3. RESULTS

3.1. Overview

The aim of this thesis is to compare different common carriers and immunogen formulations in their immunostimulatory potency for peptide immunization. In pursuing this goal the project was organized into three parts which form the underlying structure of the RESULTS section (Figure 6):

I. Synthesis of peptide immunogens
II. Molecular characterization of peptide immunogens
III. Evaluation of immunogenicity of different formulations

Chapter 3.2 introduces and describes the synthesis of 3 model peptide antigens corresponding to epitopes of the human pathogenic bacterium *Neisseria meningitidis*: two 20-mer peptides derived from neisserial Opc invasin and serogroup C IgA1-protease, and a 50-mer peptide from serogroup A IgA1-protease. Peptides were synthesized by solid-phase peptide synthesis in their free forms, and as cysteine- and aminooxyacetyl variants. Cysteine-derivatives were coupled to liposomes or further assembled into protein carrier conjugated immunogens, including BSA, ovalbumin, KLH, thyroglobulin and tetanus toxoid carriers. Aminooxyacetyl peptides were condensed to form tetra-oxime type synthetic proteins. Additionally, the two 20-mers were synthesized as multiple antigenic peptides (MAPs). In all 29 different immunogens took part in this “immunogenicity rally”.

Following synthesis and assembly, peptide antigen constructs underwent molecular characterization which is the subject of **Chapter 3.3**. Synthetic peptides or peptide derivatives were analyzed by mass spectrometry. Chromatographic methods were used

for tetra-oxime characterization and circular dichroism analysis was carried out on the secondary structure of the 50 amino acid (aa) peptide IgA1-PA50. Liposomal preparations were subjected to amino acid analysis (AAA) in order to measure molar peptide concentrations, and the protein carrier conjugates were similarly analyzed to determine the peptide to carrier coupling ratio. The latter was done by applying a multiple regression, least squares analysis method [146] to amino acid analysis data.

