supplemented with 20 μg/mL ampicillin and incubated for 1 h in a 37°C shaker. The ampicillin concentration was increased to 100 μg/mL ampicillin and cultures were incubated for one additional hour at 37°C in a shaker. Fifty microliters of helper phage ($>10^{12}$ PFU/mL) was added (M13KO7 or M13VCS), and cultures were incubated another 30 min at 37°C. Medium was exchanged (by spinning at $3000 \times g$

for 10 min at RT) by 100 mL 2 × TY containing 100 μg/mL ampicillin. After 90 min incubation in a shaker at 37°C, 70 μg/mL kanamycin was added and cultures were grown o/n at 30°C in a shaker. The following day, the cultures were centrifuged at 5000 × *g* for 15 min to pellet the bacteria. The supernatants were transferred to clean centrifuge tubes containing 20 mL of 20% polyethylene glycol (PEG) −2.5 M NaCl (0.2 × volume) and incubated on ice for 30 min. The phages were pelleted by centrifugation at 5000 × *g* for 40 min. The supernatants were discarded and the phages were resuspended in 1% bovine serum albumin (BSA) in PBS supplemented with 0.1% NaN_3. The phages were filtered through 0.45 μm syringe filters (Schleicher and Schuell, Dassel, Germany), and stored at 4°C.

Screening Phage Pools and Individual Phage Clones

Phage pools from each round of panning, as well as individual phage clones obtained from these pools, were analyzed by specificity and competition ELISA. Individual colonies were picked from the titration plates representing the phage pools that were positive in ELISA analysis. Clones were grown in 96 well plates, PCR amplified using oligos PCRCs and PCRCa for the chicken derived clones and oligos LMB3 and FdSeq1 (Table 1) for the synthetic library derived clones and analyzed by Bst-NI restriction digestion (fingerprint). Clones with distinct fingerprints were infected with helper phage and analyzed for binding to their target antigens and in a competition ELISA simultaneously (Fig. 2a). Only those clones that showed good binding to the target mabs as well as good competition, with cortisol or aldosterone for binding to the target anti-cortisol and anti-aldosterone mabs, were analyzed further in a specificity ELISA (Fig. 2b).

Competition ELISA

One or four micrograms of antigen/well anti-cortisol-mab and anti-aldosterone mab, respectively, was coated to 96-well Maxisorb ELISA plates (NUNC) in PBS, pH 7.4, o/n at 4°C. The following day, the plates were blocked in 4% MPBST for 2 h at RT. After blocking, plates were washed 3 times in PBS and subsequently 50 μL of the appropriate antibodies (phage pools, phage clones, scFv or dimeric scFvs were added together with 10 μL aldosterone, cortisol or testosterone (1 pg–1 μg/mL)

in 50 μL 4% MPBST and incubated for 1.5 h at RT. In the human serum containing competition assays, the 50 μL 4% MPBST was substituted by 50 μL human serum and the coating concentration was increased to 10 μg/well to render the assay less sensitive. Plates were washed 4 times with PBST and 4 times with PBS and 50 μL horse radish peroxidase (HRP) conjugated sheep anti-M13 (1:5000), or 50 μL HRP conjugated anti-VSV-G (P5D4, anti 11 amino acid vesicular stomatitis virus derived YTDIEMNRLGK tag[7]) mab (1:1000) was added to each well. The plates were incubated for 1 h at RT and washed 4 times with PBST and 4 times with PBS and developed with 3,3′,5,5′-tetramethylbenzidine (SIGMA) and H_2O_2. Reactions were stopped by the addition of 2 M H_2SO_4 and the absorbance at 450 nm was measured.

Specificity ELISA

One microgram antigen/well anti-cortisol mabs, anti-aldosterone mabs, or control antibodies (anti-testosterone mab1, anti-testosterone mab2, anti-insulin mab), were coated to 96-well Maxisorb ELISA plates (NUNC) in PBS, pH 7.4, o/n at 4°C. The following day, the plates were blocked in 4% MPBST for 2 h at RT. After blocking, plates were washed 3 times in PBS and, subsequently, 50 μL of the appropriate phage pools from each round of panning were applied in 50 μL 4% MPBST and incubated for 1.5 h at RT. Plates were washed 4 times with PBST and 4 times with PBS and 100 μL sheep anti-M13 horse radish peroxidase (HRP) conjugated antibody (1:5000) (Amersham Pharmacia Biotech Inc., Little Chalfont, Buckinghamshire, England) was added to each well. The plates were incubated for 1 h at RT and washed 4 times with PBST and 4 times with PBS and developed with 3,3′,5,5′-tetramethylbenzidine (SIGMA) and H_2O_2. Reactions were stopped by the addition of 2 M H_2SO_4 and the absorbance at 450 nm was measured.

Expression of Mono-, and Dimeric Soluble Antibody Fragments (scFv and $scFv_2$)

Bacterial cultures, containing recombinant antibody clones in pUC119VSVHis6Sfi/Not or pUFosVH6, were induced to express soluble recombinant antibodies using 1 mM IPTG in 2 × TY medium containing 100 μg/mL ampicillin o/n at 30°C. Soluble antibodies were retrieved from the culture supernatant or could be extracted from the periplasm,

essentially as described previously.[19] All assays were performed using crude periplasmic extracts.

RESULTS

Selection of Antibodies from Nissim Library

Test of Various Selection Protocols

Phage preparations from the Nissim library were used to test the various selection methods described in detail in the Experimental section. We tested three basic selection protocols. Method A, in which the anti-cortisol-mab was coated and selection was performed without any pre-absorption; method B, in which a pre-absorption step was performed using a mouse monoclonal antibody of the same isotype as the anti-cortisol-mab to avoid binders to regions of the antibody that are not involved in binding to the hapten; method C in which pre-absorption was performed using the anti-cortisol-mab saturated with cortisol. The subsequent selection was performed with the same anti-cortisol-antibody without cortisol. The aim of the pre-absorption methods is to remove all binders from the library that are not directed against the antigen binding site of the anti-cortisol-mab (methods B and C) or that are directed against regions of the anti-cortisol-mab that undergo conformational changes upon binding of cortisol (method C). Conformational changes of antibodies induced by antigen-binding have been reported previously (e.g., Ref.[20]). During all selection procedures, recombinant anti anti-cortisol-mab phage antibodies were eluted from the anti-cortisol-mab by competition with cortisol.

Wild Type vs. Trypsin-Sensitive Helper Phage

As an alternative to the wild type M13VCS helper phage (selection methods A and B only), we tested whether the use of a trypsin-sensitive helper phage[18] would benefit these selections. The trypsin-sensitive helper phage has been designed to remove phage particles that do not display a gene-3-antibody fusion protein from the phage pool. The trypsin-sensitive gene-3 protein (derived from the helper phage) will be cleaved by trypsin at the built-in trypsin site rendering phages containing

only trypsin-sensitive gene-3-protein incapable of infecting *E. coli*. The gene-3 protein derived from the phagemid containing the gene-3-antibody sequence does not contain a trypsin cleavage site. Therefore, phages displaying one or more copies of this gene-3-fusion product remain infective.

