2. MATERIALS AND METHODS

2.1. Providers/addresses

Alexis	Läufellingen, Switzerland
Amersham/Pharmacia	Braunschweig, Germany
Applied Biosystems	(→ PE/Applied Biosystems)
Arthur H. Thomas	Philadelphia, PA, USA
Axxiom	Moorpark, CA, USA
Bachem Feinchemikalien	Bubendorf, Switzerland
Bandelin electronics	Berlin, Germany
BDH	Poole, UK
Bio-RAD Laboratories	Richmond, Ca, USA
Bio-Tek Instruments	Winooski, VT, USA
Boehringer Mannheim (now Roche)	Mannheim, Germany
Branson	Heusenstamm, Germany
Braun, Melsungen	Melsungen, Germany
Bruker-Franzen	Bremen, Germany
Calbiochem-Novabiochem	La Jolla, CA, USA
Cole Parmer	Chicago, IL, USA
Dako	Glostrup, Denmark
Difco Laboratorien	Detroit, MI, USA
Dr. Bruno Lange	Berlin, Germany
Du Pont Instruments	Bad Nauheim, Germany
Eastman Kodak	Rochester, NY, USA
Eppendorf	Hamburg, Germany
Fluka	Buchs, Switzerland
Forma Scientific	Marietta, OH, USA
Gelman Sciences	Ann Arbor, MI, USA
Gibco BRL	Gaithersburg, MD, USA
Greiner	Nürtingen, Germany
Haake	Karlsruhe, Germany
Heraeus	Berlin, Germany
Hoefer Scientific Instruments	San Francisco, CA, USA
J.T. Baker	Phillipsburg, NJ, USA
Janke & Kunkel	Staufen, Germany
JASCO	Tokyo, Japan
LKB	(s. Pharmacia/LKB)
MDL Information Systems	Surrey, England, UK
Merck	Darmstadt, Germany

Mettler	Greifensee, Switzerland
Microcal Software Inc.	Northampton, MA, USA
Micrografx	Richardson, TX, USA
Microsoft	Redmond, WA, USA
Millipore	Eschborn, Germany
Neosystem	Strasbourg, France
New Brunswick Scientific	New Brunswick, NJ, USA
Novabiochem	(s. Calbiochem)
Nunc	Roskilde, Denmark
Orpegen	Heidelberg, Germany
Ovid Technologies	New York, NY, USA
Pall/Filtron	Northborough, MA, USA
PE/Applied Biosystems	Foster City, CA, USA
Perkin Elmer	(→ PE/Applied Biosystems)
PerSeptive Biosystems	(→ PE/Applied Biosystems)
Pharmacia Biotech	Uppsala, Schweden
R & D Systems	Wiesbaden, Germany
Rapp Polymere	Tübingen, Germany
Research Information Systems	Carlsbad, CA, USA
Romil Chemicals	Shepshed, UK
Roth	Karlsruhe, Germany
Sartorius	Göttingen Germany
Savant Instruments	Holbrook, NY, USA
Schleicher & Schüll	Dassel, Germany
Serva	Heidelberg, Germany
Seromed/Biochrom	Berlin, Germany
Shimadzu	Kyoto, Japan
Sigma	St. Louis, MO, USA
Sigma, Laborzentrifugen	Osterode, Germany
StatSoft	Tulsa, OK, USA
SYKAM	Gilching, Germany
Tecniplast	Buguggiate, Italy
Tierzucht Schöneweide	Berlin, Germany
UCSF	San Francisco, CA, USA
University of Wisconsin	Madison, WI, USA
Vacubrand	Wertheim, Germany
Verder	Düsseldorf, Germany
Whatman	Maidstone, UK
Wheaton	Millville, NJ, USA

2.2. Materials

Machines

Amino acid analyzer	SYKAM
CD spectrometer JASCO J-720	JASCO
Centrifuges	
– Sorvall RC-5B	Du Pont
– 5414S	Eppendorf
– Heraeus Biofuge 13	Heraeus
– Sigma 3MK	Sigma Laborzentrifugen
CO_2-Incubators	
– Forma Scientific	Forma Scientific
– Heraeus	Heraeus
FPLC	Pharmacia

Genequant spectrophotometer	Pharmacia
HPLC	Pharmacia/LKB
HPLC columns Pep-S 5 μm and Pep-S 15 μm	Pharmacia
ImmunoWasher NK-350	Nunc
Klett-Summerson photoelectric colorimeter	Arthur H. Thomas
Macrosep 10 K ultrafiltration unit	Pall/Filtron
mass spectrometers	
– PerSeptive Biosystems Voyager-DE Biospectrometry Workstation 5414S	PerSeptive Biosystems
– PerSeptive Biosystems Voyager Elite Biospectrometry Research Station	PerSeptive Biosystems
– Reflex II	Bruker-Franzen
– TSQ 700 electrospray triple quadrupole mass spectrometer	Finnigan
Microferm fermentor	New Brunswick Sc.
Microplate reader EL320	Biotek Instruments
Mighty Small II gel racks and transfer unit TE 22	Hoefer Scientific
Mini-Sette-System, 10 kDa cut-off	Filtron
peptide synthesizer ABI433A	Applied Biosystems
Roto-Torque	Cole Parmer
sonication bath	Bandelin electronics
Ultra-Turrax blender	Janke & Kunkel
ventilated racks (isolators)	Tecniplast

Supplies

filter paper 3MM	Whatman
Gelman Supor Acrodisc 13 or 25 filter	Gelman
HiTrap Desalting column	Pharmacia
Macrosep 10 K ultrafiltration unit	Pall/Filtron
microtiter plate NUNC Maxisorp	Nunc
NC membranes	Sartorius
sample vial, 4ml, glass, screw cap with rubber liner	Wheaton
sample bottle, 8 ml, glass, snap cap	Wheaton
Econo-column, 5 ml disposable column	Bio-RAD

2.3. Programs/software applications

Access 2.0	Microsoft
CONTIN	S. W. Provencher, Uslar-Schlarpe, Germany (http://www.provencher.de/contin.html)
Designer 7	Micrografx
Excel 7.0	Microsoft
GCG, Wisconsin Package Version 10.0	University of Wisconsin Genetics Computer Group (GCG)
ISIS Draw 2.1.3d	MDL Information Systems
Origin 5.0	MicroCal Software
Ovid 7.8	Ovid Technologies http://www.ovid.com/
Picture Publisher 7	Micrografx
Protein Prospector 3.2.1	UCSF (http://prospector.ucsf.edu/)
Pyramide 1.009	Axxiom
Reference Manager 8.01 N	Research Information Systems
Statistica 5.0 A	StatSoft
SWISSPROT	http://www.ebi.ac.uk/ebi_docs/swissprot_db/swisshome.html

Variable Selection	W. C. Johnson, Oregon State University, OR, USA (ftp: ucs.orst.edu, directory: pub/user/varselec)
Word 7.0	Microsoft

2.4. Chemicals/reagents

2.4.1. Commercial reagents

(Unlisted reagents were of p.a. quality from Merck)

acetonitrile	J.T. Baker
acetic anhydride	Fluka
acrylamide	Kodak
affinity adsorbent	Boehringer Mannheim
agarose (SDS-PAGE)	Eastman Kodak
aminooxy acetic acid	Sigma
AMP (AMP-buffer)	Sigma
anisole	Aldrich
aqua pro injectione	Braun
bacto-agar	Difco
bacto-tryptone	Difco
bacto-yeast extract	Difco
BCIP	Sigma
bisacrylamide	Bio-Rad laboratories
Boc-Ser(tBu)*DCHA	Orpegen
Boc-AOA-NSu	Neosystem
4-carboxybenzaldehyde	Fluka
cholesterol	Sigma
Click-RPMI	Biochrom/Seromed
Coomassie G 250	Serva
L-cysteine	Calbiochem
DHB	Aldrich
2, 3-dimercaptopropanol	Sigma
diethanolamine	Sigma
DMF	Romil
EDT	Du Pont
1,2-ethandithiol	Du Pont
ethanolamine	Fluka
ethylacetate	Fluka
FCS, mycoplasma/virus screened	Gibco BRL
Fmoc-Ala-OH	Novabiochem
Fmoc-Cys(Trt)-OH	Novabiochem
Fmoc-Asp(OtBu)-OH	Applied Biosystems/Perkin Elmer
Fmoc-Glu(OtBu)-OH	Bachem
Fmoc-Phe-OH	Bachem
Fmoc-Gly-OH	Bachem
Fmoc-His(Trt)-OH	Bachem
Fmoc-Ile-OH	Bachem
Fmoc-Lys(Boc)-OH	Bachem
Fmoc-Leu-OH	Bachem
Fmoc-Met-OH	Bachem
Fmoc-Asn(Trt)-OH	Applied Biosystems/Perkin Elmer
Fmoc-Pro-OH	Bachem
Fmoc-Gln(Trt)-OH	Applied Biosystems/Perkin Elmer
FmocArg(Pmc)-OH	Applied Biosystems/Perkin Elmer

Fmoc-Ser(tBu)-OH	Bachem
Fmoc-Thr(tBu)-OH	Bachem
Fmoc-Val-OH	Bachem
Fmoc-Trp-OH	Bachem
Fmoc-Tyr(tBu)-OH	Bachem
Freund's complete/incomplete adjuvant	Difco
GC medium base	Difco
L-glutamine	Sigma
GM-CSF, mouse, recombinant	R & D Systems
Hanks' Salts	Biochrom/Seromed
4-HCCA	Sigma
hemocyanin, keyhole limpet	Boehringer Mannheim
HOBt	Sigma
Hepes	Gibco
IL-4, mouse, recombinant	Sigma
iodine	Fluka
MBS	Pierce
β-mercaptoethanol	Serva
MPB-PE	Sigma
MPLA	Sigma
Na-bicarbonate, ACS grade	Gibco
NBT	Serva
NEA	Seromed/Biochrom
4-nitrophenyl phosphate	Boehringer Mannheim
Octyl-POE	Alexis
ovalbumin	Calbiochem
PC	Sigma
Phenyl Sepharose 6 Fast Flow high sub, HIC	Pharmacia
Pierce protein kit	Pierce
piperidine	Fluka
PG	Sigma
polymyxin B sulfate	Sigma
Proteose Peptone III	Difco
SDS	BDH
serum albumin, bovine	Sigma
skimmed milk	Difco
sodium metaperiodate	Fluka
SOURCE 15 S, IEC	Pharmacia
resins, SPPS	Rapp Polymere
– Rapp TentaGel R RAM, 0.15 mmol/g	
– Rapp TentaGel S RAM, 0.15 mmol/g	
– Rapp TentaGel S RAM, 0.20 mmol/g	
– Rapp TentaGel S RAM, Cys(Trt), 0.19 mmol/g	
– Rapp RA 2306 TentaGel R RAM Cys(Trt), 0.17 mmol/g	
– Rapp cleavable PEG MAP 4-branch, J1002 TentaGel PAP, 0.7 mmol/g	
Superose 12 HR 10/30	Pharmacia
TEMED	Bio-Rad laboratories
TFA	Romil
TFE	Fluka
thioanisole	Du Pont
thyroglobulin, bovine	Sigma
thriethylsilane	Fluka
TLCK	Sigma
Tris HCl/Tris Base	Sigma

Triton X-405	Sigma
Trypan-blue	Biochrom/Seromed
Tween 20/Tween 80	Serva
vancomycin	Sigma
Zwittergent 3-14	Calbiochem

2.4.2. Synthetic peptide antigens and peptide derivatives

Synthetic peptides and peptide derivatives are summarized in Chapter 2.8.

2.4.3. Antibodies and sera

Monoclonal antibodies

Monoclonal antibodies and their characteristics are listed in Table 2; hybridomas B306 and AH623 had been characterized previously. MAb AH623, specific for murine epitope 4 of meningococcal serogroup A IgA1-protease, was isolated by Morelli *et al.* [150]. Opc loop 2-specific B306 was isolated by Achtman *et al.* [151].

Hybridoma	Sys. No.	Specificity	Species, Isotype	Comment	Dilution ELISA, ELISPOT (WB)	Ref.
AH623	S3573	serogroup A IgA1-protease, epitope 4	murine IgG2b	purified by protein A-Sepharose from culture supernatant	1000 (1000)	[150]
B306	S3575	Opc loop 2	IgG1	purified by protein A-Sepharose from culture supernatant	12000 (3000)	[151]

Table 2: Monoclonal antibodies

Sera

Sera are listed in Table 3.

Serum *αIgA1-PA50* is a peptide IgA1-PA50-specific, affinity purified serum derived from serum 782. Serum 782 had been originally provided by Marie-Jose Quentin Millet at Pasteur Mérieux Connaught. It stems from rabbit immunized with peptide D35 corresponding to aa 558-587 of IgA1-protease, serogroup C, ET-37 (but it is cross-reactive with serogroup A derived peptides spanning the same region). D35 corresponds to the first 30 aa of the IgA1-PA50 N-terminus, except for a substitution in aa position 583 ($Q_{583,\ \text{IgA1-PA50}}/V_{583,\ \text{D35}}$). Immunoaffinity purification was carried out in this study and is described in Chapter 2.7.