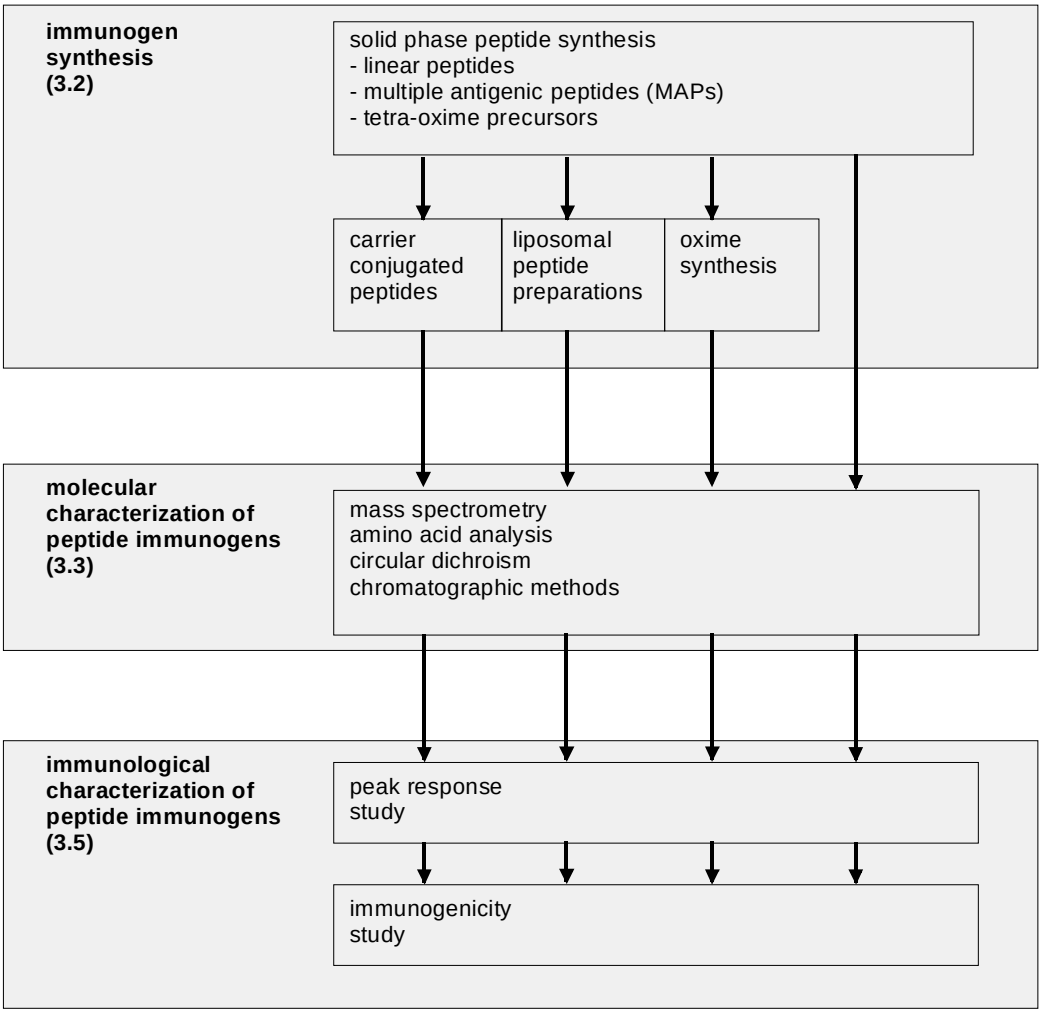


Figure 6: Project overview. Numbers in brackets refer to Chapter No. in RESULTS.

Chapter 3.5 covers the immunological characterization of the peptide immunogens, i.e. the evaluation of peptide immunogenicity. Mice were immunized with the constructed peptide immunogens, and their potency to stimulate an antigen-specific antibody response was measured. Chapter 3.5 is subdivided into two parts, (1) a *Response Peak Study* and (2) an *Immunogenicity Study*.

The ***Response Peak Study*** **(3.5.1)** first introduces a murine BALB/c model to the project. The *Response Peak Study* was designed as a pilot study and was conducted in

order to characterize the time course of the murine immune response. It focused on the evaluation of two dynamic parameters of the murine immune response, splenocyte activation and antibody titer fluctuation. Mice were immunized with a partial set of 8 peptide immunogens. Peptide antigen immunogenicity was assessed by monitoring two main parameters: (1) the induction of antigen-specific serum antibodies and (2) the stimulation of splenocytes secreting antigen-specific antibodies. Titers of serum antibodies were measured by ELISA. Splenocyte activity was estimated by an ELISPOT assay, a technique which is described in Chapter 3.4. The *Response Peak* pilot study provides the basic parameters needed to design the subsequent main study, the *Immunogenicity Study*.

The ***Immunogenicity Study*** **(3.5.2)** is the focus of the project. Here, the entire set of 29 peptide immunogens is tested. Mice were immunized with peptide antigen, at equimolar concentration following a standardized immunization protocol. Immunogens were administered with Complete (initial immunization) and Incomplete (all subsequent immunizations) Freund's Adjuvants. Additionally two peptides and one tetra-oxime formulation were co-administered with cytokines. Immunogen potencies were again monitored at the serum level (antibody concentrations) and at the cellular level (splenocyte activity) for both synthetic peptide-specific antibody response and reactivity with native protein.

3.2. Immunogen synthesis

3.2.1. Synthetic peptide antigens

Three model peptide antigens derived from virulence factors of *N. meningitidis* were utilized for this study.

The peptides were: (1) a 20-mer representing loop 2 of the neisserial Opc invasin, (2) a 20-mer of serogroup C IgA1-protease and (3) a 50 amino acids stretch of meningococcal serogroup A IgA1-protease. The peptide antigens were designated Opc loop 2, IgA1-PC20 and IgA1-PA50, respectively. Their sequences are given in Table 8.