Because phage selection from the Nissim library using methods A and B in combination with wild type M13VCS helper phage did not result in any significant enrichment of phages reactive with the anti-cortisol-mab as monitored by ELISA (not shown), method C was only applied with the trypsin-sensitive helper phage. The results in Table 2 show that a clear phage enrichment was observed for selection method C (100 fold in the third round). In selection method A trypsin treatment resulted in case of the trypsin-sensitive helper phage in a reduction of the phage background (from 10^4 particles in the first, 10^2 particles in the second and 10 particles in the third round) compared to the non-trypsin treated samples (Table 2 compare method A M13K07 before trypsin and M13K07 after trypsin). Phage background titres were determined by performing simultaneous phage selections on immunotubes that were blocked with MPBST only (no antigen coated).

Selection Results

The ligand competition ELISA (Fig. 1a) revealed that selection methods B and C resulted in polyclonal phage pools, the reactivity of which was reduced in the presence of cortisol (200 ng/mL cortisol; 24 and 27% competiton for phages from methods B and C, respectively), whereas no competition was observed for the phage pool derived from method A. The polyclonal phage ELISA (Fig. 1a) demonstrated that the highest ELISA signals were obtained with the phages from selection method C (compare rounds 3). These analyses suggested that the superior selection strategy for the selection of anti-idiotypic recombinant anti anti-cortisol-mab phage antibodies is selection method C in combination with trypsin-sensitive helper phages. Therefore, only individual clones derived from round 3 of this selection procedure were analyzed in more detail. Of 96 individual clones tested 66 displayed binding to the anti-cortisol antibody, and the binding efficiency of 62 of these was (strongly) reduced in the presence of cortisol. The latter clones could be subdivided into 8 fingerprint groups. Phage clones CS (reactive with anti-Cortisol-mab; from Synthetic library)-A8, CS-C7, CS-C10, CS-E8, CS-G6, CS-G12, CS-H4, and CS-H9 representing the various fingerprint groups were chosen for further analysis (Fig. 2a).

Table 2. Phage titres and enrichment factors obtained with different selection methods.

		M13K07 before trypsin			M13K07 after trypsin		
		Method A	Method B	Method C	Method A	Method B	Method C
Round 1	Background (BG)	1,00E + 09	nd	nd	3,00E + 05	nd	nd
Round 2	Background (BG)	3,00E + 09	3,00E + 09	5,00E + 04	4,00E + 07	1,00E + 06	1,00E + 04
Round 3	Background (BG)	9,00E + 06	5,00E + 05	5,00E + 05	2,00E + 05	2,00E + 05	2,00E + 04
Round 1	Phage titre	3,00E + 08	nd	nd	4,00E + 04	nd	nd
Round 2	Phage titre	3,00E + 09	5,00E + 09	2,00E + 05	3,00E + 07	8,00E + 06	5,00E + 04
Round 3	Phage titre	4,00E + 06	6,00E + 06	7,00E + 06	3,00E + 05	4,00E + 05	2,00E + 06
Round 1	Enrichment factor (phage titre/BG)	0	nd	nd	0	nd	nd
Round 2	Enrichment factor (phage titre/BG)	1	2	4	1	8	5
Round 3	Enrichment factor (phage titre/BG)	0	12	14	2	2	100

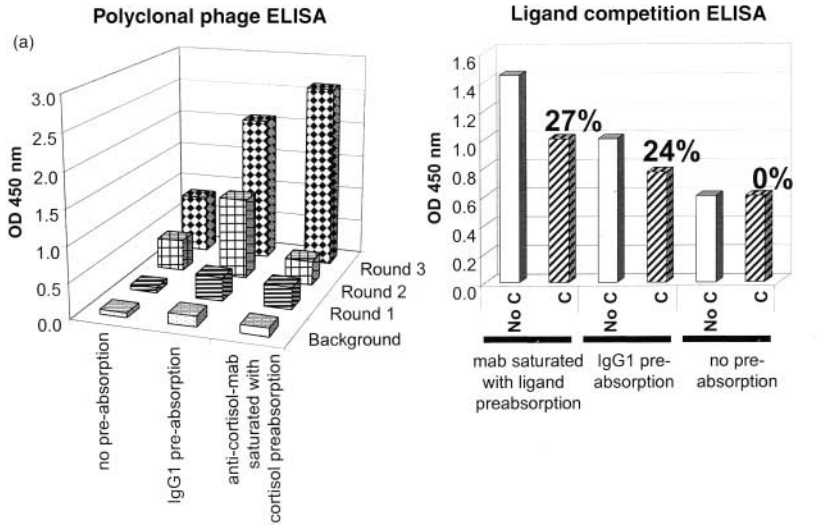


Figure 1. Selection of recombinant antibody phage clones with anti-cortisol-mab and with anti-aldosterone-mab. (a) Synthetic library selections: *Polyclonal phage ELISA* of phage pools resulting from selections (3 rounds) with the various selection methods tested (no pre-absorption (method A), IgG1 pre-absorption (method B), anti-cortisol-mab saturated with cortisol pre-absorption (method C)). Trypsin-sensitive helper phage (M13K07 + trypsin) was used during these selections. The anti-cortisol-mab was used as antigen in the ELISA (coating concentration 1 μg/well). *Ligand competition ELISA* of phage pools resulting from the third selection rounds (anti-cortisol-mab; synthetic library). The coating concentration of the anti-cortisol-mab was 1 μg/well and as competitor 200 ng/mL cortisol was used. Competition for phage binding was observed with the phage pools of selection methods B and C (compare lanes No C, no competitor, with lanes C, competitor added). The levels of competition (%) are indicated in the graph; (b) Chicken library selections: *Polyclonal phage ELISA* of phage pools resulting from selections (3 rounds) with selection method C. Wild type M13VCS helper phage was used during these selections. Either the anti-cortisol-mab or the anti-aldosterone-mab was used as antigen in the ELISAs (coating concentration 1 μg/well). *Ligand competition ELISA* in phage pools from the third selection round of selection methods. The coating concentration the anti-cortisol-mab and the anti-aldosterone-mab was 1 μg/well, and as competitor 200 ng/mL cortisol or aldosterone was used. Competition for phage binding was observed with both phage pools (compare lanes No C, no competitor, with lanes C, competitor added). The levels of competition (%) are indicated in the graph.

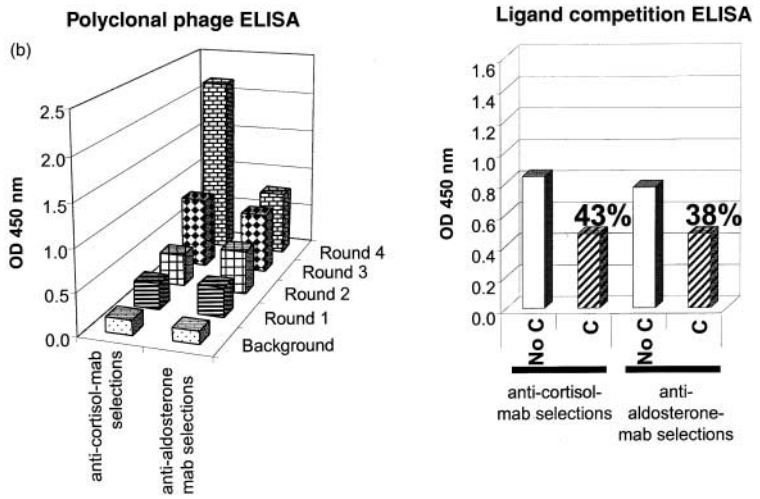


Figure 1. Continued.