Standard sera “pms Opc loop2/IgA1-PC20/IgA1-PC50” and “pms2 Opc loop2/IgA1-PC20/IgA1-PC50” were prepared by blending equal amounts of serum samples collected from mice immunized with different formulations of the same peptide antigen. For the former serum aliquots collected during the *peak response*

study were blended from 2 mice per group immunized with free peptide, and BSA-, ovalbumin- and thyroglobulin-peptide conjugates. For the latter serum aliquots from the *Immunogenicity Study* were blended from 5 mice per group immunized with free peptide, and BSA-, KLH-, ovalbumin-, thyroglobulin- and tetanus toxoid-peptide conjugates. For details on immunization and serum collection, refer to Chapter 2.12.

Designation (Sys. No., Aliquot)	Specificity	Species, Isotype	Comment	Dilution ELISA, ELISPOT (WB)
serum *αIgA1-PA50* (S3939)	peptide D35, correspnd. to PNmC, aa 558-587	rabbit	affinity purified from serum 782 against peptide D77B (IgA1-PA50)	2000 (1000)
serum pms Opc loop2	Opc loop 2	mouse	standard serum *peak response study* Opc loop 2 immunization	-
serum pms IgA1-PC20	IgA1-PC20	mouse	standard serum *peak response study* IgA1-PC20 immunization	-
serum pms IgA1-PA50	IgA1-PA50	mouse	standard serum *peak response study* IgA1-PA50 immunization	-
serum pms2 Opc loop2	Opc loop 2	mouse	standard serum *Immunogenicity Study* Opc loop 2 immunization	
serum pms2 IgA1-PC20	IgA1-PC20	mouse	standard serum *Immunogenicity Study* IgA1-PC20 immunization	
serum pms2 IgA1-PA50	IgA1-PA50	mouse	standard serum *Immunogenicity Study* IgA1-PA50 immunization	

Table 3: Sera

Conjugated antibodies

Conjugated antibodies and their characteristics are listed in Table 4.

Original designation	Specificity	Species	Sys. No. Aliquot	Provider	Comment	Dilution ELISA, ELISPOT (WB)
conjugated affinity isolated goat immunoglobulins	Fc, rabbit	goat	S3827	Dako	AP-conjugated	1500 (1500)
conjugated affinity purified rabbit immunoglobulins	mouse immuno-globulins	goat	S3752	Dako	AP-conjugated	1500 (1500)
anti-mouse IgA, whole antibody	IgA, mouse	goat	S3852	Amersham	biotynilated antibody	8000
rat anti-mouse IgE-AP	IgE	goat	S3983	Southern Biotechnology	AP-conjugated	1000
anti-mouse IgG, whole antibody	IgG	goat	S3850	Amersham	biotynilated antibody	8000
anti-mouse IgG1, whole antibody	IgG1	goat	S3854	Amersham	biotynilated antibody	16000
anti-mouse IgG2a, whole antibody	IgG2a	goat	S3852	Amersham	biotynilated antibody	2000
anti-mouse IgM, whole antibody	IgM	goat	S3851	Amersham	biotynilated antibody	4000
streptavidin alkaline phosphatase conjugate	-	-	S3988	Amersham	strepavidin AP	3000

Table 4: Conjugated antibodies

2.5. Solutions and media

2.5.1. ELISPOT

AMP-buffer, 0.5 l

In 300 ml H_20 were dissolved:

$MgCl_2$	75 mg
Triton X-405	50 µl
NaN_3	0.5 g
AMP	47.9 ml

The pH was adjusted to 10.25 with ammonia. Then H_20 was added to a final volume of 500 ml, readjusting the pH to 10.25.

ELISPOT color reagent (BCIP/AMP-buffer)

BCIP was dissolved at 1 mg/ml in AMP (30-60 min. at 37°C) and filtered through a paper filter (Schleicher & Schüll).

Hanks' Salts (BSS), cell culture medium

Hanks' Salts (BSS) was prepared in pyrogen-free water (aqua pro injectione):

Hanks' Salts	49.3 g
Penicillin/Streptomycin 10^4 U/ml	50 ml
HEPES buffer	50 ml
H_2O, aqua pro injectione	4.9 l

The culture medium was stirred for 4 h. The pH was then adjusted to 7.2 - 7.4 with Na-bicarbonate ($NaHCO_3$) and the volume adjusted to 5 l. Hanks' Salts (BSS) was devided into 500 ml aliquots by sterile filtration. Prior to use 10 ml FCS (inactivated for 30 min. at 56°C) was added to one aliquot.

CLICK-Medium, cell culture medium

CLICK-Medium was prepared in pyrogen-free water (aqua pro injectione):

Click RPMI	56.7 g
Penicillin/Streptomycin 10^4 U/ml	50 ml
HEPES buffer	50 ml
Na-bicarbonate ($NaHCO_3$)	78.5 ml
H_2O, aqua pro injectione	4.0 l

The culture medium was stirred for 4 h. The pH was then adjusted to 7.2 - 7.4 with Na-bicarbonate ($NaHCO_3$) and the volume adjusted to 5 l. CLICK-Medium was

devided into 450 ml aliquots by sterile filtration. Prior to use the following sterile supplements were added to one aliquot:

FCS, inactivated (30 min, 56°C)	50 ml
L-glutamine, 200 mM	5 ml
NEA, 100x	5 ml
Na-pyruvate, 100 mM	5 ml
β-mercaptoethanol, 10 mM	2.5 ml

2.5.2. IgA1-protease purification

1x/5x Proteose Peptone III (PPIII)

For 5x PPIII concentrate, 450 g Proteose Peptone III were dissolved in 6 l H_2O. The solution was filtered in a tangential-flow filtration unit (flow pressure 1.5 bar, 10 kDa cut-off, Mini-Sette-System, Filtron).

1x PPIII aliquots were made by dilution. For storage 1xPPIII and 5xPPIII preparations were autoclaved (20 min, 121°C).

Salt solution, 5x

For 10 l fermentation medium, the following salt stock solution was prepared in 2 l H_2O and autoclaved (40 min, 121°C).

$K_2HPO_4 \cdot 3H_2O$	52 g
KH_2PO_4	10 g
NaCl	50 g
H_2O	2 l

Supplement

For 10 l fermentation medium, the following supplement solution was prepared in 50 ml H_2O:

$Fe(NO_3)_3 \cdot 9H_2O$	340 mg
L-glutamine	1 g
L-cysteine	3 g
Polymyxin B	75 mg
Vancomycin	30 mg

After sterile filtration (0.02 micron), Nystatin (20 mg in 70% EtOH), 125 ml glucose solution (40%, autoclaved) and 250 ml yeast extract (autoclaved) were added aseptically.

GC agar plates

72 g GC medium and 4 g agar were dissolved in 2 l H_2O and autoclaved. 1/5 of the above supplement preparation was prepared for GC agar medium and added after autoclaving (20 min. 121°C) prior to pouring (at ca. 60°C).

GC fermentation medium, 10 l

The fermentor was filled with 5x PPIII (2 l), 5x salt solution (2 l), prepared as above, and 6 l H_2O before being autoclaved *in toto* (40 min, 121°C). After that the fermentor was cooled down and pre-adjusted to 37°C. Prior to inoculation supplement was added aseptically.

Buffer A (Phenyl Sepharose)

Reagent/stock solution		final conc.
$(NH_4)_2SO_4$	132.14 g	200 mM
Trizma, pH 9.0 (4°C), 1 M	250 ml	50 mM
EDTA	186.12 g	100 mM
H_2O	5 l	

The buffer was adjusted to pH 9.0 (4°C) with ammonium hydroxide.

Buffer B (Phenyl Sepharose)

Reagent/stock solution		final conc.
Na-acetate, 1 M	100 ml	50 mM
EDTA	74.45 g	100 mM
Octyl-POE, 20%	100 ml	1% (v/v)
H_2O	2 l	

The buffer was adjusted to pH 6.0 (4°C) with ammonium hydroxide.

Buffer C (Source S)

Reagent/stock solution		final conc.
Na-acetate, 1 M	50 ml	50 mM
EDTA	37.22 g	100 mM
H_2O	1 l	

The buffer was adjusted to pH 4.0 (4°C) with acetic acid.

Buffer D (Source S)

Reagent/stock solution		final conc.
Na-acetate, 1 M	25 ml	50 mM
EDTA	18.61 g	100 mM
NaCl	20.45 g	700 mM
H_2O	500 ml	

The buffer was adjusted to pH 6.0 (4°C) with acetic acid/NaOH.

2.5.3. Opc protein purification

Resuspension buffer I, 250 ml

Reagent/stock solution		final conc.
Trizma pH 7.2 (RT), 1 M	12.5 ml	50 mM
EDTA, 500 mM	5 ml	10 mM
Zwittergent, 10%	125 ml	5%

The buffer volume was adjusted to 250 ml with H_20.

Resuspension buffer II, 10 ml

Reagent/stock solution		final conc.
Trizma pH 8.0 (RT), 1 M	0.5 ml	50 mM
EDTA, 500 mM	0.2 ml	10 mM
Zwittergent, 10%	4 ml	4%
NaCl, 5M	0.4 ml	200 mM

The buffer volume was adjusted to 10 ml with H_20.

Buffer A, 2 l

Reagent/stock solution		final conc.
Trizma pH 7.2 (RT), 1 M	100 ml	50 mM
EDTA, 500 mM	40 ml	10 mM
Zwittergent, 10%	100 ml	0.5%

The buffer volume was adjusted to 2 l with H_20.

Buffer B, 1 l

Reagent/stock solution		final conc.
Trizma pH 7.2 (RT), 1 M	100 ml	50 mM
EDTA, 500 mM	40 ml	10 mM
Zwittergent, 10%	100 ml	0.5%
NaCl, 5 M	200 ml	1 M

The buffer volume was adjusted to 1 l with H_20.

Buffer C, 2 l

Reagent/stock solution		final conc.
Trizma pH 8.0 (RT), 1 M	100 ml	50 mM
EDTA, 500 mM	40 ml	10 mM
Zwittergent, 10%	100 ml	0.5%
NaCl, 5M	80 ml	200 mM

The buffer volume was adjusted to 2 l with H_20.

2.5.4. ELISA

Diethanolamine buffer

9.7% (v/v) diethanolamin, 0.02% (w/v) NaN_3, 0.01% (w/v) $MgCl_2 \cdot 6H_2O$. The pH was adjusted to 9.8 with conc. HCl.

Substrate solution

1 mg/ml 4-nitrophenyl phosphate in diethanolamine buffer adjusted to 37°C.

2.5.5. Western Blot

Transfer buffer

0.028 M Tris, 0.188 M glycine. A 5x concentrated stock solution was prepared of 17.5 g Tris and 70.5 g glycine in 1 l H_20.

Substrate solution

5 mg of NBT were dissolved in 5 ml DMF and 2.5 mg BCIP were dissolved in 0.5 ml DMF. The NBT solution was added to 45 ml diethanolamine buffer and mixed with the BCIP solution.

2.5.6. LPS silver staining

Fixing reagent I (II)

40% EtOH, 5% acetic acid, 0.7% periodic acid.

Silver-nitrate color reagent

$AgNO_3$ (20%) was added dropwise under rapid stirring to a mixture of 1.75 ml 25% NH_4-solution and 28 ml 0.1 M NaOH 5 ml. The mixture was then diluted with 115 ml H_2O.

Developing reagent

50 mg citric acid, 0.5 ml 37% formaldehyde in 1 l H_2O.

2.5.7. Immunoaffinity chromatography

Glycine buffer

0.751 g/100 ml glycine (0.1 M), pH adjusted to 2.8 with 1 M HCl.

Na-phosphate buffer

1 M stock solutions of $NaH_2PO_4 \cdot H_2O$ (13.79 g in 100 ml) and $Na_2HPO_4 \cdot 2H_2O$ (17.79 g in 100 ml) were prepared and mixed such that the pH was adjusted to 6.8. For 20 mM Na-phosphate buffer the stocks were diluted 50x prior to mixing.

Thiocyanate buffer

388.72 g thiocyanate (CKNS) in 1 l H_2O.

2.5.8. Other

20x PBS

160g NaCl (2.8 M), 28.8 g Na_2HPO_4 (162 mM), 4.0 g KH_2PO_4 (28 mM) per liter H_2O.

PBS

PBS was made by diluting the “20x PBS” concentrate with H_2O resulting in the following concentrations: 0.14 M NaCl, 1.4 mM KH_2PO_4, 8.1 mM Na_2HPO_4.

PBST20/(PBST80)

PBS/0.05% Tween 20 (or Tween 80)

Coomassie brilliant blue solution

0.6% (w/v) Coomassie G 250, 6% (v/v) perchloric acid in water.

2.6. Protein methods

2.6.1. Protein-PAGE

Acrylamide-bisacrylamide mixture

45 g acrylamide and 1.2 g bisacrylamide in 100 ml were filtered through 3MM filter paper to prepare solutions in a 45:1.2 ratio. 30 g acrylamide and 0.8 g bisacrylamide were used for solutions in a 30:0.8 ratio.