Opc loop 2

Peptide *Opc loop2*, 20 aa in length, is derived from the meningococcal outer membrane protein Opc. Opc is hypervariable in expression and mediates adhesion to, and invasion of, endothelial and epithelial cells [172,173]. Opc invasin is immunogenic in humans and stimulates bactericidal antibodies [174].

Peptide *Opc loop 2* represents amino acids 61-80 of the mature protein and spans the surface exposed loop 2 according to a 2-dimensional structural model developed by Merker *et al.* [175]. Loop 2 of Opc protein appears to be highly conserved. Only 18 Opc allelic variants were found in a representative set of 152 strains of *N. meningitidis*, of which only one allele showed a single aa substitution in this stretch: $N_{78} \rightarrow D_{78}$ [176].

Surface exposure has been demonstrated by inserting a foreign epitope from Simliki Forest virus into loop 2. This insertion eliminated reactivity of loop 2 with reference antibodies and resulted in reactivity with Simliki Forest virus-specific MAbs in whole cell preparations when expressed in *E. coli* [175]. Furthermore, monoclonal antibodies specific to loop2 inhibited invasion.

Three monoclonal antibodies have been described as being reactive with loop 2 and their binding motifs have been mapped by pins-ELISA [175]. Of these, B306 [151] was selected as an Opc loop 2-specific reference antibody for the present study.

Opc loop 2 as a model antigen:

As evident from pins- and inhibition-ELISA analysis [175], various peptides corresponding to stretches of loop 2 of Opc protein (including peptide *Opc loop 2*) are reactive with MAbs derived from Opc immunization. In contrast, nothing is known about whether peptide *Opc loop 2* by *itself* is immunogenic, i.e. able to confer a specific antibody response.

IgA1-PC20 and IgA1-PA50

Peptide IgA1-PC20, 20 aa in length, is derived from amino acids 584-603 of meningococcal serogroup C IgA1-protease (ET-37 complex). IgA1-PA50 represents a 50 amino acids stretch (aa 557-606, mature protein) of meningococcal serogroup A, IV-1 IgA1-protease [150]. Their relationships are shown in Figure 7.

IgA1-proteases (IgA1-Pep) are a family of bacterial enzymes that specifically cleave human IgA1, the immunoglobulin that provides antibody defense on mucosal surfaces. These proteases are a unifying characteristic of a clinically important group of bacteria responsible for human infections of many kinds, setting them in contrast to taxonomically related bacteria that are IgA1-protease negative and do not cause disease [177,178]. Besides *N. meningitidis*, prominent members of this group are *Neisseria gonorrhoeae* and *Haemophilus influenzae*. Proteases with similar specificity to other immunoglobulin isotypes (IgE, IgG, IgM etc.) are not known, a phenomenon which is still not understood.

Meningococcal IgA1-protease is able to confer a strong and persistent IgG response upon infection or even asymtomatic nasopharyngeal carriage [171,179]. Comparing the human and the murine immune response on immunization with IgA1-protease, intriguing differences were found: Most B-cell epitopes recognized by murine monoclonal antibodies differ from epitopes recognized by human sera, as demonstrated in epitope mapping and inhibition ELISA analysis [150,180,181]. There is one exception however, murine epitope 4, part of which is also recognized by human antibodies.

Peptide IgA1-PA50 is derived from murine epitope 4 of serogroup A protease, spanning aa 557-606 of the mature protein. Epitope 4 is a discontinuous or conformational epitope and is recognized by the murine MAb AH623. This MAb also reacts well with a long 104 aa synthetic peptide of epitope 4, fairly well with the IgA1-PA50 50-mer, and loses reactivity upon further C- or N-terminal truncation. Moreover, it was impossible to map epitope 4 using the Geysen technique of scanning with short pin-bound 12-mers [180].