Chicken Libraries

Chicken Library Construction

After optimizing the selection procedure for the synthetic library, we wanted to investigate whether this procedure also works for other antibody libraries and for other haptens. In addition, we wanted to compare recombinant phage antibodies derived from synthetic libraries (expected low to moderate affinity) with those from immunized animal derived libraries (expected moderate to high affinity). Therefore, we generated phage libraries from chickens immunized with either the anti-cortisol-mab or an anti-aldosterone-mab. Sera from all immunized animals showed good reactivities with the respective antigens in ELISA (not shown). Libraries were constructed as described in the materials and methods section and the complexity of these libraries was analyzed by titration after transformation in TG1 cells. The total complexity of the libraries (mix of long and short linker libraries) was 2.9×10^9 and 1.9×10^9 for the anti-cortisol-mab and the anti-aldosterone-mab library, respectively. The use of short linker scFvs is expected to promote diabody formation on the phage, thereby enhancing the avidity, whereas long linker scFvs favor the monomeric format (for detailed overview see Ref.[14]).

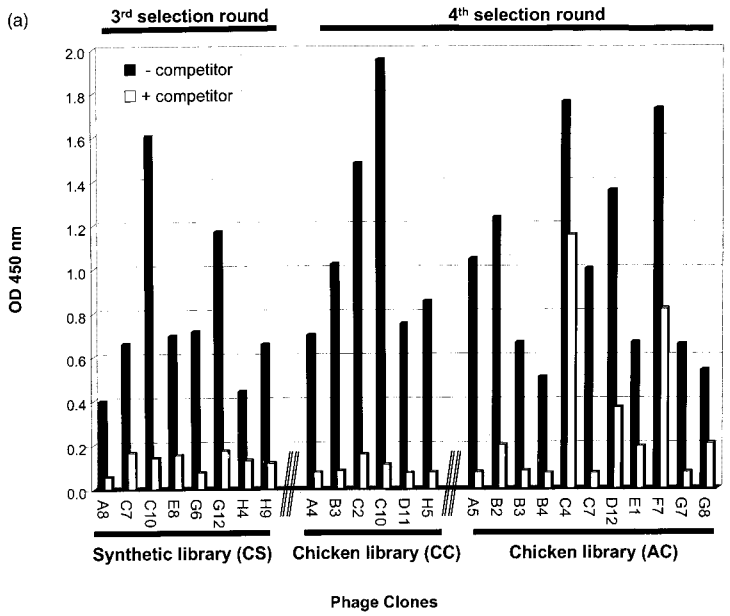


Figure 2. Specificity of individual phage clones. (a) *Ligand competition ELISA* with individual phage antibody clones representing the various fingerprint groups which were isolated from the synthetic library (third selection round; selection method C; trypsin-sensitive M13K07 helper phage plus trypsin treatment) or from the chicken libraries (fourth selection round; selection method C; wild type helper phage M13VCS). Competition was performed with 200 ng/mL of the corresponding hapten competitor. Note that the binding of some of the phage clones reactive with the anti-aldosterone-mab was only partially impeded in the presence of competitor. These clones might interact with regions of the antigen that are partly overlapping the hapten binding site, or they might have a relatively high affinity which would require higher concentrations of aldosterone to achieve a higher degree of competition. Only clones that displayed efficient binding to the target mabs as well as strong competition by hapten were used for further analyzes. Distinct ELISA experiments are separated by double lines (//); (b) Reactivity of individual phage antibody clones with structurally related antigens: anti-cortisol-mab, anti-testosterone-mab 1, anti-testosterone-mab 2, mouse IgG1, anti-insulin-mab, anti-aldosterone-mab (coating concentrations 1 μg/well). All clones analyzed displayed strong competition by the respective hapten (see (a)). Note that only phage clone AC-B4 (weakly) cross-reacts with one of the other mabs, the anti-cortisol-mab. Distinct ELISA experiments with clones derived from the synthetic and chicken libraries are separated by double lines (//). CS-H4 (marked with *) results have been obtained in a separate ELISA experiment compared to the other synthetic library derived clones.

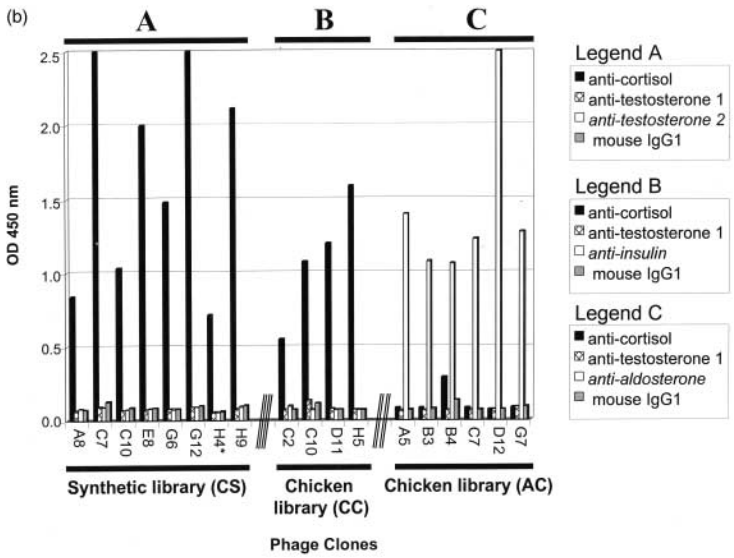


Figure 2. Continued.

Chicken Library Selections and Selection Results

The pComb3H vector (used to generate chicken-derived phage libraries) doesn't contain the full-length gene-3 sequence (which is necessary for phage infection) and, therefore, in this case, the use of the trypsin-sensitive helper phage was not possible. We used only M13VCS helper phage (expressing wild type gene-3 protein) for selection of chicken-derived phages.

The chicken scFv libraries were used for phage selections with respective target antigens according to selection method C. The reactivities of the polyclonal phage pools obtained from these libraries as monitored by ELISAs with the respective antigens are shown in Fig. 1b, *Polyclonal phage ELISA*. To analyze whether the ligands (200 ng/mL) could compete for the binding of these polyclonal phage pools, phages from all third selection rounds were tested in a competition ELISA. Competition levels of up to 43% were observed for the various phage preparations (Fig. 1b, *Ligand competition ELISA*). These results indicated that from the 3rd round onwards positive clones were selected, but the characterization of 33 individual clones from the 3rd selection round did not result in any positive clones. However, selections

with the anti-cortisol-mab resulted after the fourth round in 87 clones (out of 192 tested) binding to the anti-cortisol-mab; cortisol competed for the binding of 86 of these. These clones could be subdivided into 6 fingerprint groups.

Furthermore, selections with the anti-aldosterone-mab resulted after the fourth round in 23 clones (out of 25 tested) recognizing the target antigen; aldosterone competed for the binding of 19 of these clones, which could be subdivided into 11 different fingerprint groups.

Finally, phage clones CC (reactive with anti-Cortisol-mab, Chicken library)-C2, CC-C10, CC-D11, and CC-H5, representing various fingerprint groups from the anti-cortisol-mab chicken libraries, and AC (reactive with anti-Aldosterone-mab, Chicken library)-A5, AC-B3, AC-B4, AC-C7, AC-D12, and AC-G7 from the anti-aldosterone-mab chicken libraries were randomly chosen for further analysis (for an overview of competition results see Fig. 2a).