Separating gel

	11%	15%	
1.5 M Tris pH 8.8	5	5	ml
Acryl-bisacrylamide mixture (45:1.2)	5.1	6.9	ml
10% SDS	0.2	0.2	ml
H_2O	10.5	8.78	ml
TEMED	20	20	µl
10% APS	90	90	µl

The solution was mixed and poured between 2 glass plates of 10 x 7.3 cm^2 with 0.75 mm spacers and covered with a layer of 1 ml 0.1% SDS. The gel polymerized for 20 min. at RT.

Stacking gel

(for both 11% and 15% separating gels)

0.25 M Tris pH 6.8	5 ml
Acryl-bisacrylamide mixture (30:0.8)	0.83 ml
10% SDS	50 µl
H_2O	1.61 ml
TEMED	10 µl
10%APS	12 µl

The solution was mixed and poured on top of the separating gel after removing the layer of SDS solution. The gel polymerized for 20 min. at RT.

Running buffer

Tris	3.025 g
Glycine	14.264 g
SDS	1 g

The pH was adjusted to 8.3

2.6.2. Polyacrylamide gel electrophoresis

A modified method from Laemmli [152] was used. Electrophoresis was performed at 20 mA/gel in Mighty Small II chambers for approx. 45 min. The samples were visualized by either staining with Coomassie brilliant blue or Western-blotting.

2.6.3. Coomassie staining

Gels were incubated in Coomassie brilliant blue staining solution on a horizontal shaker at RT. Destaining was carried out with 3 changes of 5% (v/v) acetic acid.

2.6.4. LPS silver staining

All steps were done at RT in gel size TupperWare boxes. Gel-fixing was carried out in fixing reagent I for 2-3 h followed by fixing reagent II (5 min). Gels were washed with H_2O 3 times for 10 min. before silver-nitrate color reagent was added (10 min. incubation in the dark). Three further washing steps were carried out and developing reagent added (1-5 min. incubation). Gels were washed again and fixed with 5% acetic acid.

2.6.5. Western-blotting

All subsequent incubation and washing steps were carried out on a shaker. The gel-to-membrane transfer was performed using a TE 22 Mighty Small transfer unit at 150 mA for 1.5 h in transfer buffer. The membranes were blocked with 50 ml PBS/2% BSA o.n. and washed with 50 ml PBST20 for 5 min. Antibodies were diluted in PBST20/0.05%, 1% BSA. The membranes were incubated for 90 min. at RT and washed three times with 50 ml PBST20 for 5 min. Conjugate solution diluted in PBST20/1% BSA was added and incubated for another 90 min. at RT. Five 5 min. washes with 5 ml PBST20 and one wash with 50 ml diethanolamine buffer followed before the membranes were finally immersed in 50 ml Western-blot substrate. After another incubation of approx. 2 min. in the dark at 37°C color development was stopped by washing with H_2O.

2.7. Immunoaffinity chromatography (IAC)

A portion of the polyclonal rabbit serum preparation 782 (Chapter 2.4.3, Sera) was affinity purified against peptide D77B (peptide reference in Table 8, Chapter 2.8) in order to yield serum with high specificity for peptide IgA1-PA50.

Column preparation

1 g affinity adsorbens (Silicate matrix, glutardialdehyde-activated, Boehringer Mannheim) was washed with 0.9% NaCl and suspended in 5 ml 0.9% NaCl containing 3 mg synthetic peptide D77B (IgA1-PA50). The mixture was degassed, sealed in a screw cap reagent tube (13 ml glass tube, Greiner) and agitated for 4 h at RT (Roto-Torque, $\nu = 2\ min^{-1}$).

The matrix was transferred to a disposable 5 ml column (Econo-column, Bio-RAD) and thoroughly washed in open column technique (1.5% NaCl) until the eluate appeared protein free ($OD_{280} < 0.005$, Genequant). Final column volume: 2.4 ml.

Remaining free aldehyde functions were inactivated by incubation with 0.3 M ethanolamine/HCl solution (pH 7.6), 1 h, RT. Inactivation was followed by a final washing procedure with 0.9% NaCl, followed by 0.5 M propionic acid and again 0.9% NaCl.

Purification

Immunoaffinity purification was carried out batch-wise.

Polyclonal rabbit serum 782 (sys. No.: S3895) was prepared: 1.8 ml S3895 was centrifuged, filtrated and the salts replaced by Na-phosphate buffer pH 6.8 by FPLC (HiTrap desalting column, Pharmacia, flow rate 5 ml min^{-1}, 1 ml fraction size). Chromatogram peak fractions were collected, confirmed by Genequant and pooled (3.55 ml). The 5 ml column, prepared as described above, was equilibrated with 10 bed volumes of Na-phosphate buffer pH 6.8 (flow rate 0.45 ml min^{-1}). The pooled fractions were added to the open column, and the column disconnected and sealed. The column was mounted on a Roto-Torque and agitated for 10 h at RT ($\nu = 2\ min^{-1}$).

After incubation, the column was washed with 10 bed volumes of 20 mM Na-phosphate (0.45 ml min^{-1}). Then, serum immunoglobulins were eluted batch-wise with 0.1 M glycine buffer, pH 2.8. For that, the matrix was dispersed in 5 ml glycine buffer and agitated for 2 min. on a Roto-Torque. The entire column volume was drained into sample tubes (= 1 batch) containing 1/20 vol. (250 µl) of 1 M Na-phosphate pH 8.0 to quickly dilute the glycine buffer and re-establish physiologic buffer conditions. The elution was repeated 20 times and the collected batches were tested in ELISA against peptide IgA1-PA50 as described (Chapter 2.13.1). Peak batches (batches No. 1-9) were pooled.

Serum (sys. No.)	serum protein conc./ELISA unit [µg/ml]
serum 782, pre IAC (S3895)	1.9
serum *αIgA1-PA50*, post IAC (S3939)	0.3

Table 5: Immunoaffinity purification of rabbit serum 782. Specific ELISA activity of serum 782 and of affinity purified serum *αIgA1-PA50*. An ELISA unit is that concentration yielding OD_{405}=1.0 (peptide IgA1-PA50 coating; for ELISA conditions refer to Chapter 2.13.1).

The pooled peak fractions were transferred to PBS and concentrated using a centrifugal concentrator (10K Macrosep, Pall/Filtron). The resulting IgA1-PA50-specific immunoglobulin preparation showed a 6-fold increased specific ELISA reactivity as compared to the original serum preparation (Table 5).

The column was regenerated with 5 bed volumes of 2 M thiocyanate buffer.

2.8. Synthesis of peptide antigens and immunogens

2.8.1. Solid phase peptide synthesis

Synthetic peptides, which are described in detail at the beginning of Chapter 3, were synthesized by FastMoc chemistry with an automated peptide synthesizer (ABI433A, Applied Biosystems). Fmoc protected amino acid derivatives were used with Boc, PMC, tBu or Trt side protection (Table 6). Peptides were synthesized on various resins (Table 8). Activation and coupling was done in the presence of HBTU/diisopropylethylamine with double coupling at critical positions. At the end of double coupling cycles, free amino groups were blocked by acetylation with acetic anhydride. Prior to each coupling Fmoc was removed by piperidine. For Peptides D100 and D101 (tetra-oxime precursors) the N-terminal aminooxyacetyl (AOA) was introduced by SPPS as a Boc-derivative. For peptide D92X the AOA modification was carried out manually with Boc-AOA-NSu (refer to Chapter 2.8.2).

Basically, variants of three synthetic peptide antigens were synthesized which can be grouped into three functional categories (Table 8):

Synthetic peptides corresponding to stretches of 3 neisserial pathogenicity factors:

1. A 20 aa motif corresponding to loop 2 of the meningococcal Opc protein, GenBank accession M80195 (peptides D65, D89, D101).
2. A 20 aa motif derived from aa 584-603 of serogroup C, ET 37 IgA1-protease, GenBank accession AF012208 (peptides D68, D90, D100).

3. A 50 aa motif corresponding to aa 557-660 of serogroup A IgA1-protease, GenBank accession AF012203 (peptides D77B, D77C, D92X).

Functional categories:

1. Cys-variants with N- or C-terminal cysteine modifications for coupling to liposomal and protein-carriers (peptides D65, D68, D77C).
2. AOA-variants with N-terminal aminooxyacetyl modifications for tetra-oxime assembly (peptides D92X, D100, D101).
3. Multiple antigenic peptides, MAPs (peptides D89, D90).

Besides these, D99 was synthesized as a precursor for the circularized tetra-oxime template, and D77B as an unmodified peptide antigen for coating in ELISA and for immunoaffinity purification.

aa	derivative		
A	Fmoc-Ala-OH	N	Fmoc-Asn(Trt)-OH
C	Fmoc-Cys(Trt)-OH	P	Fmoc-Pro-OH
D	Fmoc-Asp(OtBu)-OH	Q	Fmoc-Gln(Trt)-OH
E	Fmoc-Glu(OtBu)-OH	R	FmocArg(Pmc)-OH
F	Fmoc-Phe-OH	S	Fmoc-Ser(tBu)-OH
G	Fmoc-Gly-OH	T	Fmoc-Thr(tBu)-OH
H	Fmoc-His(Trt)-OH	V	Fmoc-Val-OH
I	Fmoc-Ile-OH	W	Fmoc-Trp-OH
K	Fmoc-Lys(Boc)-OH	Y	Fmoc-Tyr(tBu)-OH
L	Fmoc-Leu-OH		Boc-AOA-OH
M	Fmoc-Met-OH		

Table 6: Amino acid derivatives for solid phase peptide synthesis. *t*-butyloxycarbonyl (Boc), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (PMC), O-*t*-butyl (tBu) and trityl (Trt) side protection.

Cleavage from the resin support/side chain deprotection

After final Fmoc deprotection with piperidine, all peptides except for the AOA-variants were treated with acetic anhydride for N-terminal acetylation. Side chain deprotection and cleavage from the resin support was done as follows: After synthesis the reaction vessel was removed from the synthesizer. The upper lid of the vessel was unscrewed, the body mounted on a vacuum filtration flask connected to a water-jet aspirator, and the resin washed 2 times both with DCM and *t*-butyl methyl ether. After thorough drying (high vacuum, 2 h) peptides were cleaved with different procedures based on the type of peptide or protection groups present (Table 7, Table 9):

	cleavage mix No.				
Reagents [Reference]	1 [153]	2 [153]	3 [154]	4 [155]	5 [155]
H_2O, ml	0.5	0.25			
TFA, ml	10	9.5	9.5	8	9
phenol, g	0.75				
EDT, ml	0.25	0.25			0.3
thioanisole, ml	0.5			2	0.5
anisole, ml			0.5		0.2

Table 7: Peptide cleavage from resin support: Cleavage mixes.

Peptides were incubated with the cleavage mixture for 3 h (magnetic stirring) in 8 ml disposable glass bottles (sample bottle, Wheaton). The reaction was started on ice with ice-cold cleaving mixture and continued at RT after 10 min. The resin was removed by filtration through a glass-wool stuffed Pasteur glass-pipette (small quantities) or with a fritted glass funnel (No. 3 - fine porosity, connected to a vacuum filtration flask) for preparative scale. The filters were rinsed with small amounts of TFA. In case of the cysteine peptides (D65, D68, D77C) triethylsilane was now added dropwise to the filtrate until the dark brown color of the solution changed to pale yellow. The mixture was incubated a further 3 h with shaking.

Peptides were precipitated with 10 volumes of ice-cold *t*-butyl methyl ether (12 ml disposable glass tube, Greiner). After centrifugation (Sigma 3MK centrifuge, 5000 rpm, ca. 4000 g, 20 min), the peptides were washed 2 times with ice-cold *t*-butyl methyl ether.

Cleavage with mix 3 differed slightly from the other procedures: After incubation, the resin preparation (0.1 g resin in 2 ml TFA/anisole) was filtered through a glass-wool stuffed Pasteur glass-pipette and washed 5 times with 1 ml TFA each. The TFA/peptide solution was concentrated using a gentle stream of nitrogen. The mix was precipitated by dropwise addition to 20 ml of stirred dry ether. Stirring was continued for an additional 10 min. The peptide was separated by centrifugation as described. The peptide was dissolved in a small amount of CH_3Cl, precipitated two more times as above and lyophilized.

Crude peptide preparations were dried for 5 min. in a vacuum desiccator (connected to a water-jet aspirator), sealed with perforated aluminum-foil and dried in high vacuum, RT, o.n..

The AOA-peptides D92X, D100 and D101 additionally underwent adduct-cleavage as described in 2.8.2.