Both, the 104 amino acid peptide and peptide IgA1-PA50 are highly immunogenic in mice (data not shown). A cysteine variant of the 104-mer has been successfully tested as a peptide carrier for peptide-polysaccharide conjugates [182], potentially offering its services in bacterial polysaccharide vaccines. It could be shown that this 104-mer is as good a carrier as a "classical" diphtheria toxoid carrier protein.

```
                1          11         21         31         41         50
IgA1-PA50 LYYKNYRYYA LKSGGSVNAP MPENGQTENN DWILMGSTQE EAKKNAMNHK
IgA1-PC20                              TENN DWVFMGYTQE EAKKNA
consensus                              TENN DW--MG-TQE EAKKNA
```

Figure 7: Alignment of IgA1-protease derived synthetic peptide antigens (IgA1-PA50: aa 557-606 of serogroup A IV-1 IgA1-protease; IgA1-PC20: aa 584-603 of meningococcal serogroup C ET-37 IgA1-protease; "-" indicates no consensus).

About 30 amino acid positions from the N-terminus of IgA1-PA50, three polymorphic amino acids result in the absence of epitope 4 in serogroup C protease.

Hence, serogroup C protease is not recognized by the "conformational" MAb AH623. Interestingly, exposure to serogroup C meningococci seems to stimulate antibodies to an epitope of sufficient sequence homology to allow inhibition of binding of Mabs to serogroup A epitope 4 [150].

Scanning serogroup C IgA1-protease with human sera by Geysen's pepscan technique (12-mers) unveiled human antibodies that recognize a motif (*NNDWVFMGYTQEEAK*) lying within a stretch corresponding to epitope 4 (human) and epitope 9 (murine) of serogroup A IgA-P (Figure 7, [180]).

IgA1-PC20 and IgA1-PA50 as model antigens:

In preceding experiments, peptide IgA1-PA50 had been shown to be highly immunogenic and to induce anti-peptide antibodies cross-reactive with the cognate structure, serogroup A IgA1-protease. Apparently, IgA1-PA50 mimics a conformational epitope which, at least in part, seems to be present in native IgA1-protease. In this study, peptide IgA1-PA50 was compared with a truncated peptide, a 20 aa peptide motif lying within IgA1-PA50 (Figure 7) encoding an epitope in serogroup C IgA1-protease that is recognized by some human sera.

IgA1-PC20 and IgA1-PA50 were considered to be appealing candidates for investigating the influence of carriers and their context on the immunogenicity of peptide antigens: Two peptides of a similar origin but of distinct immunological background (murine/human epitope) and different physiological features (size, structure, sequence polymorphism).

Peptide synthesis

Peptide antigens were synthesized by solid-state Fmoc chemistry as described in MATERIALS AND METHODS. Following SPPS, peptides and peptide derivatives were confirmed by mass-spectrometry. Additional structural analysis by circular dichroism was performed with IgA1-PA50.

Peptides were tested for immunogenicity in various formulations which were grouped into 4 categories regarding their mode of antigenic peptide presentation.

1. unconjugated
2. conjugated to protein carriers
3. coupled to liposomes
4. synthesized as MAPs and tetra-oximes

In the first group immunogen formulations consisted of free synthetic peptides only. Their molecular weights were calculated from the amino acid sequence and confirmed by mass spectrometry.

Mass-spectrometry

The molecular weights of the antigenic peptides were 2444 g/mol for the Opc loop 2 20-mer and 2520 g/mol for the serogroup C IgA1-protease 20-mer (IgA1-PC20). Two variants were available for IgA1-PA50: An unmodified variant was used for immunization with free peptide (5798 g/mol) and a cysteine-derivative (5901 g/mol) for coupling to carriers (Table 17, p. 90). Synthetic peptides and their derivatives are described in Chapter 2.8, p. 41 (Synthesis of peptide antigens and immunogens). Their

mass-spectrometry measurements are summarized in Table 8, p. 44 of MATERIALS AND METHODS. A typical mass-spectrum is depicted in Figure 8.