Specificity of Recombinant Phage Clones

To analyze the specificity of the selected phage clones, ELISA plates were coated with a panel of non-related, though structurally similar, antigens (mouse mab IgG1; anti-testosterone-mab 1, anti-testosterone-mab 2; anti-cortisol-mab; anti-aldosterone-mab, anti-insulin-mab). The reactivity of a representative clone from several fingerprint groups with these antigens was analyzed by ELISA. The results in Fig. 2b show that all selected clones displayed a high reactivity with the respective target antigen, whereas almost all clones did not detectably react with the other antigens. Only phage clone AC-B4, which was isolated by virtue of its binding to the anti-aldosterone-mab, showed some reactivity with one of the other mabs, the anti-cortisol-mab. These results indicated that in general the selected phage clones display a high specificity for their target antigen.

Competition Assays Using Individual Recombinant Phage Antibodies

To investigate the applicability of the selected phage clones in a ligand competition ELISA, the potential influence of solvents that are routinely used to solubilize the control ligands, on the sensitivity of the assay were determined.

Influence of Solvents Used in Competition ELISA

For these analyzes, one representative phage clone from each selection procedure was randomly chosen (CS-G12, CC-C10, and AC-G7). The influence of the presence of ethanol, which was used for dissolving testosterone and aldosterone, or DMF, which was used for dissolving cortisol on the reactivity of these phage clones was analyzed. The results showed that neither ethanol nor DMF (concentrations up to 10%) had any significant effect on the binding of these phage clones (Fig. 3).

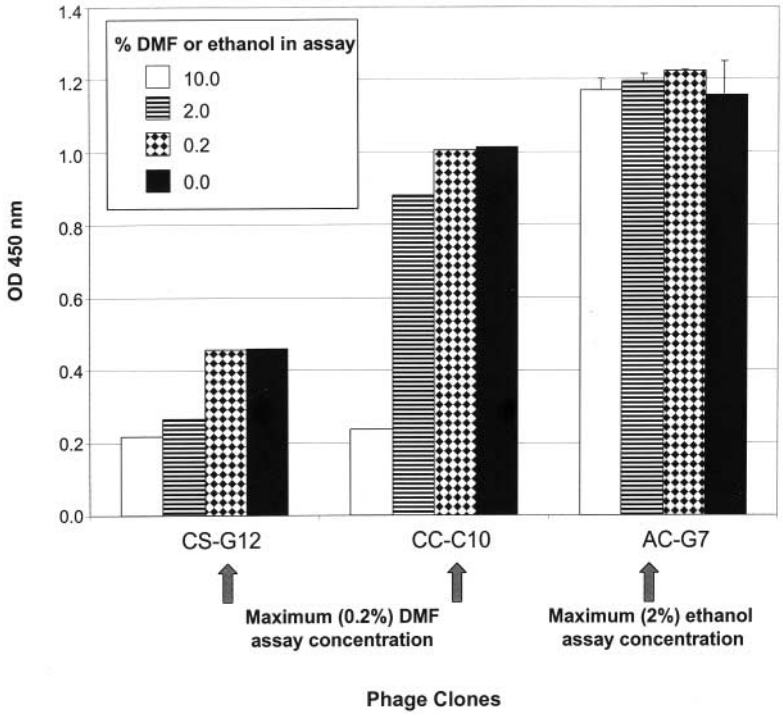


Figure 3. Influence of ethanol and dimethylfluoride on phage antibody reactivity. ELISA analysis of the influence of ethanol (average of triplicate assay; chicken clone directed to anti-aldosterone-mab, AC-G7) or DMF (one point assay; synthetic library clone directed to anti-cortisol-mab, CS-G12, and chicken library clone directed to anti-cortisol-mab, CC-C10) on the binding of phage clones to their cognate antigens. Note that for both ethanol and for DMF, at the maximal assay concentrations used (2% ethanol, 0.2% DMF), no effect was observed. Antigens were coated at a concentration of 1 μg/well.

Because the maximum concentration of these solvents in case of control ligand competition does not exceed 2%, we conclude that the presence of these solvents does not affect the reliability of the data.

Sensitivity of Displacement by Haptens in Competition Assays

To analyze the sensitivity of hapten detection in a competition assay, as well as the dynamic range of the assay, we tested a series of representative recombinant antibody phage clones. For these experiments phages were used directly from the bacterial culture supernatant. The coating concentration was optimized for the various antigens (1 μg/well for the anti-cortisol-mab and 4 μg/well for the anti-aldosterone-mab). The capacity of haptens to compete for phage binding to these antigens was studied by incubation in the presence of a range of hapten (cortisol/aldosterone/testosterone) concentrations for 1 h at RT. Bound phages were detected by an HRP-conjugated anti-M13 antibody in combination with TMB substrate and absorbance measurements at OD 450 nm.

These experiments demonstrated that for the clones derived from the synthetic libraries, a dynamic range of 2–250 ng/mL cortisol was obtained (Figs. 4a, 5a). As a control hapten the influence of testosterone (normal serum levels 0.15–10 ng/mL[21]) was determined. The results showed that, although for several randomly chosen CS clones, competition by testosterone was observed, this occurred only at concentrations that are not physiologically relevant (>20 ng/mL testosterone) (Fig. 5a). For clones derived from the chicken libraries, the dynamic range was 2–40 ng/mL cortisol (Fig. 4b). The binding of chicken phage clone CC-H5 to the anti-cortisol-mab was not detectably affected by testosterone within the concentration range analyzed (Fig. 5b). For aldosterone a dynamic range of 0.1–100 ng/mL aldosterone was observed (Fig. 4c). As competitor hapten controls for two phage clones reactive with the anti-aldosterone-mab both testosterone (normal serum levels 0.15–10 ng/mL) and cortisol (normal serum levels 30–250 ng/mL[21]) were used. Within the concentration range tested for both competitor haptens no competition for binding of these phage clones to the anti-aldosterone-mab was observed (Fig. 5c).

Competition Assays Using Mono and Dimeric scFv

Because soluble scFv fragments not associated with phage particles may be superior to phage-bound scFv fragments in these types of assays,