Source	No.	Sequence MW	Resin HPLC gradient (%ACN, v/v)	Type/comment
Nm Opc invasin, "loop2", aa 61-80	D65	Ac-*KN**I**INLETDENKLGK**TKNVK**C*-NH_2 M calc. 2443.9	Rapp TentaGel S RAM, Cys(Trt) 0.19 mmol/g 15-30%	C-terminal cysteine
	D89	[Ac-*KN**I**INLETDENKLGKTKN**VK**]$_4$-*K*$_3$-PEG M calc. ca. 12683	Rapp cleavable PEG MAP 4-branch, J1002 TentaGel PAP 0.7 mmol/g	MAP
	D101	**AOA**-*KN**I**INLETDENKLGKTKN**VK***-NH_2 MH^+ calc. 2372.7, found 2371.6	Rapp TentaGel S RAM 0.20 mmol/g 0-30%	oxime precursor, D65 aminooxyacetyl der.[2]
Nm IgA1-protease, serogroup C, ET-37,aa 584-603	D68	Ac-*TENNDWVFMGYTQEEAKKNAC*-NH_2 MH^+ calc. 2520.8, found 2521.3	Rapp TentaGel S RAM, Cys(Trt) 0.19 mmol/g 15-40%	C-terminal cysteine
	D90	[Ac-*TENNDWVFMGYTQEEAKK**NA***]$_4$-*K*$_3$-PEG M calc. ca. 12987	Rapp cleavable PEG MAP 4-branch, J1002 TentaGel PAP 0.7 mmol/g	MAP
	D100	**AOA**-*TENNDWVFMGYTQEEAKK**NA***-NH_2 MH^+ calc. 2448.6, found 2447.4	Rapp TentaGel S RAM mmol/g 10-40%	oxime precursor, D68 aminooxyacetyl der.[2]
Nm IgA1-protease, serogroup A, IV-1,aa 557-606	D77B	Ac-*LYYKNYR**YY**ALKSGGS**V**NA**PM**PENGQTE**NN**DW**I**LMGST**Q**EE**A**KKN**A**MN**HK***-NH_2 MH^+ calc. 5798.5 found 5797.8	Rapp TentaGel S RAM mmol/g 10-35%	
	D77C	Ac-*CLYYKNYR**YY**ALKSGGS**V**NA**PM**PENGQTE**NN**DW**I**LMGST**Q**EE**A**KKN**A**MN**HK***-NH_2 MH^+ calc. 5901.6, found 5897.3	Rapp TentaGel S RAM mmol/g 10-40%	N-terminal cysteine
	D92X	AOA-*C**L**YYKNYR**YY**ALKSGGS**V**NA**PM**PENGQTE**NN**DW**I**LMGST**Q**EE**A**KKN**A**MN**HK***-NH_2 M 5828.5 calc., found 5827.8	Rapp TentaGel R RAM mmol/g 10-45%	oxime precursor, D77 aminooxyacetyl der.[1]
Oxime template,	D99-O	Ac-*CKAKPGKAKC*-NH_2 MH^+ (monoisotopic) calc. 1072.6 found 1072.5	Rapp RA 2306 TentaGel R RAM Cys(Trt), 0.17 mmol/g 0-20%	oxime precursor, circularized, unmodified

Table 8: Overview of peptides and precursors synthesized by solid phase peptide synthesis. Peptides were synthesized by FastMoc chemistry with an automated peptide synthesizer. Bold letters indicate double coupling at critical aa positions. M represents molecular weight, either calculated from the sequence (calc.) or measured by mass spectrometry (found). MH^+ is the protonated mass as seen in MALDI MS (+1 Da). Synthesis, MS and HPLC purification are described in the text

([1]: aminooxyacetyl introduced post SPPS as Boc-AOA-NSu; [2]: aminooxyacetyl introduced in SPPS as Boc-NH-O-CH2-COOH. **Ac-**: N-terminal acetylation; **AOA**: aminooxyacetyl; **-NH_2**: C-terminal amid; **PEG**: polyethylenglycole).

	peptide									
	D65	D68	D77B	D77C	D89	D90	D92X	D99-O	D100	D101
cleavage mix No.	2	1	1	1	3	3	5	4	1	2
dissolved in	H_2O /M	H_2O /M	A50	D50 /M	PBS	PBS	H_2O	H_2O	H_2O	H_2O

Table 9: Key to cleavage mixes and solvation buffers. H_2O: H_2O/0.1% TFA; D50: 50% DMSO; A50: 50% acetic acid; /M: 50 mM N-methylmorpholin, 50 mM DTT in H_2O/0.1% TFA. Peptides D89 and D90 (MAPs) were not HPLC purified and were only solubilized in PBS (For a key to the peptides, refer to Table 8, p. 44).

After drying crude peptides were dissolved in H_2O/0.1% TFA and sonicated. If necessary (poor solubility), the peptides were dissolved in 50% acetic acid or 50% DMSO and then diluted to 10% solutions with H_2O/0.1% TFA (Table 9). Cysteine-peptides (D65, D68, D77C) were treated under nitrogen atmosphere with 50 mM N-methylmorpholin/DTT, o.n., RT to reduce disulfides and to prevent free SH-groups from oxidation during solubilization. In this case the pH was adjusted to 3.5 with 10% TFA prior to RP-HPLC.

RP-HPLC

RP-HPLC was carried out using an ACN/H_2O buffer system with 0.1% TFA. HPLC gradient parameters were determined by analyzing small aliquots with analytical HPLC using a gradient of 0-50% ACN (Pep-S 5 µm C_2/C_{18} column, Pharmacia, 100 Å pore size, 5 µm particle size, 4 x 250 mm, bed vol. ca. 3.2 ml, 0.8 ml min^{-1}). After that preparative scale HPLC was used with an appropriate ACN gradient and a slope of 0.5% ACN/min. (Pep-S 15 µm C_2/C_{18} column, Pharmacia, 100 Å pore size, 12-13 µm particle size, 22.5 x 250 mm, bed vol. ca. 100 ml, 15 ml min^{-1}). The individual peptide specific HPLC conditions are listed in Table 8. Preparative scale sample volumes of more than 2 ml were loaded with 10 or 50 ml Superloops (Pharmacia) at 10 ml min^{-1}. After loading columns were washed with 3 bed volumes at 0% ACN.

Chromatography was monitored with a conductivity monitor and a UV-monitor/OD. After peak collection, peptide preparations were frozen o.n. at -80°C and lyophilized.

The size and composition of all peptides and peptide derivatives were confirmed by mass spectrometry except for the MAPs (D89, D90) and D65. MAPs cannot be analyzed by MS. D65 was shown to be reactive with the reference antibody B306 αOpc loop 2.

2.8.2. Tetra-oxime synthesis

The three peptide antigens Opc loop 2, IgA1-PC20 and IgA1-PA50 were also synthesized as tetra-oxime variants [155]. A general representation of oxime synthesis

is depicted in Figure 3. The tetrameric oximes are derived from 2 precursor structures, an antigenic "arm" which is introduced as four identical copies to a cyclized carrier called "template".

The individual primary structures of synthesis precursors and the three final tetra-oxime products are depicted in Table 8 (AOA-peptides) and Figure 4 (template and tetra-oximes).

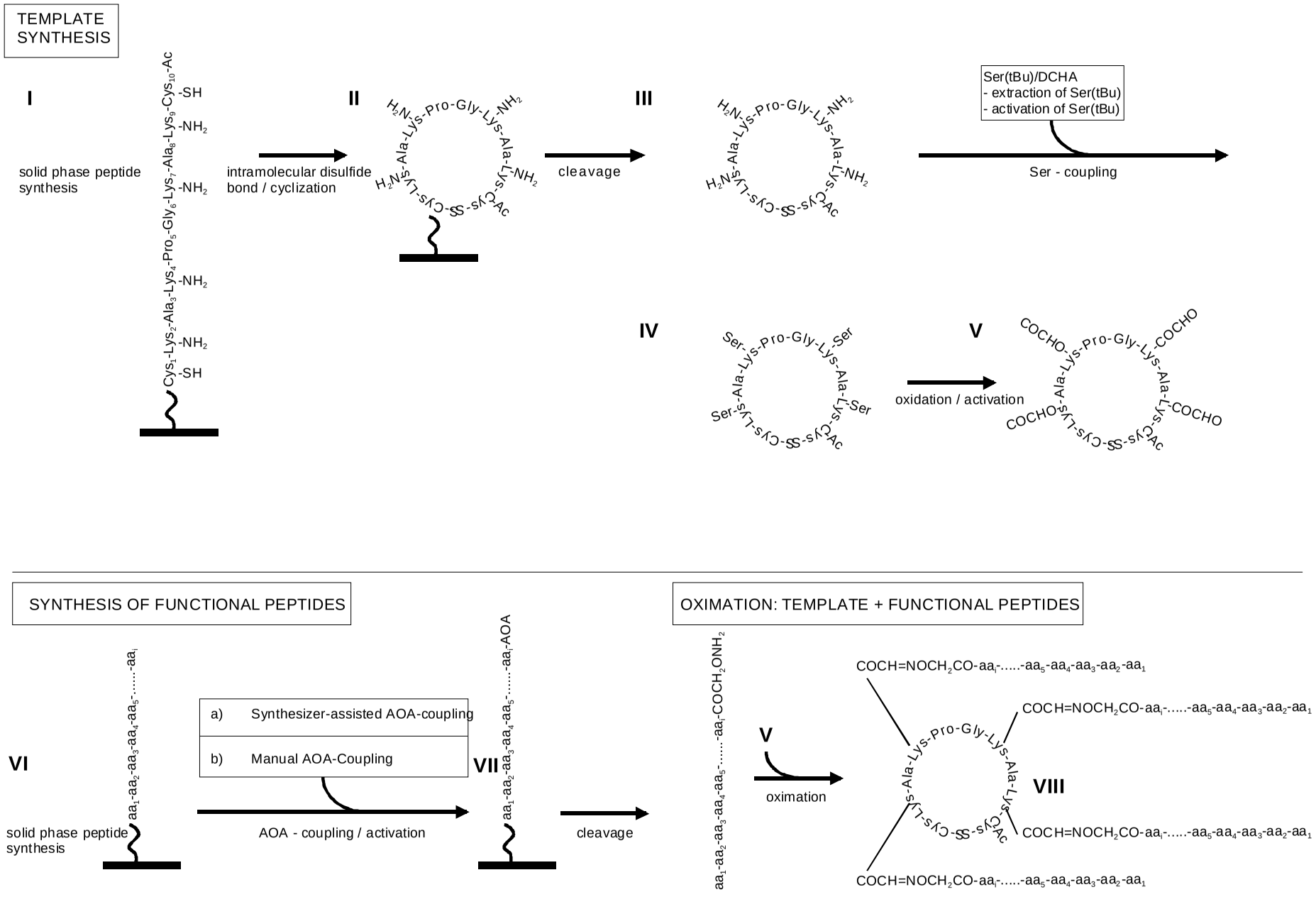
Modification of antigenic peptides

AOA-attachment

The linear antigenic peptides Opc loop 2 (D101), IgA1-PC20 (D100) and IgA1-PA50 (D92X) were modified N-terminally with aminooxyacetyl (AOA). For peptides D100 and D101 this modification was introduced as a Boc-derivative in the final step of the automated solid phase Fmoc synthesis. The 50-mer IgA1-PA50 (D92X) was modified manually (using an aliquot of resin-bound, Fmoc-protected and unmodified IgA1-PA50 from a previous synthesis):

After final deprotection of the N-terminal Fmoc group, the aminooxyacetyl group was attached using Boc-aminooxyacetyl N-hydroxysuccinimide ester (Boc-AOA-NSu, [156]. To 40 µmol resin bound peptide (4 ml sample vial, Wheaton), 100 µl Boc-AOA-NSu were added (1 ml, 0.1 M in dry DMSO, i.e. 2.5 equivalents per resin-bound amino group). The pH of the 0.1 M DMSO solution had been adjusted to pH 8-9 with N-methyl morpholine previously by spotting on humidified pH paper to determine the apparent pH. The reaction mixture was incubated for 2 h at RT. The resin was washed in nitrogen atmosphere (2x NMP, 2x DCM), dried (2 h, high vacuum) and the peptide cleaved using cleavage mix 5 (Table 7). The peptide was precipitated, washed and dried as described above.

Figure 3: Oxime synthesis, general representation. Two precursors, an activated ring template (V) and linear functional ligands (VII), are synthesized which are then condensed to form a tetra-oxime (VIII). The template is characterized by two terminal cysteines (Cys_1, Cys_{10}) and a turn-inducing central proline (Pro_5). The template was circularized on resin after automated SPPS by oxidizing the cys-thiols to form an intramolecular disulphide bridge (II). After introduction of 4 serines to the ε-NH_2 of the 4 lysine functions (Lys_2, Lys_4, Lys_7, Lys_9) in step IV, the template was activated creating four aldehyde functions by oxidation (cis-diol fission) of the serine residues with periodate (V). The linear peptides were synthesized by SPPS and N-terminally modified with aminooxyacetyl (AOA), as seen in VI and VII. After each step intermediates were purified by HPLC and their composition was confirmed by mass-spectrometry. The procedure is described in the text in detail.