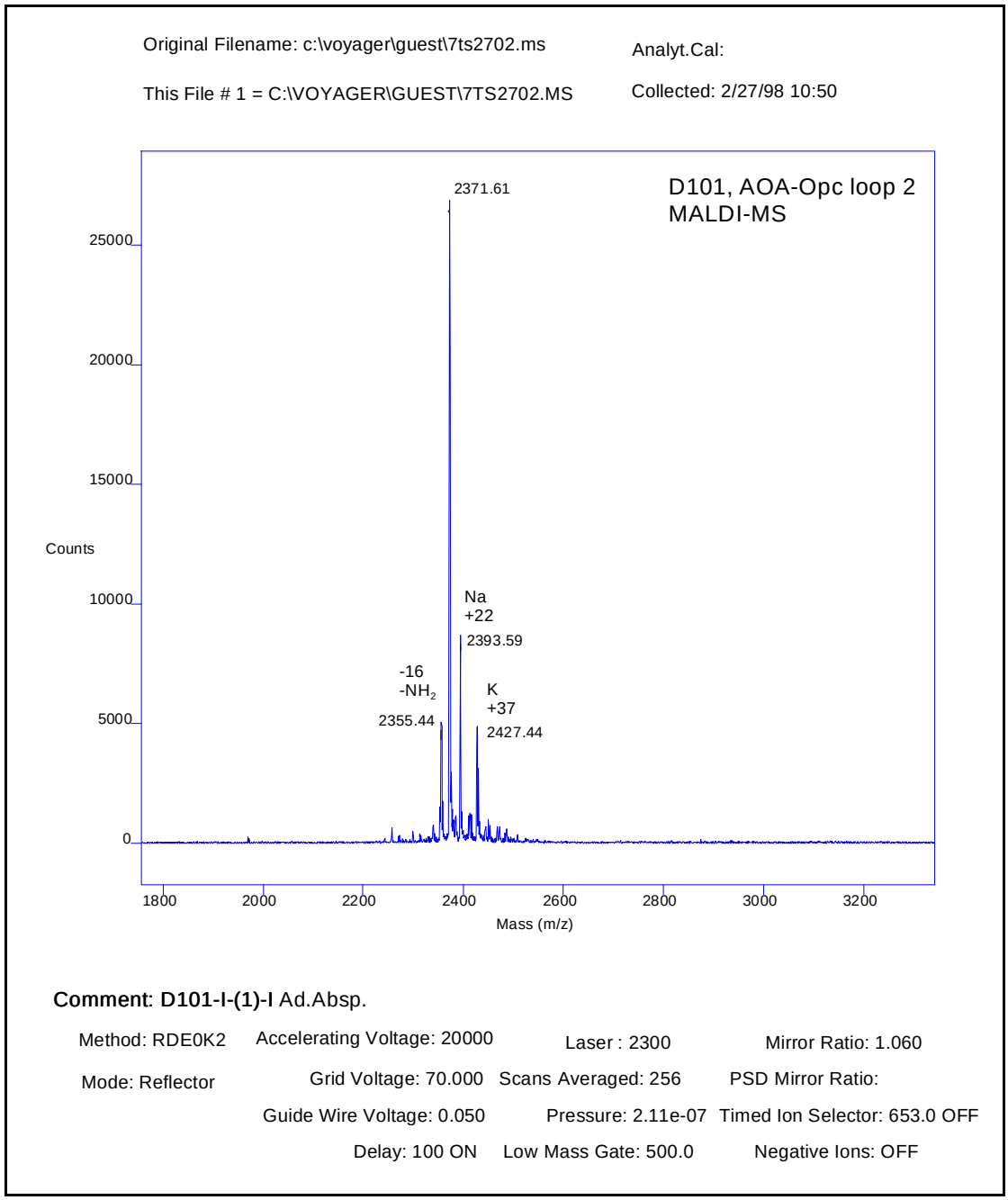


Figure 8: Mass-spectrum of peptide AOA-Opc loop 2, MH^+ calc. 2372.7, found 2371.6.