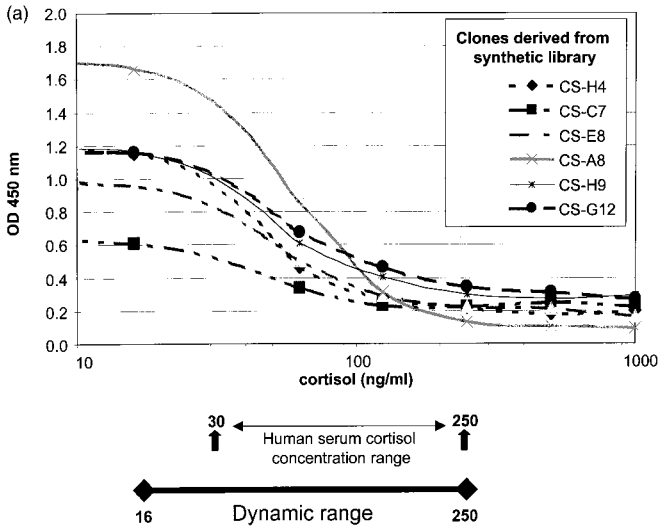
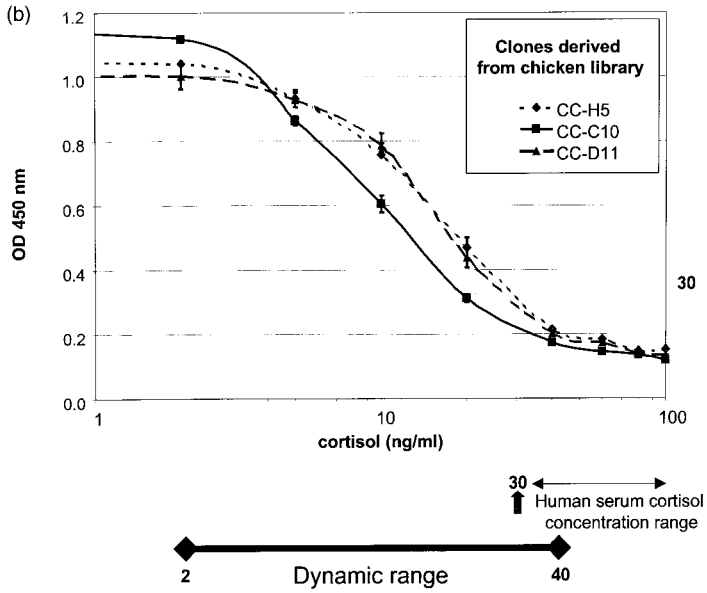
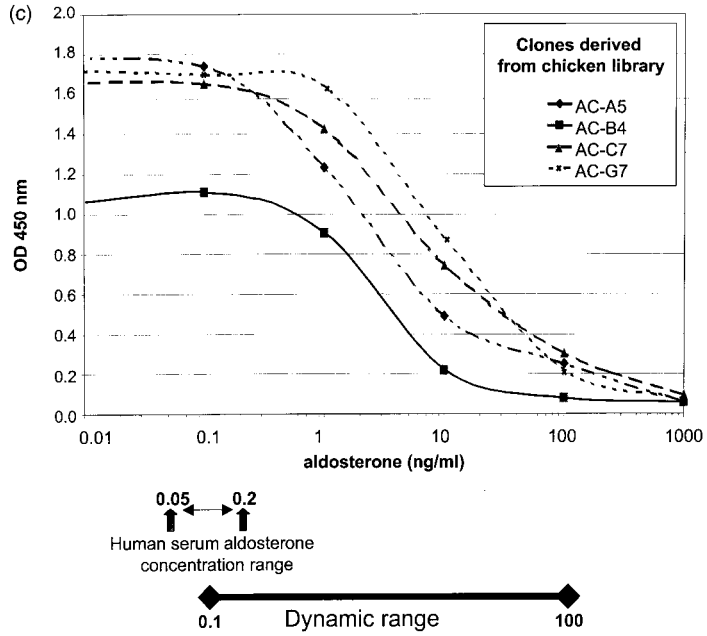


Figure 4. Dynamic range of assays with various phage clones. The dynamic range of hapten assays with various phage clones was determined using concentration ranges of both haptens in competition ELISAs. Figure 4a, clones from the synthetic library reactive with the anti-cortisol-mab: CS-H4, CS-C7, CS-E8, CS-A8, CS-H9, and CS-G12. Figure 4b, clones from the chicken library reactive with the anti-cortisol-mab: CC-H5, CC-C10, and CC-D11. Figure 4c, clones from the chicken library reactive with the anti-aldosterone-mab: AC-A5,AC-B4, AC-C7, and AC-G7. These analyzes were performed using ELISA plates coated with 1 μg anti-cortisol-mab/well ((a) and (b)) or 4 μg anti-aldosterone-mab/well (c). The reproducibility of these experiments was determined by repeating the complete experiment for a number of phage clones 2–4 times. The variations in the cortisol or aldosterone concentrations leading to 50% inhibition of recombinant antibody binding (IC50) were determined: IC50 CS-H4 = 80 ± 50 ng/mL cortisol ($n = 3$); IC50 CS-A8 = 58 ± 13 ng/mL cortisol ($n = 3$); IC50 CC-H5 = 12 ± 3 ng/mL cortisol ($n = 4$); IC50 CC-C10 = 9 ± 4 ng/mL cortisol ($n = 3$); IC50 CC-D11 = 9 ± 4 ng/mL cortisol ($n = 3$); IC50 AC-A5 = 3 ± 2 ng/mL aldosterone ($n = 2$). n = number of repeat experiments.

(*continued*)

we tested the reactivity of the corresponding recombinant antibody fragments. Surprisingly, although scFv expression levels were normal, none of the monomeric scFvs derived from the synthetic library and directed to the anti-cortisol-mab exhibited significant binding to the antigen (only 4 of these showed weak binding). Therefore, scFvs were not further assessed. As an alternative approach some of the scFvs (randomly chosen) were recloned to allow production in a dimeric format (CS-A8





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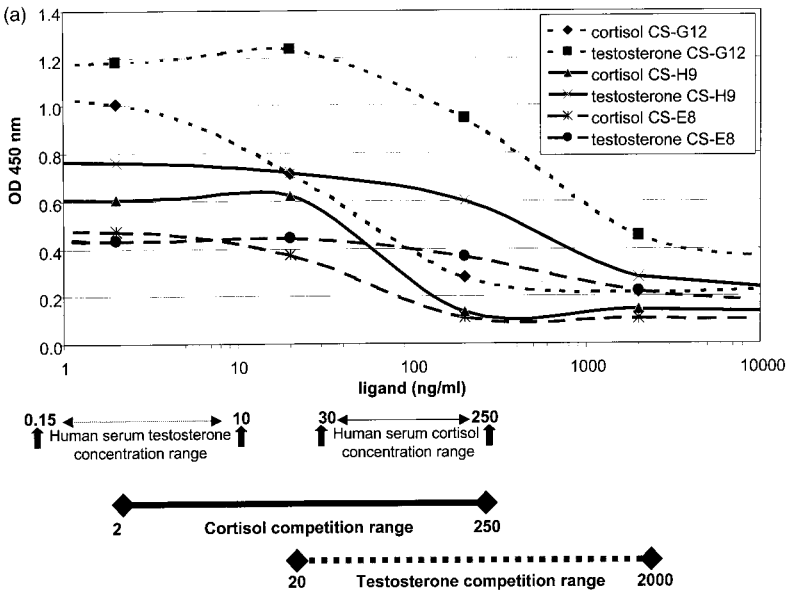
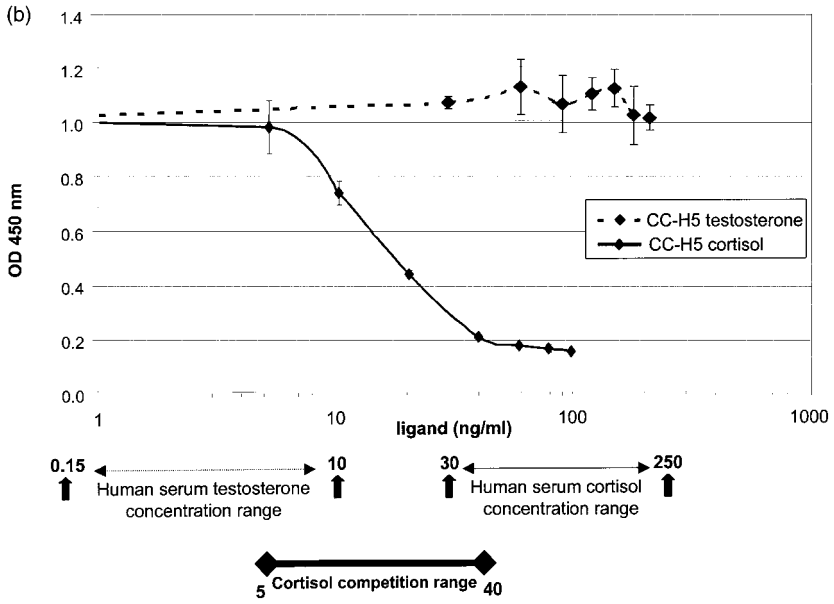