TEMPLATE SYNTHESIS
I
solid phase peptide synthesis
Cys1-Lys2-Ala3-Lys4-Pro5-Gly6-Lys7-Ala8-Lys9-Cys10-Ac
-SH
-NH2
-NH2
-NH2
-NH2
-SH
intramolecular disulfide bond / cyclization
II
H2N-Lys-Pro-Gly-Lys-Ala-Lys-NH2
H2N-Ala-Lys-Lys-NH2
Ac-Cys-SS-Cys-NH2
cleavage
III
H2N-Lys-Pro-Gly-Lys-Ala-Lys-NH2
H2N-Ala-Lys-Lys-NH2
Ac-Cys-SS-Cys-NH2
Ser(tBu)/DCHA
- extraction of Ser(tBu)
- activation of Ser(tBu)
Ser - coupling
IV
Ser-Lys-Pro-Gly-Lys-Ala-Lys-Ser
Ser-Ala-Lys-Lys-Ser
Ac-Cys-SS-Cys-NH2
oxidation / activation
V
COCHO-Lys-Pro-Gly-Lys-Ala-Lys-COCHO
COCHO-Ala-Lys-Lys-COCHO
Ac-Cys-SS-Cys-NH2
SYNTHESIS OF FUNCTIONAL PEPTIDES
VI
solid phase peptide synthesis
aa1-aa2-aa3-aa4-aa5-..... -aai
a) Synthesizer-assisted AOA-coupling
b) Manual AOA-Coupling
AOA - coupling / activation
VII
aa1-aa2-aa3-aa4-aa5-.....-aai-AOA
cleavage
OXIMATION: TEMPLATE + FUNCTIONAL PEPTIDES
aa1-aa2-aa3-aa4-aa5-..... -aai-COCH2ONH2
V
oximation
VIII
COCH=NOCH2CO-aai-.....-aa5-aa4-aa3-aa2-aa1
Ac-Cys-SS-Cys-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-
COCH=NOCH2CO-aai-.....-aa5-aa4-aa3-aa2-aa1
COCH=NOCH2CO-aai-.....-aa5-aa4-aa3-aa2-aa1
COCH=NOCH2CO-aai-.....-aa5-aa4-aa3-aa2-aa1

Adduct cleavage

After AOA attachment (manually or machine-assisted) AOA-activated peptides showed an unexpected HPLC behavior: Instead of eluting as a single major peak after synthesis, an elution pattern showing multiple scattered peaks was observed. Therefore, it was impossible to recover "single peak" purified AOA-peptides.

The problem was solved by 1 M HCl-acidolysis subsequent to peptide cleavage. HCl-acidolysis was carried out in disposable glass vials (4 ml sample vial, Wheaton). Peptide and 1 M HCl were mixed at 1mg peptide/100 µl HCl and agitated (magnetic stirring) for 1 h at RT. The reaction mixture was diluted 10-fold with H_2O/0.1% TFA and purified by semi-preparative HPLC.

This approach led to the expected products, aminooxyacetyl-derivatives of peptides Opc loop 2, IgA1-PC20 and IgA1-PA50. Presumably, this reversible adduct formation was caused by the high intrinsic reactivity of the aminooxyacetyl function. One reasonable, but speculative explanation would be the formation of ester adducts via the aminooxyacetyl terminus upon peptide cleavage.

Template synthesis (D99)

The peptide Ac-Cys-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Cys-NH_2 [157], designated D99, was synthesized as described in Chapter 2.8.1. After final Fmoc deprotection and acetylation, the resin was washed (DMF and methanol) and dried as described (Chapter 2.8.1). Subsequent to assembly the peptide was circularized *in-situ*, i.e. resin-bound. The following steps were performed manually under argon atmosphere.

Intramolecular disulfide bond (D99 → D99-O)

1.256 g acetylated peptide resin (corresponding to 1 g resin starting weight, i.e. 170 mmol) in 40 ml DMSO/DMF (2:1, v/v) was chilled in an ice-water bath (100 ml Erlenmeyer). To this was added a pre-chilled solution of 1.52 g iodine (5.2 mmol) in 24 ml DMSO/DMF (2:1, v/v). After 1 h shaking the resin was washed first with a saturated solution of vitamin C to remove excess iodine (3 x 40ml, 120 g/200 ml), then with DMSO (2 x 40ml), then DMF (2 x 40ml), then methanol (2 x 40ml), and dried for 1 h in high vacuum. The resin (1.122 g) was transferred to a 8 ml disposable glass bottle (sample bottle, Wheaton) and the peptide cleaved from the resin with cleavage mix 4 as described (Chapter 2.8.1). The resulting peptide D93-O was purified by HPLC as described in 2.8.1. It was shown to be the acetylated cyclized template (MALDI-MS, protonated mass MH^+ (monoisotopic) calc. 1072.6, found 1072.5).

Serine attachment (D99-O → D99-O-$(Ser)_4$)

Prior to use, Boc-Ser(tBu) was extracted from its DCHA salt. DCHA extraction was monitored by TLC (Silica gel 60 plates, Merck) in AcOEth:MetOH:H_2O = 10:2:1 with ninhydrin (1% v/v in acetone).

1 g Boc-Ser(tBu)*DCHA (MW 442.6) was dissolved in 100 ml ethylacetate in a 250 extraction funnel. After 18-fold extraction (1:1 v/v with 0.1 M HCl) the solvent appeared to be free of DCHA. Extraction was continued with 3x H_2O (1:1, v/v). The

organic phase was dried with 1g $MgSO_4$ and briefly centrifuged. The supernatant was evaporated at 40°C and subsequently dried o.n. in high vacuum over di-phosphorus pentoxide (P_2O_5). The yield was 287 mg (1.1 mmol), i.e. 48.6% based on a MW of 261.32 for pure Boc-Ser(tBu) contents.

Boc-Ser(tBu) (1.1 mmol) was then converted into an active ester derivative. Boc-Ser(tBu) and HOBt (0.149 g) were dissolved in chilled 400 µl DMF in a disposable glass vial (4 ml sample vial, Wheaton) and stirred for 5 min. with a magnetic stirrer at 0°C. 1.01 mmol (0.383 g) HBTU, 4.43 mmol DIEA (0.574 g or 0.774 ml) and more DMF were added (final vol. 2.2 ml). Agitation was continued for 10 min. at 0°C and for 30 min. at RT.

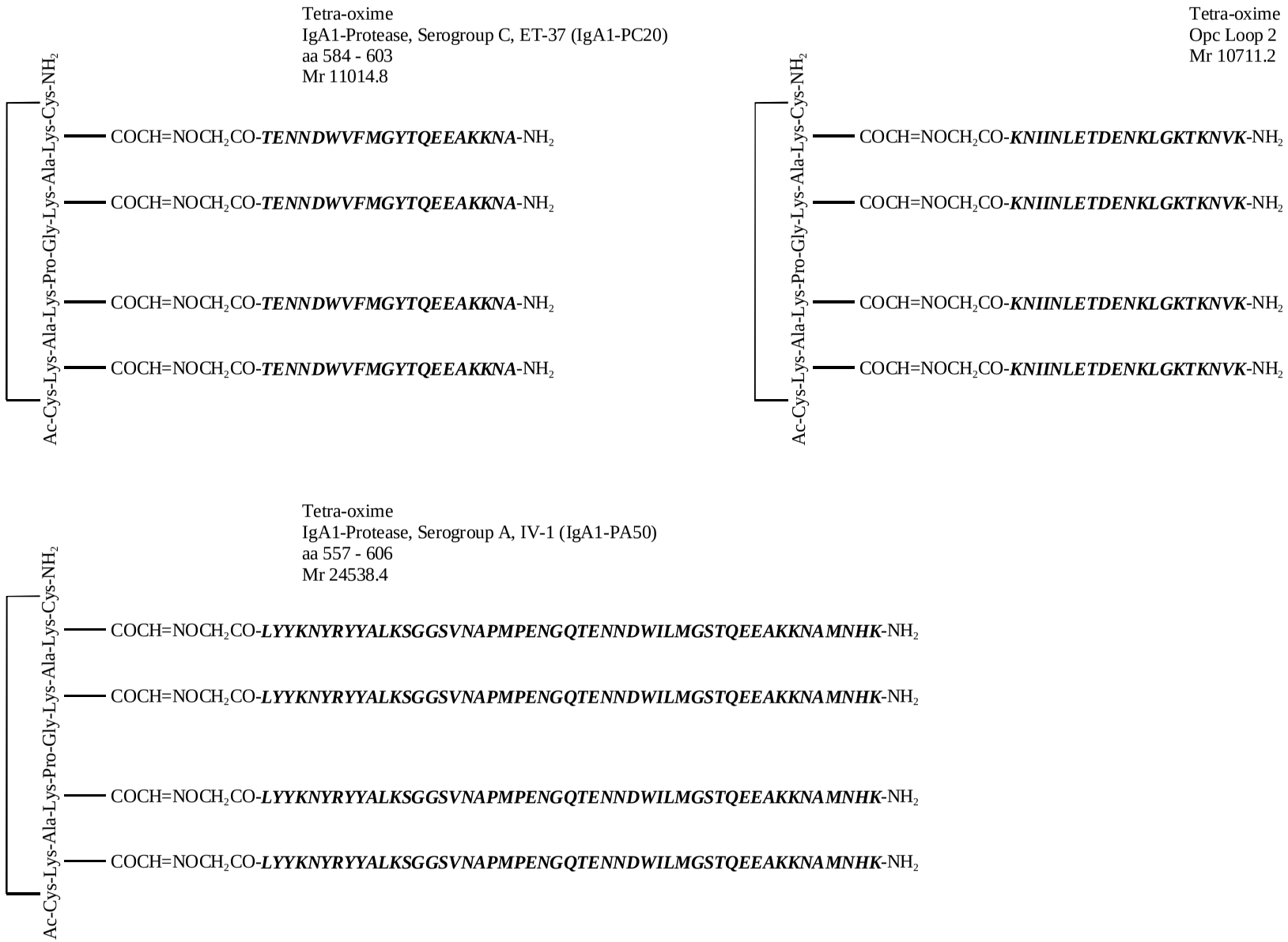
Boc-Ser(tBu) coupling: 34.6 µmol (37.1 mg) template D99-O was dissolved in 2 ml DMF and added to the Boc-Ser(tBu) reaction mixture. Turnover was monitored by analytical HPLC. The reaction was stopped at 5.5 h after the D99-O peak had completely been replaced by a more hydrophobic peak. To stop the reaction, the mixture (volume 4.2 ml) was diluted 1:1 with 90% acetonitrile/0.1% TFA (v/v in H_2O). Then 2.1ml of H_2O/0.1% TFA was added. After semi-preparative HPLC 41.9 mg dried product were obtained (20.49 µmol; yield 59% based on a MW of 2045 for D99-O-(Boc-Ser(tBu))$_4$).

Boc and tBu cleavage: 41.9 mg D99-O-(Boc-Ser(tBu))$_4$ was dissolved in 2 ml TFA and stirred for 1 h at RT (magnetic stirring; 4 ml sample vial, Wheaton). TFA was evaporated by a gentle stream of N_2 and the mixture concentrated to ca. 100 µl. The mixture was diluted with 3 ml H_2O/0.1% TFA and HPLC purified. The D99-O-(Ser)$_4$ compound again showed a rather hydrophilic retention behavior and eluted much earlier than D99-O-(Boc-Ser(tBu))$_4$. After drying 18.2 mg (12.8 µmol) D99-O-(Ser)$_4$ was collected. The desired template was HPLC purified and lyophilized before it was further oxidized.

Creation of aldehyde groups on the template
(D99-O-(Ser)$_4$ → D99-O-(HCOCO)$_4$)

The template in its serine form, prepared as above, was oxidized with periodate [158]. For this, 10 mg template was diluted with 9.4 ml imidazole buffer (50 mM, pH 6.95 with HCl), then 560 µl sodium metaperiodate (100 mM in H_2O) was added with mixing. After 5 min, the oxidation was quenched by adding 1.125 ml ethylene glycol solution (100 mM in H_2O). The oxidized template (D99-O-(HCOCO)$_4$) was isolated by semi-preparative HPLC and characterized by MALDI-MS (MH+ (monoisotopic) calc. 1296.5, found 1296.3) corresponding to Ac-Cys-Lys(HCOCO)-Ala-Lys(HCOCO)-Pro-Gly-Lys(HCOCO)-Ala-Lys(HCOCO)-Cys-NH_2.