3.2.2. Protein carrier-conjugated peptides

BSA , KLH , ovalbumin, thyroglobulin and tetanus toxoid were chosen as carriers. The selection of the protein carriers was based on a literature survey. It appeared that BSA and KLH are the most frequent protein carrier structures used for peptide immunization [75], along with ovalbumin [145]. Other prominent carrier structures are thyroglobulin and tetanus toxoid [75,148].

In this study the bifunctional crosslinker MBS was utilized for coupling antigenic peptides to protein carriers. The procedure requires a free sulfhydryl group on the synthetic peptide and free amino groups on the carrier protein. Although MBS is less flexible than non-aromatic linkers and is itself immunogenic [145], this method has the advantage of complete control of the geometry of peptide coupling to the carrier. Cysteine modifications allow the peptides to be coupled to MBS activated proteins. Other commonly used coupling reagents such as glutaraldehyde, bis(imido) esters, toluene diisocyanate and carbodiimides are not as suitable since they react mainly by coupling amino groups with amino or carboxyl groups and can result in extensive cross-coupling of proteins.

The peptides Opc loop 2, IgA1-PC20 and IgA1-PA50 were coupled to protein carriers through an additional terminal cysteine as described in MATERIALS AND METHODS. Following coupling, the conjugate immunogens were subjected to amino acid analysis. The amino acid analysis data output was analyzed by a computational analysis employing a least squares multiple regression method [146] in order to determine the peptide to carrier coupling ratio (MATERIALS AND METHODS).

3.2.3. Liposome associated peptides

The third group is liposome associated peptides. Peptides Opc loop 2, IgA1-PC20 and IgA1-PA50 were surface bound to small unilamellar vesicle (SUV) type liposomes [99]. The cysteine-peptides were conjugated to the liposomes via the amino-function bearing membrane component MPB-PE. These constructs were synthesized by Barica Kusecek (AG Achtman, Max-Planck-Institut für molekulare Genetik). To form SUVs, PC, PG, MPB-PE, cholesterol and MPLA (all from Sigma) were dissolved and mixed 65:20:15:50:10 in chloroform. The lipids were dried under vacuum, resuspended in 2 ml (to 10mmol lipid/ml, 10 mM Hepes, pH 6.5, 145 mM NaCl), covered with an argon layer and sealed (10 ml glass tube, Schütt). The liposome-preparations were then sonicated for 30 min. at 50°C. 1 ml of the preparation was subjected to extrusion (15 times, 2 Hamilton syringes connected by an extruder). Peptides were then coupled via their terminal cysteine to the liposomal MPB-PE. For that, 1.5 mg peptide in 500 µl 10 mM Hepes, pH 6.5, 145 mM NaCl, was mixed with 1 ml liposome-preparation and incubated (1 h, on ice, under argon). 10 µl β-mercaptoethanol were added to derivatise remaining maleimide groups. The liposomes were dialyzed at 4°C o.n. against two changes of 5 l PBS. They were stored at -80°C and restored by extensive vortexing and 15 min. of sonification.

3.2.4. MAPs

MAPs [61] were synthesized by SPPS. Only two of the three neisserial peptide antigens, Opc loop 2 and IgA1-PC20, are introduced as MAPs [183] as the SPPS of a functional MAP version of the large 50 amino acid IgA1-PA50 failed. It is known that Tam's multiple antigenic peptide technique experiences synthesis limitations for peptides exceeding a certain length [73,155].