Figure 5. Influence of other steroid haptens on the competition assays. The effects of other steroids on the binding of the phage clones to either the anti-cortisol-mab or the anti-aldosterone-mab were determined using concentration ranges of these steroids in competition ELISAs. Fig. 5a, clones from the synthetic library reactive with the anti-cortisol-mab: CS-E8, CS-H9, and CS-G12 (one point assays); Fig. 5b, clone CC-H5 from the chicken library reactive with the anti-cortisol-mab (average values from duplicate determinations); Fig. 5c, clones from the chicken library reactive with the anti-aldosterone-mab: AC-A5, and AC-G7 (one point assays). Note that no competition by testosterone was observed for the chicken library derived phage antibodies, whereas for phage antibodies from the synthetic library competition by testosterone was observed, but only with concentrations much higher than physiologically relevant. These analyzes were performed using ELISA plates coated with 1 μg anti-cortisol-mab/well ((a) and (b)) or 4 μg anti-aldosterone-mab/well (c).

(*continued*)

and CS-H4). During expression these scFvs form dimers in the periplasm due to the incorporation of Fos dimerization domains. In these experiments dimerization was suspected,[11] but not experimentally confirmed. In contrast to the monomeric scFvs described above, these scFv dimers (used as crude periplasmic extracts) readily bound to the antigen in ELISA and thus could be evaluated in competition assays. The dynamic range for cortisol in assays with these dimeric antibody fragments



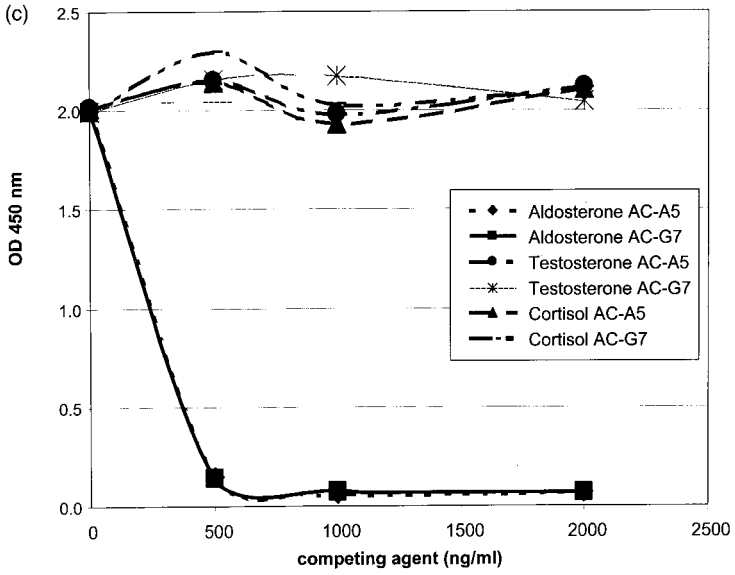


Figure 5. Continued.

was 0.15–20 ng/mL (Fig. 6). Thus, in comparison with the phage format, the use of dimeric antibody fragments resulted in this case in an approximately ten-fold higher sensitivity.

Competition Assay Using Spiked Patient Serum and Dimeric Anti anti-cortisol-mab Antibody

To analyze the influence of serum components we used the cortisol competition assay with synthetic library derived dimeric clone CS-H4 as a model system. The coating concentration of the anti-cortisol-mab was raised ten-fold which resulted in a twentyfour-fold decrease of sensitivity

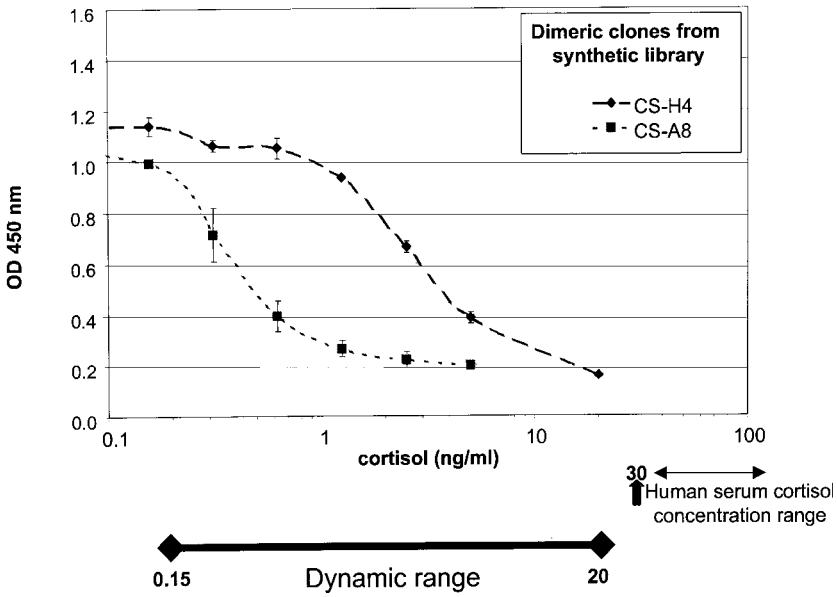


Figure 6. Competition ELISA with non-phage associated dimeric scFv antibodies. A competition ELISA was performed using dimeric recombinant antibody fragments derived from phage clones CS-A8 and CS-H4 (reactive with anti-cortisol-mab). The data represent the mean values of duplicate determinations with a coating concentration 1 μg anti-cortisol-mab/well. The reproducibility of these determinations, expressed in IC50 values as described in the legend to Fig. 5, were IC50 CS-A8 = 0.3 ± 0.1 ng/mL cortisol ($n = 2$); IC50 CS-H4 = 2.3 ± 0.4 ng/mL cortisol ($n = 2$). Note that the use of a dimeric scFv antibody instead of the corresponding phage clone led to an increase in sensitivity of at least one order of magnitude (compare with Fig. 4a).