Figure 4: Tetra-oximes Opc loop 2, IgA1-PC20 and IgA1-PA50


Tetra-oxime
IgA1-Protease, Serogroup C, ET-37 (IgA1-PC20)
aa 584 - 603
Mr 11014.8
Ac-Cys-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Cys-NH2
COCH=NOCH2CO-TENNDWVFMGYTQEEAKKNA-NH2
COCH=NOCH2CO-TENNDWVFMGYTQEEAKKNA-NH2
COCH=NOCH2CO-TENNDWVFMGYTQEEAKKNA-NH2
COCH=NOCH2CO-TENNDWVFMGYTQEEAKKNA-NH2
Tetra-oxime
Opc Loop 2
Mr 10711.2
Ac-Cys-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Cys-NH2
COCH=NOCH2CO-KNIINLETDENKLGKTKNVK-NH2
COCH=NOCH2CO-KNIINLETDENKLGKTKNVK-NH2
COCH=NOCH2CO-KNIINLETDENKLGKTKNVK-NH2
COCH=NOCH2CO-KNIINLETDENKLGKTKNVK-NH2
Tetra-oxime
IgA1-Protease, Serogroup A, IV-1 (IgA1-PA50)
aa 557 - 606
Mr 24538.4
Ac-Cys-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Cys-NH2
COCH=NOCH2CO-LYYKNYRYYALKSGGSVNAPMPENGQTENNDWILMGSTQEEAKKNAMNHK-NH2
COCH=NOCH2CO-LYYKNYRYYALKSGGSVNAPMPENGQTENNDWILMGSTQEEAKKNAMNHK-NH2
COCH=NOCH2CO-LYYKNYRYYALKSGGSVNAPMPENGQTENNDWILMGSTQEEAKKNAMNHK-NH2
COCH=NOCH2CO-LYYKNYRYYALKSGGSVNAPMPENGQTENNDWILMGSTQEEAKKNAMNHK-NH2

Oxime formation

For oxime formation, I introduced a guanidine HCl modification to the original method described by Rose *et al.* [155]. 10 mM solutions of the oxidized template (in H_2O) and 10 mM solutions of the aminooxyacetyl peptides (in 0.1 M Na-acetate buffer, 6 M guanidine HCl, adjusted to pH 4.6 with acetic acid) were prepared. In detail, 9 mg of peptide D92X (1.6 µmol) was dissolved in 400 µl buffer, 5 mg (2 µmol) peptide D100 in 400 µl and 5 mg peptide D101 (2.3 µmol) in 300 µl buffer. Oxime formations were initiated by addition of 0.13 µmol (13 µl), 0.16 µmol (16 µl) and 0.19 µmol (19 µl) template. The peptide derivatives were thus present in a 16-fold excess over the template or a 4-fold excess over each aldehyde group on the template. The chemistry of oxime formation is depicted in Figure 5.

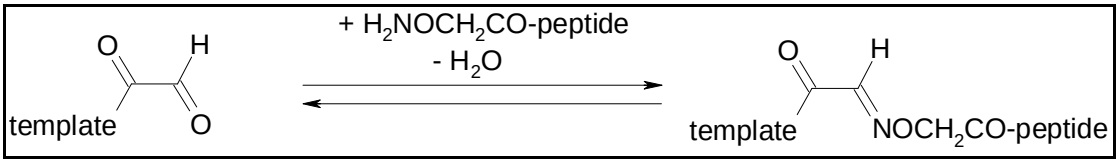


Figure 5: Oxime bond formation. The primary reaction is a nucleophilic attack of the H_2N- amino terminus of the AOA-peptide directed to the template aldehyde C-atom which is positively polarized due to the -I-effect of the proximal oxygens.

The reaction was carried out under argon in parafilm-sealed 2 ml Eppendorf safe-locks and monitored by analytical HPLC. HPLC chromatograms were characterized by a newly emerging, sharp product peak eluting at more hydrophobic conditions as compared to the AOA-precursors. After 15 h no further changes were detected and tetra-oximes were purified by shallow gradient semi-preparative HPLC and lyophilized. For the Opc loop 2 tetra-oxime (OX-Opc loop 2) 1.73 mg or 161 nmol were obtained (a yield of 85% based on 190 nmol template). OX-IgA1-PC20 resulted in 0.78 mg (70.8 nmol or 44%) and OX-IgA1-PA50 yielded 2.03 mg (83 nmol or 64%).

Mass spectrometry analysis of the tetra-oximes is described in RESULTS.

2.8.3. Peptide-carrier conjugation

Peptides were coupled to protein carriers by the *m*-maleimidobenzoyl *N*-hydroxysucccinimide ester (MBS) method [21,159].

In neutral aqueous solution, MBS reacts first by acylation of amino groups (here: on the protein carrier) via the active *N*-hydroxysuccinimide ester followed by formation of a thioether bond through addition of a thiol group (terminal cysteine on peptide) to the double bond of the maleimide [160].

Carrier proteins were dissolved at 5 mg/ml and peptides at 1-5 mg/ml in argon purged 50 mM phosphate buffer pH 7.4 and filtered through 0.2 µm filters (Gelman

Supor Acrodisc). Phosphate buffer was used instead of PBS which led to precipitation in some cases.

MBS was dissolved at 10 mg/ml in DMF. 1 ml carrier protein and 100 µl MBS solution were mixed in a 4 ml sample vial (Wheaton), covered with an argon layer and stirred for 30 min. on a magnetic stirrer at RT.

Excess MBS was removed by gel filtration with a HiTrap desalting column, at a flow rate of 5 ml/min. The absorbance at 278 nm was monitored and the peak fractions were pooled. The pH was adjusted to 7.0-7.5 if necessary. The activated carrier protein (MB-protein) was mixed with peptide in a molar ratio of peptide:protein of 30-40:1 under argon in a 8 ml sample bottle (Wheaton). After 3 h reaction at RT with stirring the conjugate preparations (vol. ca. 5 ml) were dialyzed at 4°C against PBS with 8 changes in 36 hours. After dialysis the size of the conjugates was analyzed by gel filtration (Superose 12, 1 cm × 30 cm, 0.8 ml/min, Pharmacia). Aliquots were stored at -20°C.

The coupling was confirmed by Western Blot analysis of the protein conjugates using MAb B306 (Opc loop 2 conjugates) and serum *αIgA1-PA50* (IgA1-PA50 conjugates), and against polyclonal serum preparations collected during the immunization work (IgA1-PC20 conjugates).

2.8.4. Cytokines as adjuvants

For peptides Opc loop 2 and IgA1-PA50 as well as for the tetra-oxime of IgA1-PA50 two cytokines have been co-administered as adjuvants along with Incomplete and Complete Freund's Adjuvant (IFC/CFA) in order to augment the immune response. For this purpose Interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been selected. The former has been shown to induce the proliferation and differentiation of B lymphocytes, the latter to have a systemic effect on primary and secondary responses [116,121,122].

2.9. Characterization of peptide antigens and immunogens

2.9.1. Mass spectrometry

Peptides and peptide derivatives were analyzed by MALDI mass-spectrometry, as summarize in Table 10 (PerSeptive Biosystems *Voyager-DE* or *Voyager Elite*, Bruker-Franzen *Reflex II*). Tetra-oximes were also tested by ESI-MS (Finnigan *TSQ 700* electrospray triple quadrupole).

MALDI-MS

The PerSeptive Voyager-DE/Elite and Bruker-Franzen *Reflex II* MALDI mass spectrometers were equipped with a N_2 laser (337 nm) and delayed extraction technology. The accelerating voltage was +20 kV (*Voyager-DE/Elite*) or +30 kV (*Reflex II*). For peptides of a molecular weight smaller than 3 kDa, mass spectra were recorded in linear and reflector mode. Molecules larger than 3 kDa were recorded in linear mode only. Spectra were obtained by summing over 256 (*Voyager-DE/Elite*) or 40 (*Reflex II*) laser pulses. *Reflex II* mass spectra were acquired and processed using the Bruker *Xtof* software package version 3.1 (on a SUN SPARC5 workstation). The *Voyager-DE/Elite* mass spectrometers were equipped with the Biospectrometry Workstation software package version 3.07 (PC, Windows 3.11).

Peptides were dissolved at concentrations of 10 pmol/µl in H_2O. Sample aliquots of 2-3 µl (containing at least 500-1000 fmol) were co-crystallized with 4-HCCA or DHB matrix. For that, samples were diluted with ACN or MetOH to give a concentration of 500 pmol/µl. A 0.3 to 1 µl portion of a 1:1 mixture of peptide and matrix (dissolved in ACN, 10 mg/ml) was deposited onto the target and airdried.

ESI-MS

Ionspray mass analyses were performed on a Finnigan *TSQ 700* LC-MS. The potential of the electrospray needle was placed at +4.5 kV to produce positive ions. The potential of the tube lens voltage was set to 80-160 V. Sheath gas pressure (N_2) was set to 50 psi.

Synthetic peptide samples were prepared at 1 mg/ml and diluted to 10 pmol/µl in aqueous solutions of 50% ACN, 1% acetic acid. Samples were injected at 2 µl/min. with a Harvard syringe pump connected to the electrospray interface via a Rheodyne 8125 injector. Mass analysis was conducted by scanning the mass-to-charge range of interest (m/z 300-1800) in 4 s. Data acquisition and processing was carried out with the ICSIS software package (version 8.2.1, Finnigan MAT) on a Digital DEC 3000 workstation.

Mass spectrometer	Type	Peptide		
		Designation		Reference
1	MALDI	D99-O	(tetra-oxime precursor)	Chapter 2.8.2
		D99-O-Ser_4	(tetra-oxime precursor)	Chapter 2.8.2
		D99-O-$(HCOCO)_4$	(tetra-oxime precursor)	Chapter 2.8.2
		D100	(tetra-oxime precursor)	Table 8, Chapter 2.4.2
		D101	(tetra-oxime precursor)	Table 8, Chapter 2.4.2
2	MALDI	D68		Table 8, Chapter 2.4.2
		D77B		Table 8, Chapter 2.4.2
		D77C		Table 8, Chapter 2.4.2
		D99-O		Chapter 2.8.2
3	ESI	D92X	(tetra-oxime)	Chapter 2.8.2
		D100	(tetra-oxime)	Chapter 2.8.2
		D101	(tetra-oxime)	Chapter 2.8.2
		D92X	(tetra-oxime precursor)	Table 8, Chapter 2.4.2

Table 10: Synthetic peptide antigens and mass spectrometers (1: PerSeptive Biosystems *Voyager-DE/Elite*, 2: Bruker-Franzen *Reflex II*, 3: Finnigan *TSQ 700.*

2.9.2. Sequence based secondary structure prediction

Sequence based secondary structure predictions (Chou-Fasman [161] and Garnier-Osguthorpe-Robson [162]) were calculated using the *pepstructure* module of the GCG software package (University of Wisconsin).

2.9.3. Circular dichroism

The structure of peptide IgA1-PA50 was assessed by Far-UV CD spectroscopy on a thermostat-equipped JASCO J-720. Spectra were recorded under nitrogen atmosphere (the boil-off from a nitrogen tank was used at a flow rate of 16 l/min). Parameter settings are summarized in Table 11.

CD-spectra were obtained at various peptide concentrations and buffer conditions. Cell path lengths were d=0.05 mm for 5mg/ml, d=0.1 mm for 1 mg/ml and d=0.05 mm for 0.1 mg/ml peptide concentration.

Samples were pre-incubated for 30 min. in order to reach temperature equilibrium for recording. Reported spectra are expressed in terms of mean residue ellipticities $[\Theta]_{MRW}$, which were calculated using a mean residue weight of 115.3.

Computer-aided spectrum analysis (Variable Selection, CONTIN) was carried out with the help of Dr. Klaus Gast, Max-Delbrück-Centrum für Molekulare Medizin. The programs Variable Selection and CONTIN were retrieved from W. C. Johnson, Oregon State University (ftp: ucs.orst.edu, directory: pub/user/varselec) and S. W. Provencher, Uslar-Schlarpe, Germany (http://www.provencher.de/contin.html), respectively. CONTIN contains the ridge regression procedure of Provencher and Glöckner [163], Variable Selection is based on the variable selection method of Manavalan and Johnson [164].

Parameter settings, Scan mode	
Temperature	25°C
Band width	1.0 nm
Slit width	Auto
Sensitivity	50 mdeg
Response	4 s
Start wavelength	260 nm
End wavelength	181 nm
Step resolution	0.5 nm
Accumulate	3
Alternator	off

Additional parameters, Temperature scan mode	
Start temperature	25°C
End temperature	80°C
Ramp rate:	50°C/h

Table 11: Circular dichroism. Jasco J-720 parameter settings.

2.9.4. Amino acid analysis

Amino acid composition (protein conjugates) and protein or peptide concentration (protein conjugates and liposomes) was determined by amino acid analysis. Sample preparation and amino acid analytical measurements were carried out by Gerlinde Grelle, Max-Delbrück-Centrum für Molekulare Medizin, Berlin.

Conjugate sample concentrations were adjusted to 0.5 mg protein/ml as estimated by a Genequant spectrophotometer. 2-4 µl samples, corresponding to 1-2 µg protein, were transferred to a glass vial and centrifuged under vacuum till dry. After drying, the samples were subjected to vapor phase hydrolysis [165] with 6 N HCl/7% thioglycolic acid [166] for 24 h at 110°C (*Dabs* hydrolysis vessel, Beckman). Dried hydrolysates were dissolved and diluted in 50 µl sodium citrate buffer (0.15 M, pH 2.2), such that 0.05-0.1 µg protein in 50 µl were applied to AAA. Samples were analyzed in a SYKAM analyzer (equipped with a SYKAM No. LCAK 06 cation exchanger) with post column derivatisation with orthophtaldialdehyde (OPA) reagent.

Fluorometry derived chromatograms (RF 535 Fluorescence HPLC monitor, Shimadzu) were processed using the program *Pyramide* (Axxiom).

2.9.5. Determination of peptide to carrier molar ratio

Peptide to carrier coupling ratio was calculated according to Shuler *et al.* [146]. The molar ratio of peptide to protein was determined by applying a least squares minimization method using the amino acid analysis data entered onto a Microsoft Excel worksheet template. The coupling ratio was obtained by comparing the conjugate composition with that of both the protein carrier and peptide analyzed separately. Amino acid analysis was performed with both carrier protein and conjugate in duplicate and the resulting values averaged.