3.2.5. Tetra-oximes

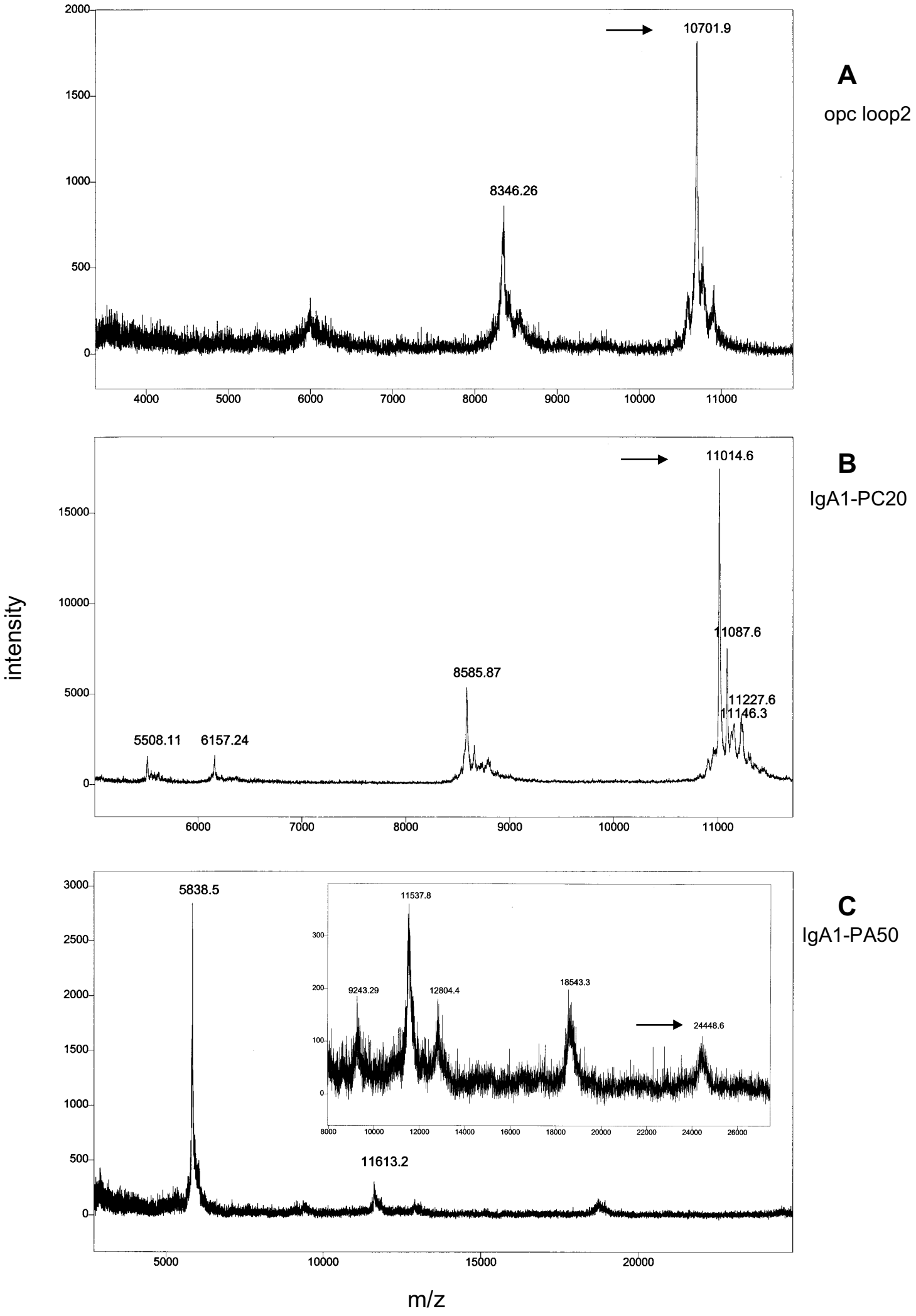
Quite recently an innovative approach to assemble synthetic peptide epitopes into homogenous artificial proteins was described by Rose and co-workers [10,155]: Based on oxime bonds it allows a number of unprotected synthetic or native polypeptides to be brought together in modular fashion to produce a well-defined, high purity macromolecule. The peptides are attached to a core frame called "template". The template provides activated aldehyde functions as coupling sites which bind aminooxyacetyl ligands under oxime-bond formation (Figure 5, p. 52). It is possible to attach at least 8 ligands to a template. Furthermore, non-peptide ligands can be utilized and different coupling chemistries included in a combinatorial chemistry approach [11,109,184,185]. Thus, different functional ligands can be selectively addressed to form a defined polyfunctional macro-structure.