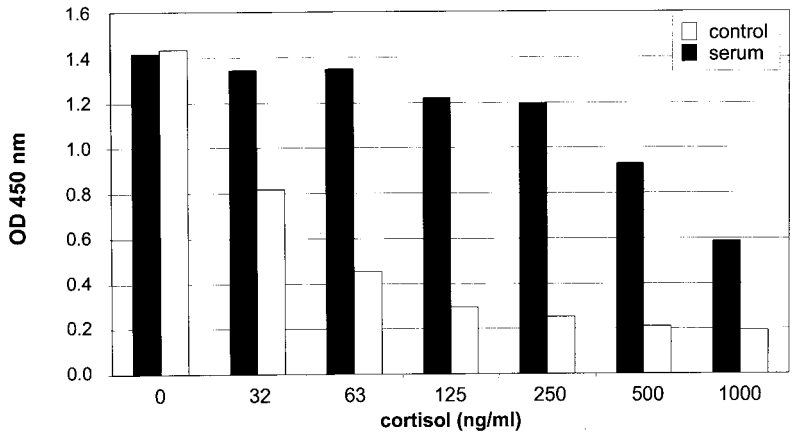


Figure 7. Influence of human serum on the cortisol competition assay. Human serum was spiked with cortisol and the influence of serum components on the cortisol competition assay with dimeric scFv antibody CS-H4 was determined (one point assay). Anti-cortisol-mab was coated at a concentration of 10 μg/well, h reduces the sensitivity of the competition assay 24-fold in comparison with a coating concentration of 1 μg/well (see Fig. 4, CS-H4). Note that the presence of serum reduces the sensitivity of displacement by haptens in the competition assay approximately 20-fold.

(compare Fig. 6, H4 and Fig. 7, control). This was necessary to avoid the influence of the cortisol present in the human serum. Human serum was spiked with a concentration range of cortisol and compared with the same cortisol concentration range in MPBST buffer. The results in Fig. 7 demonstrate that the sensitivity of hapten detection in the serum-containing samples is approximately twenty-fold lower than that of the cortisol samples in MPBST buffer. The most likely cause of this difference is the presence of cortisol-binding globulin (CBG) in human serum that interacts with the cortisol added, thereby reducing the concentration of free cortisol that can compete with CS-H4 for binding to anti-cortisol-mab. Therefore, cortisol quantification in serum samples using this competition assay would probably benefit from CBG blockage prior to cortisol determination.

DISCUSSION

Accurate determination of the concentration of haptens such as cortisol and aldosterone in body fluids of individuals provides vital infor-

mation for clinical and nutritional diagnosis. In this article, we describe a panning procedure that enables the selection of recombinant phage antibodies that specifically interact with hapten-binding molecules in the absence of the hapten, but not when the hapten-binding molecules are bound by the hapten. Because the binding of recombinant (phage) antibody is highly specific and dependent on the hapten concentration, these reagents can be applied to develop assays for the quantitative determination of haptens in body fluids.

In recent years recombinant antibody technology has been used for creating new tools for biologic agent detection,[22–25] and for optimizing antibody specificity.[1,2] Various studies showed that recombinant antibodies can be successfully generated from a range of animal species.[23,24,26–29] Our results demonstrate that the recombinant antibody phage display technology is an extremely powerful tool for the generation of anti-idiotypic recombinant antibodies for use in diagnostic assays.

Initially we used a synthetic library to test various ''guided'' selection protocols to select recombinant antibodies for our hapten assays. Pre-absorption with a control antibody of the same isotype as the antigen, or even better with hapten-saturated antigen, improved the selection efficiency of antibodies that compete for binding of the hapten. The use of trypsin-sensitive helper phage enabled the rapid selection of anti-idiotypic antibodies from synthetic libraries. Similar studies have shown that this is caused by enhancing the specific phage enrichment during the initial selection rounds, which reduces the number of selection rounds required to isolate the desired phages. The selection strategy described in this article may be more generally applicable for generating recombinant antibodies with similar specificities, although more experimentation will be required to establish this.

With both the anti-cortisol-mab and the anti-aldosterone-mab based chicken libraries we were able to select recombinant anti-idiotypic antibodies similar to those isolated from the synthetic library. A randomly chosen subset of the many recombinant antibodies that were obtained
was subjected to more detailed analyzes to demonstrate that the approach described in this article can be applied for the design and construction of immunoassays for the quantification of haptens in solution.

The normal concentration range of cortisol in human serum is 30–250 ng/mL (80–690 nmol/L), whereas that of aldosterone is 50–200 pg/mL (140–560 pmol/L) and that of testosterone is 0.15–10 ng/mL (0.5–35 nmol/L).[21] The initial selection with the synthetic library generated a number of clones that, when tested in a ''crude'' assay, displayed a dynamic range (2–250 ng/nL, 5.5–690 nmol/L cortisol) that covered the

cortisol serum concentration range. It should be noted, however, that for the determination of the total cortisol concentration in human serum by this assay, the CBG present in the serum should be blocked or removed, since it binds to cortisol thereby rendering it incapable to compete for binding to the anti-cortisol-mab.

The synthetic library derived recombinant antibodies in a monomeric (scFv) format appeared to bind only very weakly to their target antigen and were therefore not suitable for the development of competition assays. However, the same recombinant antibodies in the dimeric scFv format efficiently bound to the antigen and when these were used in a competition assay, this improved the sensitivity by one order of magnitude compared to the assay in which the phage-associated antibodies were used. The improvement of the sensitivity is most likely due to differences in the avidity of phage antibodies in comparison with the corresponding dimeric antibodies. This may be caused by differences in protein folding and in the number of antibody fragments displayed on the phage particles.

Using the chicken library approach we generated phage antibodies reactive with the anti-cortisol-mab, all of which displayed a dynamic range of 2–40 ng/mL (5.5–110 nmol/L) cortisol in a competition assay. In contrast, only one of the tested, synthetic library derived antibodies displayed a similar sensitivity (2 ng/mL; 5.5 nmol/L). For the phage antibodies recognizing the anti-aldosterone-mab, the sensitivity of the assay was 1 ng/mL (2.8 nmol/L). By increasing the coating concentration of the anti-aldosterone-mab we could enhance the sensitivity by one order of magnitude, which for measuring the aldosterone concentration in plasma is still one order of magnitude too low. Consequently, the development of a recombinant antibody based aldosterone assay requires further optimization, e.g., by the selection of other recombinant antibodies that lead to a higher sensitivity in the competition assay.

Whereas the sensitivity of these assays is dependent on the interaction of phage antibodies with the anti-hapten mouse monoclonal antibodies, the specificity of these assays is introduced by the mouse monoclonal antibodies that bind specifically to cortisol or aldosterone. Because in body fluids, many different, though structurally related haptens exist, the effect of cortisol, aldosterone and testosterone on the competition assays was analyzed. Testosterone did not affect the anti-cortisol and anti-aldosterone immunoassays and cortisol did not interfere with the anti-aldosterone immunoassay, which substantiates their specificity. We also found that the solvents ethanol and DMF, used for solubilisation of the analytes, did not interfere with the immunoassays.

ABBREVIATIONS

scFv	Single chain antibody fragment
Vh	Heavy chain variable region
Vl	Light chain variable region
mab	Monoclonal antibody
ab	Antibody
ELISA	Enzyme-linked immunosorbent assay
PCR	Polyerase chain reaction
o/n	Over night
RT	Room temperature
PEG	Polyethylene glycol
BSA	Bovine serum albumin
HRP	Horse radish peroxidase
DMF	Dimethylformamide
MPBST	5% Marvel (non fat dry milk) and 0.05% Tween-20 in PBS
MPBS	5% Marvel (non fat dry milk) in PBS
PBST	0.05% Tween-20 in PBS

ACKNOWLEDGMENTS

We would like to thank Prof. Winter and Dr. Nissim for the use of the synthetic library. We thank Dr. Kristensen for the trypsin-sensitive helper phage. We thank the CSHL phage display course team (Carlos Barbas, Don Siegel, and Gregg Silverman) for providing us with both the knowledge for the construction of the chicken phage display libraries as well as for the pComb3H vector system for cloning the chicken libraries. We thank Future Diagnostics B.V., Wijchen, The Netherlands, for financial support. This work was partially funded by BTS (Bedrijfsgerichte Technologische Samenwerking) grant 97247, and the Royal Netherlands Academy of Arts and Sciences (KNAW).