The amino acid analysis values for cysteine, threonine, serine, methionine, tyrosine, and tryptophan have not been included in the calculation, since they may be partially destroyed by oxidation during the hydrolysis. Proline was excluded because of Kanda and Dunham's observation of simultaneous elution of proline and cysteine derivatives [146]. Histidine was not included in some calculations which showed a relatively high variation of histidine values.

The experimental amino acid composition for KLH was extrapolated to give values reflecting the number of mol of each residue per mol of KLH, based on an assigned MW of 3 MDa for KLH. For the purposes of defining the composition of peptide-KLH conjugates, setting the KLH MW to a particular value has no effect on the calculated mass ratios of peptide to KLH (i.e. the determined peptide concentration) even though the apparent molar ratio will vary with the assigned MW for KLH. Thus, for KLH, the reported weight concentration of the commercial stock solution becomes the reference point for quantification.

For BSA, ovalbumin, thyroglobulin and tetanus toxoid, amino acid compositions were calculated from the sequences of the SWISSPROT protein database entries (protein carrier/SWISSPROT accession No.): BSA/P02769, OA/P01012, TG/P01267, TT/P04958. Calculations were based on the corrected sequences of the mature proteins. In detail, for BSA the signal- (aa 1-18) and pro-peptide (aa 19-24) were subtracted from the precursor sequence for calculations. For TG the signal-peptide aa 1-19 was subtracted. Ovalbumin does not undergo proteolytic processing. Tetanus toxoid was based on the sequence of the tetanus toxin precursor which is cleaved before proline 2 and serine 458 to form a light chain/heavy chain dimer (light chain: aa 2-257, heavy chain: aa 461-1315; tetanus toxoid is prepared from tetanus toxin by formaldehyde inactivation).

Multiple regression, least squares analysis

In order to assess the peptide to carrier molar ratio of the protein conjugates, a multiple regression, least squares analysis was carried out as described by Shuler *et al.* [146].

A detailed derivation of equation (1) and an example using this calculations is given in *Appendix: Amino acid analysis*. In brief, the ProCOR (X_1) column represents the experimentally determined amino acid composition for 1 mol of the respective protein carrier. The PepCOR (X_2) column represents the theoretically determined values for 1 mol of antigenic peptide. The ProPep (Y) column is the experimentally determined amino acid composition (nmol) of the peptide-protein conjugate.

The amino acid analysis raw data were converted to experimentally determined amino acid residues (X_1) by fitting ΣX_1 to the number of residues based on the amino acid sequence.

In the determination of the molar ratio of peptide to carrier protein within a conjugate, the experimentally obtained value for the *i*th of *n* amino acid residues of the conjugate (Y_i) is considered as being dependent on the value for the *i*th of *n* amino acid of the carrier (X_{1i}) and peptide (X_{2i}) in some manner (see chapter 13 of Snedecor and Cochran [167]). The amino acid compositions of the conjugates are obtained by best-fit of multiple regressional analysis of Y_i, using the formula

(1) $$\ddot{Y}_i = a + b_1 X_{1i} + b_2 X_{2i}$$

where $\ddot{Y}_i$ is the unbiased predicted value for the *i*th residue of the conjugate, b_1 is the amount of carrier and b_2 the amount of peptide found in the conjugate sample (both in [nmol]).

b_1 and b_2 are used to express the molar ratio of peptide to carrier in the conjugate

(2) $$\frac{b_2}{b_1} = \text{"}coupling\ ratio\text{"}$$

In the course of the least squares fitting, the values for a, b_1 and b_2 are determined by minimizing the quantity $\Sigma(Y_i - \ddot{Y}_i)^2$, the sum of squares of the differences between n experimental and predicted Y values.

The determined peptide to protein coupling ratios of the individual conjugates are summarized in Table 17 (RESULTS, Chapter 3.3.3).

2.10. Purification of protein antigens

2.10.1. Purification of IgA1-protease

Serogroup A IgA1-protease was purified as described by Blake and Eastby (1991) [168] and modified by Morelli *et al.* [150].

As an exo-enzyme IgA1-protease was produced in a 10 l scale batch fermentation. The inoculum was prepared as follows: On 6 supplemented GC agar plates [169] strain Z6367 (*Nm* serogroup A, ET 67, IV-1) was grown for 14 h (37°C, humidified CO_2-incubator: 5% CO_2, 100% humidity, Forma Scientific). The cells were harvested and suspended in 100 ml 1x Proteose peptone III.

A 14 l fermentor (Microferm Fermentor, New Brunswiek Scientific) containing 10 l of supplemented GC medium was inoculated and cultivated at 37°C (low/medium level aeration, rotor speed 150 rpm) to late-exponential phase. Aeration was increased to “high” near the end of the fermentation. Foam formation was controlled by dropwise addition of 20% sterile Antifoam. The fermentation was stopped after 155 min. (Klett = 100, Klett photoelectric colorimeter). Since IgA1-protease is auto-proteolytic, it requires rapid down-stream processing at low temperature. Further operations were performed at 4°C.

The fermentate was centrifuged in 230 ml portions in a Sorvall RC-5B centrifuge (Type GSA rotor, 1,3 x 10^4 rpm/2.75 x 10^4 g, 20 min, 4°C). The cell debris was discarded, the supernatant collected on ice and solid EDTA added (final conc. 100 mM). The pH was adjusted to 9.0 with NH_4OH. $(NH_4)_2SO_4$ was added to a final concentration of 200 mM and the pH re-adjusted to 9.0.

The culture supernatant was applied to hydrophobic interaction chromatography (HIC) on a Phenyl Sepharose 6 Fast Flow high sub column (200 ml bed vol., 5 cm ∅, XK50 fast flow, Pharmacia): The column was equilibrated with 5 bed volumes of buffer A, and the supernatant loaded and washed with 1 l buffer A (flow rate 100 ml min^{-1}, peristaltic pump Peristaltic 2005, Verder). Column operations were switched to FPLC and washing was continued o.n. at 2 ml min^{-1}.

Elution was performed at 4 ml min^{-1} with a 800 ml linear gradient (buffer A/B) monitoring the absorbance at OD 0.5 (λ=280 nm). 10 ml fractions were collected. IgA1-protease eluted as a large peak followed by a second, smaller peak interconnected by a shoulder. The first peak was pooled (430 ml).

HIC was followed by FPLC ion exchange chromatography (IEC) on a SOURCE 15 S column (10 ml bed vol., type HR10/10 column, Pharmacia) which had been equilibrated with buffer C, o.n., 1 ml min^{-1}. The pooled protease preparation was adjusted to pH 4.0 (HCl), loaded and washed with 100 ml buffer C (5 ml min^{-1}). A linear gradient (buffer C/D) was applied over 90 ml (5 ml min^{-1}) and 2 ml fractions collected. Mature IgA1-protease eluted at 250-300 mM NaCl and was pooled (44 ml). After concentration by 2 h centrifugal ultrafiltration (Sorvall RC-5B centrifuge, type SS34 rotor, 6.4 x 10^3 rpm, 5 x 10^3 g; Macrosep 10 K, 10 kDa cut-off, Pall/Filtron), 8.6 ml of concentrated IgA1-protease were obtained. The protein concentration was determined to 0.39 mg/ml (3.44 mg total protein) by PIERCE. The IgA1-protease preparation was mixed with one volume of glycerol, designated FPLC 3539 and stored at -70°C.

Throughout the purification, sample aliquots were collected, precipitated in saturated $(NH_4)_2SO_4$ and analyzed by 11% acrylamide SDS-PAGE (Coomassie staining).

Serogroup C IgA1-protease had been purified by Norbert Brieske (AG Achtman, Max-Planck-Institut für molekulare Genetik).

2.10.2. Purification of Opc

Neisserial Opc outer membrane protein was purified from strain Z6310 as described by Achtman *et al.* [151] with modifications: TLCK (Sigma) was used as protease inhibitor instead of PMSF, and Mono Q/S resins were substituted by Source Q/S resins (Pharmacia) in ion exchange chromatography (IEC). All steps not otherwise described were carried out at RT.

Strain Z6310 was plated on a supplemented GC agar plate and grown o.n. as described under 2.10.1. The cells were harvested, transferred to 50 ml GC medium and plated out on 150 pre-incubated supplemented GC agar plates (square format, 12 x 12 cm^2, 50 ml, Greiner). These plates were incubated as before for 16 h and the bacterial lawn was scraped together with a glass slide (yield: 92.4 g wet weight).

Bacterial cells were resuspended in 11.1 ml 1 M Na-acetate, pH 4.0 (5 ml/50 g) containing 44.3 µl 50 mM 2,3-dimercaptopropanol (24 µl/50 g). Cells were homogenized using an Ultra-Turrax blender (5 min, RT). 45 ml 1 M $CaCl_2$ and 45 ml 10% Zwittergent were added to the bacterial suspension and dispersed again (Ultra-Turrax, 5 min, RT, final volume ca. 190 ml). Ice cold EtOH was added (50 ml, final conc. 20% v/v) for precipitation (5 min, on ice). The precipitate was removed by centrifugation (Sorvall RC-5B centrifuge, type GSA rotor, 15 min, 4°C, 1.3 x 10^4 rpm/2.7 x 10^4 g).

The pellet was discarded and the clear supernatant (150 ml) adjusted to 80% EtOH (450 ml ice cold 100% EtOH) and precipitated for 1 h on ice. After precipitation much of the supernatant volume was removed by vacuum suction. The preparation was centrifuged as above and the pellet collected (7.64 g). The sediment was thoroughly resuspended with a syringe in 400 ml resuspension buffer I. This suspension was stirred for 30 min. at RT. 30 mg TLCK was dissolved in 1 ml EtOH and added. The mixture was stirred at 65°C for 15 min. and cooled to 30°C. After centrifugation, the supernatant was filtered through a GVWP membrane (Millipore GV, 0.22 µm).

The Opc protein preparation was subjected to IEC. The material was applied to a tandem set-up of a Source Q followed by a Source S column (10 ml bed vol., type HR10/10 column, Pharmacia). The column matrices were equilibrated with buffer A and the sample loaded (6 ml min^{-1}). After the OD_{280} had returned to the baseline level, the Source S column was detached from the Source Q column and eluted with a NaCl gradient (buffer A/B: linear 750 ml gradient 0-20% B, followed by a linear 150 ml 20-100% gradient). The collected fractions (size 5 ml) were separately precipitated (80% EtOH, o.n., on ice) and analyzed by SDS-PAGE. Peak fractions were pooled and centrifuged (Sorvall RC-5B, type GSA rotor, 15 min. 1.3 x 10^4 rpm, 4°C).

The pellet was dissolved in 2.5 ml resuspension buffer II. This preparation was again centrifuged (Sorvall RC-5B, type SS34 rotor, 15 min. 1.5 x 10^4 rpm, 2.7 x 10^4 g), and the supernatant filtered (Gelman Supor Acrodisc 25) and applied to gel filtration on a Superose 12 column (300 ml bed vol., Pharmacia) which had been equilibrated o.n. (buffer C, 1 ml min^{-1}, 1.2 ml/fraction). The peak fractions were precipitated (80% EtOH, o.n., on ice), centrifuged as above at 4°C, and resuspended in 1 ml buffer C. Three Opc preparations were collected and designated FPLC 3137 through 3139. Preparations were confirmed to be free of LPS contamination by SDS-PAGE silver nitrate staining. Protein concentration was determined according to Schaffner and Weissman [170] and gave 5.35 mg/ml (FPLC 3137), 2.77 mg/ml (FPLC 3138) and 4.33 mg/ml (FPLC 3139) Opc protein (total yield 12.45 mg).

Throughout the purification sample aliquots were collected, precipitated in 80% EtOH (o.n., on ice) and analyzed by 11% acrylamide/3 M Urea SDS-PAGE (Coomassie staining).

2.11. Animals

Pathogen-free female BALB/c mice were obtained from Tierzucht Schöneweide (Berlin, Germany). Mice of the *peak response study* were kept in conventional open rack cages. Mice of the *Immunogenicity Study* were maintained in sterile cages

(isolators/ventilated cages and racks by Tecniplast) in groups of five, and provided sterile food and water *ad libitum*. They received the first immunization at 8 to 10 wk of age.

2.12. Immunization, serum and spleen collection

2.12.1. Summary of immunogens

immunogen/carrier		**peptide antigen**	
	Opc loop 2 20 aa	**IgA1-PC20** 20 aa	**IgA1-PA50** 50 aa
peptide, unconjugated	Pep	Pep	Pep
peptide, unconjugated + cytokines	CP	-	CP
multiple antigenic peptides	MAP	MAP	-
tetra-oxime	OX	OX	OX
tetra-oxime + cytokines	-	-	COX
liposomes	L	L	L
protein carrier	BSA OA KLH TG TT	BSA OA KLH TG TT	BSA OA KLH TG TT

Table 12: Summary of immunogens ("-" indicates "not prepared"; Immunogen abbreviations: free synthetic peptides (Pep), peptides co-administered with cytokines (CP), MAPs, tetra-oximes (OX), tetra-oximes co-administered with cytokines (COX), peptides associated with liposomes (L), coupled to bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OA), thyroglobulin (TG) and tetanus toxoid (TT).)

Various antigenic peptide formulations were synthesized with peptides Opc loop 2, IgA1-PC20 and IgA1-PA50 (Table 12). These included both free peptides (Pep) and diverse carrier-conjugated peptides: Peptides were coupled to protein carriers BSA, ovalbumin (OA), KLH, thyroglobulin (TG) and tetanus toxoid (TT) via C- or N-terminal cysteines. Aminooxyacetyl derivatives were assembled to tetra-oximes (OX). The peptides were also attached to liposomes (L) and the 20-mers were synthesized in the form of tetra-lysine multiple antigenic peptides (MAP).

Mice were immunised 3 times with all of these constructs Three immunogens were administered together with cytokines IL-4 and GM-CSF (peptides Opc loop 2 and

IgA1-PA50, CP; tetra-oxime IgA1-PA50, COX). As described in the following chapter, immunizations were performed in the presence of Freund's complete and incomplete adjuvant, except for liposomes.

2.12.2. Immunization

Peak response study

The *peak response study*, a pilot-study to the subsequent *Immunogenicity Study*, was performed with 8 of the immunogen formulations listed in Table 12. Two peptide antigens, Opc loop 2 and IgA1-PA50, were each tested as free peptide and conjugated to BSA, OA and TG. Immunogens were emulsified in Freund's complete (CFA) and incomplete adjuvants (IFA) as described for the *Immunogenicity Study* (next paragraph). Mice were immunized simultaneously on days 0, 14 and 28, and pairs of mice sacrificed on each of five consecutive days after the final injection at day 28. One cohort comprising of mice which had received Opc loop2 conjugates, was analyzed on days +3 to +7 post-immunization. The other cohort (IgA1-PA50 immunogens) was analyzed on days +2 to +6 post-immunization (Table 13).

Immunogen		**conc. per injection**		**immunization**			**analysis**
		peptide antigen	peptide or protein (total)	**1.** day 0	**2.** day 14	**3.** day 28	
peptide antigen	carrier	[pmol/inj.]	[µg/inj.]	site/adjuvants			days post immunization
Opc loop 2	Pep	204.6	0.5	c/CFA	i.p./IFA	i.p./IFA	3-7
	BSA	88.2	1.5				
	OA	124.3	2.0				
	TG	78.7	2.2				
IgA1-PA50	Pep	203.3	1.2	c/CFA	i.p./IFA	i.p./IFA	2-6
	BSA	54.1	1.4				
	OA	147.9	5.4				
	TG	45.4	1.1				

Table 13: Immunization schedule, peak response study. "pmol/inj." indicates the peptide antigen concentration per injection (abbr.: Pep, free peptide; BSA/ OA/ TG, peptide conjugated to BSA/ ovalbumin/ thyroglobulin; c, caudal; i.p., intraperitoneal; CFA/ IFA, 50% Complete/Incomplete Freund's Adjuvants in PBS).

In contrast to the *Immunogenicity Study* (next paragraph), the immunogens were standardized only for protein contents rather than for the amount of peptide antigen. Immunogens were (1-5 µg peptide or protein per injection). Thus, peptide antigen concentrations varied between 45.5 and 263.8 pmol/inj. (as determined later during the molecular characterization of the immunogens).

In this part of the work 82 mice were analyzed: 2 mice per immunogen and per day of analysis were tested, including one control group immunized with PBS.

Immunogenicity study

Mice of the *Immunogenicity Study* received normalized amounts of immunogen (peptide antigen conc.: 200 pmol/injection; refer to Chapter 3.3.4: Molar normalization of immunogens). Immunogens in 50 µl PBS were emulsified with 50 µl FCA or IFA (injection volume 100 µl), except for liposomal antigens which were applied in PBS only. The emulsion was mixed with a 2 ml disposable syringe until a stable, viscous emulsion had formed. Cytokine preparations contained 110 ng each of recombinant murine GM-CSF and murine IL-4 per injection. Both cytokine preparations contained supplemented BSA for stabilization: GM-CSF 5.5 µg BSA/injection, IL-4 0.11 µg BSA/injection.

All immunogen formulations in Table 12 were tested. Groups of 5 mice were immunised 3 times on days 0, 14 and 28. They were all analyzed on day 3 post-immunization.

For 29 immunogen formulations a total of 150 mice was tested: 5 mice per immunogen, including one control group immunized with PBS.

2.12.3. Blood sampling

Mice were bled prior to being sacrificed for spleen excision from the tail vein. Samples were allowed to coagulate for 30 min. Blood samples were centrifuged at 2500 g for 5 min. and the serum supernatant was stored at -80°C.

2.12.4. Spleen excision

Mice were killed by cervical dislocation. Spleen excision was carried out under sterile conditions in a horizontal laminar flow cabinet. Spleens were immediately transferred to Hanks' Salts (BSS) cell culture medium and processed as described in 2.13.2.

2.13. Immunoglobulin measurements

2.13.1. ELISA

Murine sera were analyzed by ELISA.

Coating, blocking

96 well microtiter plates (Nunc Maxisorp) were coated with synthetic peptide (Opc loop 2, IgA1-PC20, IgA1-PA50: "peptide coating") or purified protein antigens

(Opc protein, serogroup A/C IgA1-protease: “protein coating”). Peptides and proteins were prepared in carbonate buffer pH 9.6. Peptides were taken from 1 mg/ml stock solutions and sonicated for 1 min. after thawing. IgA1-protease was inactivated prior to incubation (5 min, 96°C) in order to avoid auto-proteolysis. Plates were incubated with 50 µl/well (4°C, o.n., wrapped in plastic). Peptides were coated at 1 µg/ml, except for IgA1-PA50 which was 3 µg/ml. Proteins were coated at 2 µg/ml.

After coating, plates were washed 3 times with PBST80 with an automatic plate washer (ImmunoWasher NK-350) and blocked with 200 µl/well PBS/Tween 80, 0.1% for 1 h (RT, horizontal shaker).

Diluted sera were added and incubated for 90 min, RT, and washed 3 times as described above. For the color reaction, plates were incubated at 37°C with 100 µl/well substrate solution. The color reaction was stopped by adding 100 µl/well of 1 M NaOH and OD_{405} was measured with a Microplate reader.

The plates contained duplicate standard curves of reference sera for calibration (twofold serial dilution). Test serum samples were analyzed in duplicate in 10-fold serial dilutions ranging from 1 x 10^2 to 1 x 10^5 for detection of antigen-specific IgM and from 1 x 10^3 to 1 x 10^6 for IgG quantification.

Two coated wells, incubated with buffer instead of serum or antibodies, were included on each plate as blanks for both the standard curve and the test sera. Their average values were subtracted from all standard curve and test sera.

ELISA serum standard

The standard curves were fitted to a sigmoidal curve after four-parameter fitting [171] using the equation

$$OD = \text{Min } OD + \frac{\text{Max } OD - \text{Min } OD}{1 + \left(ED_{50} / c\right)^{slope}} \quad (8)$$

where *OD* is the measured optical density and *c* is the concentration of antibodies in that well. The four parameters were fitted. Min *OD*, Max *OD*, ED_{50} and *slope* were calculated by nonlinear best fitting using the program Origin.

Serum sample values were calculated by interpolation from the standard curves obtained on the same plate. This interpolation was based on the following converted expression of (8):

$$c_{calc} = dilution \times \left(ED_{50} \times \left(\frac{\text{Max } OD - OD}{OD - \text{Min } OD} \right)^{-1/\,slope} \right) \quad (9)$$

where c_{calc} is the calculated concentration. *Dilution* is the dilution of the tested serum sample and the other parameters were those calculated above.

Serum standard calibration

Three standard sera, designated pms2 Opc loop 2, pms2 IgA1-PC20 and pms2 IgA1-PA50 were prepared by pooling serum aliquots from the final bleedings of the mice tested (pms, **p**ooled **m**urine serum **s**tandard). These standard sera were calibrated

with respect to their peptide- or protein-antigen-specific IgG concentration against an external standard of known IgG concentration (MAb S3141/A222):

For each of the 3 peptide and 3 protein antigens three independent calibrations were performed. Duplicate two-fold serial end-point dilutions of the IgG standard and the pms standard sera were placed in parallel on one plate. The plates were incubated and developed as described above.

Differences in affinity of the IgG standard and the pms standard sera were corrected by a factor *F* as follows:

After incubation with the IgG standard or the pms standard sera and just before washing and adding conjugate, the contents of each well was transferred to a fresh coated and blocked plate which was then treated identically to the original plate. For each external standard and standard serum, the average ratio for all wells within the dilution series was calculated between the *OD* values on the second plate and the corresponding wells on the first plate:

(10) $R = OD_{2.} / OD_{1.}$.

Then the factor F was calculated for each standard serum as

(11) $$F = \frac{1 - R_s}{1 - R_t}$$

where R_s is the average ratio for the external standard curve on that plate and R_t is the average ratio for the standard serum to be calibrated.

Standard serum	IgG [µg/ml] peptide specific	protein specific
pms-Opc loop 2	285.5	-
pms-IgA1-PC20	1531.9	760.2
pms-IgA1-PA50	1484.9	873.9

Table 14: Pms standard sera. Peptide- and protein-antigen-specific IgG concentrations.

The IgG standard OD curve was then fitted as described above (equation (8)). The resulting parameters were used to calculate antibody concentrations in the pms standard dilution series (equation (9) which were corrected by *F*. If consistent values were obtained, the IgG concentration of the individual pms standard serum was calculated as the geometric mean of the three independent measurements (Table 14).

Pms-Opc loop 2 α-protein could not be calibrated due to unspecific reactivity of the detection antibody with Opc protein.

It was not possible to calibrate IgM standards, because of different binding kinetics of the murine standard sera with commercially available IgM standards. Therefore, IgM standards arbitrarily were set to 10^5 U (arbitrary units). Hence, IgM titers can be compared within each of the groups tested against the same antigen only.

2.13.2. ELISPOT

Cell preparation

All buffers and media were prepared in pyrogen free water (aqua pro injectione, Braun) except for the procedures subsequent to cell incubation.

After excision (2.12.4), spleens were transferred to 3 ml Hanks' Salts (BSS) (Falcon, 15 ml tubes). Splenocytes were harvested by passing the spleens through a high-grade steel sieve into a Petri-dish using the base of a disposable syringe piston. The cell suspension was transferred back to the 15 ml Falcon tube. Piston, sieve and Petri-dish were rinsed 3 times with 3 ml of BSS. Cells were centrifuged at 400 g (Sorvall RC-5B RT6000, 1400 rpm) for 5 min. (4°C) and the supernatant discarded. To lyse erythrocytes the cell pellet was resuspended in 1 ml 0.15 M NH_4Cl, pH 7.2 and incubated for 2 min. at RT (lysis of erythrocytes by osmotic shock). 9 ml BSS was added and the cell preparation again centrifuged as described (wash I). The cells were washed two more times and resuspended to a volume of 10 ml BSS (wash II, III).

Wash III resuspended splenocytes were counted as Trypan blue-excluding ("viable") cells in a type "Bürker" chamber and centrifuged as above. The pellet was finally resuspended in Click-Medium/10% FCS and adjusted to 5 x 10^6 cells/ml in 45 ml Falcon tubes.

Coating, blocking

Nunc Maxisorp 96-well plates were incubated with antigen as described for ELISA (Chapter 2.13.1), except that peptides and proteins were coated at 5 µg/ml in the immunogenicity study. Plates were incubated with 50 µl/well (4°C, o.n., wrapped in plastic).

Coating buffer was discarded and plates were washed three times with an automatic microtiter plate washer (ImmunoWasher NK-350; washing buffer: PBS/Tween 20 0.05%). 100 µl/well of blocking buffer (PBS/Tween 20 0.1%, skim milk 0.5%) was added and incubated for 1-2 h at RT.

Incubation

Blocking buffer was discarded and plates were washed 3 times again, as described above. 200 µl of the cell preparation was transferred to rows A and E of the microtiter plates. All the other wells were filled with 100 µl of complete medium. Sample were diluted in triplicates in two-fold serial dilutions from 5 x 10^5 to 0.625 x10^5 cells/well and incubated (4 h, 37°C, 5% CO_2, 100% humidity) on a flat surface without moving.

Incubation was stopped by gently rinsing the microtiter plates 4 times with H_2O, followed by three-fold washing in an automatic washer (washing buffer: PBS/Tween 20 0.05%).

ASC-detection, enumeration

Alkaline phosphatase enzyme-conjugated developing antibodies specific for murine immunoglobulin isotypes A, E, IgG, G1, G2a and M were used for the detection of antigen-specific ASC. Antibodies were diluted in PBS to the concentrations in Table 4 and 50 µl/well incubated at 4°C, o.n.

The antibody solution was discarded and plates were washed three times, as described above. Spots were developed by adding BCIP/AMP solution (50 µl/well) and incubating for 1-5 h. The color reaction was stopped by rinsing with H_2O after spots had become clearly visible.

The numbers of ASC were enumerated under a microscope (Zeiss) at 30x magnification. ASC frequencies were plotted as individual values (*peak response study*) or mean (± standard error) of 5 animals per group (*Immunogenicity Study*).