REFERENCES

1. Chames, P.; Baty, D. Engineering of an anti-steroid antibody: amino acid substitutions change antibody fine specificity from cortisol to estradiol. Clin. Chem. Lab. Med. **1998**, *36*, 355–359.

2. Chames, P.; Coulon, S.; Baty, D. Improving the affinity and the fine specificity of an anti-cortisol antibody by parsimonious mutagenesis and phage display. J. Immunol. **1998**, *161*, 5421–5429.
3. Le-Calvez, H.; Fieschi, J.; Green, J.M.; Marchesi, N.; Chauveau, J.; Baty, D. Paratope characterization by structural modelling of two anti-cortisol single-chain variable fragments produced in *E. coli*. Mol. Immunol. **1995**, *32*, 185–198.
4. Falkenstein, E.; Christ, M.; Feuring, M.; Wehling, M. Specific nongenomic actions of aldosterone. Kidney Int. **2000**, *57*, 1390–1394.
5. Lumbers, E.R. Angiotensin and aldosterone. Regul. Peptides **1999**, *80*, 91–100.
6. Garbers, D.L.; Dubois, S.K. The molecular basis of hypertension. Ann. Rev. Biochem. **1999**, *68*, 127–155.
7. Kreis, T.E. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. EMBO J. **1986**, *5*, 931–941.
8. Soldati, T.; Perriard, J. Intracompartmental sorting of essential myosin light chains: molecular dissection and in vivo monitoring by epitope tagging. Cell **1991**, *66*, 277–289.
9. Finnern, R.; Pedrollo, E.; Fisch, I.; Wieslander, J.; Marks, J.D.; Lockwood; Ouwehand, W.H. Human autoimmune anti-proteinase 3 scFv from a phage display library. Clin. Exp. Immunol. **1997**, *107*, 269–281.
10. Griffiths, A.D.; Williams, S.C.; Hartley, O.; TomLinson, I.M.; Waterhouse, P.; Crosby, W.L.; Kontermann, R.E.; Jones, P.T.; Low, N.M.; Allison, T.J.; Prospero, T.D.; Hoogenboom, H.R.; Nissim, A.; Cox, J.P.L.; Harrison, J.L.; Zaccolo, M.; Gherardi, E.; Winter, G. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. **1994**, *13*, 3245–3260.
11. de Kruif, J.; Logtenberg, T. Leucine zipper dimerized bivalent and bispecific antibodies from a semi-synthetic antibody phage display library. J. Biol. Chem. **1996**, *271*, 7630–7634.
12. Nissim, A.; Hoogenboom, H.R.; TomLinson, I.M.; Flynn, G.; Midgley, C.; Lane, D.; Winter, G. Antibody fragments from a 'single pot' phage display library as immunochemical reagents. EMBO J. **1994**, *13*, 692–698.
13. Andris-Widhopf, J.; Rader, C.; Steinberger, P.; Fuller, R.; Barbas, C.F. Methods for the generation of chicken monoclonal antibody fragments by phage display. J. Immunol. Methods **2000**, *242*, 159–181.

14. Barbas III, C.F.; Burton, D.R.; Scott, J.K.; Silverman, G.J. *Phage Display: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 2001.
15. Barbas III, C.F.; Wagner, J. Synthetic human antibodies: selecting and evolving functional proteins. Methods, a companion to Meth. Enzymol. **1995**, *8*, 94–103.
16. Rader, C.; Barbas, C.F. Phage display of combinatorial antibody libraries. Curr. Opin. Biotechnol. **1997**, *8*, 503–508.
17. Yang, W.P.; Green, K.; Pinzsweeney, S.; Briones, A.T.; Burton, D.R.; Barbas, C.F. CDR walking mutagenesis for the affinity maturation of a potent human Anti-HIV-1 antibody into the picomolar range. J. Mol. Biol. **1995**, *254*, 392–403.
18. Kristensen, P.; Winter, G. Proteolytic selection for protein folding using filamentous bacteriophages. Fold. Des. **1998**, *3*, 321–328.
19. de Wildt, R.M.; Finnern, R.; Ouwehand, W.H.; Griffiths, A.D.; van Venrooij, W.J.; Hoet, R.M.A. Characterization of human variable domain antibody fragments against the U1 RNA-associated A protein, selected from a synthetic and patient-derived combinatorial V gene library. Eur. J. Immunol. **1996**, *26*, 629–639.
20. van den Elsen, J.; Van de Putte-Rutten, L.; Kroon, J.; Gros, P. Bactericidal antibody recognition of miningococcal PorA by induced fit. Comparison of liganded and unliganded Fab structures. J. Biol. Chem. **1999**, *274*, 1495–1501.
21. Wilson, J.D.; Foster, D.W.; Kronenberg, H.M.; Larsen, P.R. *Williams Textbook of Endocrinology*. W. B. Saunders Co.: Philadelphia, 1998.
22. Emanuel, P.A.; Dang, J.; Gebhardt, J.S.; Aldrich, J.; Garber, E.A.E.; Kulaga, H.; Stopa, P.; Valdes, J.J.; Dion, S.A. Recombinant antibodies: a new reagent for biological agent detection. Biosens. Bioelectron. **2000**, *14*, 751–759.
23. Li, Y.; Cockburn, W.; Kilpatrick, J.; Whitelam, G.C. Selection of rabbit single-chain Fv fragments against the herbicide atrazine using a new phage display system. Food Agr. Immunol. **1999**, *11*, 5–17.
24. Li, Y.; Cockburn, W.; Kilpatrick, J.B.; Whitelam, I. High affinity ScFvs from a single rabbit immunized with multiple haptens. Biochem. Biophys. Res. Commun. **2000**, *268*, 398–404.
25. Strachan, G.; Whyte, J.A.; Molloy, P.M.; Paton, G.I.; Porter, A.J.R. Development of robust, environmental, immunoassay formats for the quantification of pesticides in soil. Environ. Sci. Technol. **2000**, *34*, 1603–1608.

26. Li, Y.; Kilpatrick, J.; Whitelam, G.C. Sheep monoclonal antibody fragments generated using a phage display system. J. Immunol. Meth. **2000**, *236*, 133–146.
27. Nakamura, N.; Aoki, Y.; Horiuchi, H.; Furusawa, S.; Yamanaka, H.I.; Kitamoto, T.; Matsuda, H. Construction of recombinant monoclonal antibodies from a chicken hybridoma line secreting specific antibody. Cytotechnology **2000**, *32*, 191–198.
28. Cary, S.P.; Lee, J.; Wagenknecht, R.; Silverman, G.J. Characterization of superantigen-induced clonal deletion with a novel clan III-restricted avian monoclonal antibody: exploiting evolutionary distance to create antibodies specific for a conserved V-H region surface. J. Immunol. **2000**, *164*, 4730–4741.
29. Davies, E.L.; Smith, J.S.; Birkett, C.R.; Manser, J.M.; Anderson, D.D.; Young, J.R. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. J. Immunol. Methods **1995**, *186*, 125–135.

Received June 30, 2002
Accepted August 3, 2002
Manuscript 3075