In this project, this technique was applied to the construction of three 4-ligand (→"tetra-oxime") artificial protein immunogens based on the three meningococcal peptide antigens described above.

In Figure 3 (p. 47) the principal construction of the tetra-oximes is displayed schematically. The individual primary structures of the three oxime variants (OX-Opc loop 2: 10711 Da; OX-IgA1-PC20: 11015 Da; OX-IgA1-PA50: 24530 Da) are depicted in Figure 4 (p. 50). The precursor molecules (a circularized template and linear functional peptides) were synthesized by SPPS. The template was further modified and activated. Following synthesis oxime precursors underwent mass spectrometric analysis and their functionality was confirmed in trial oximations.

The template and linear peptide precursors then react to form an oxime-bond tetramer. This condensation reaction was monitored by analytical HPLC. For each of the three tetra-oximes a sharp product peak emerged which was then purified in semi-preparative HPLC (Precursor synthesis, oxime formation and purification are described in detail in MATERIALS AND METHODS).

Figure 9: MALDI-TOF mass spectra of tetra-oximes. The expected target masses are indicated by arrows. (A) Opc loop 2 tetra-oxime, experimentally determined protonated MW 10701.9 Da (calculated 10712.2 Da), (B) IgA1-PC20 tetra-oxime, experimentally determined MW 11014.6 Da (calculated 11015.8 Da) and (C) IgA1-PA50 tetra-oxime, experimentally determined 24448.6 (calculated 24539.4 Da). For (A) and (B) the molecular ion is clearly visible, although the laser partly breaks down polyoximes [186] resulting in peaks of smaller MW. For the IgA1-PA50 tetra-oxime (C) the spectrum is dominated by low molecular weight signals (5838.5 Da, 11613.2 Da). A separate scan in the higher m/z range ($8000 \leq m/z \leq 27000$) gave better resolution and showed the single protonated target molecule of 24448.6 Da (small inset).


A
opc loop2
10701.9
8346.26
B
IgA1-PC20
11014.6
11087.6
8585.87
11227.6
11146.3
5508.11
6157.24
C
IgA1-PA50
5838.5
11537.8
9243.29
12804.4
18543.3
24448.6
11613.2
intensity
m/z

MALDI-mass spectrometry analysis

Tetra-oximes were analyzed by matrix-assisted laser desorption and ionisation mass spectrometry, MALDI-MS. In Figure 9 MALDI mass-spectra are depicted. While spectra for the Opc loop 2 and IgA1-PC20 oxime constructs showed the expected target masses and also substantial fragmentation, the 24.5 kDa target peak of IgA1-PA50 was almost negligible. Instead, predominantly fragments of lower molecular weight could be seen. Rose *et al.* have described this MALDI laser decomposition phenomenon in oxime analysis; they reported that oxime bonds disintegrate under laser impact [10]. The low molecular signals are oxime torsos which have lost one or more peptide "arms". In (B) for instance, 11014.6 Da represents the single protonated tetramer, 8585.9 Da a trimer (-1 ligand), 6157.2 Da a dimer (-2 ligands) and 5508.1 Da is the 2× protonated tetramer (z=2).

Compared with (A) and (B) the IgA1-PA50 tetra-oxime signal was almost negligible (C). This was a puzzling observation since judging from the preceding steps of the synthesis a successful tetra-oxime formation had been anticipated. On the other hand, on account of the size of the 50 amino acid peptide, it was not sure whether sterical constraints would be an obstacle to OX-IgA1-PA50 synthesis. To overcome secondary structure interference, a guanidinium/HCl modification of the synthesis protocol had been successfully introduced for oxime construction.

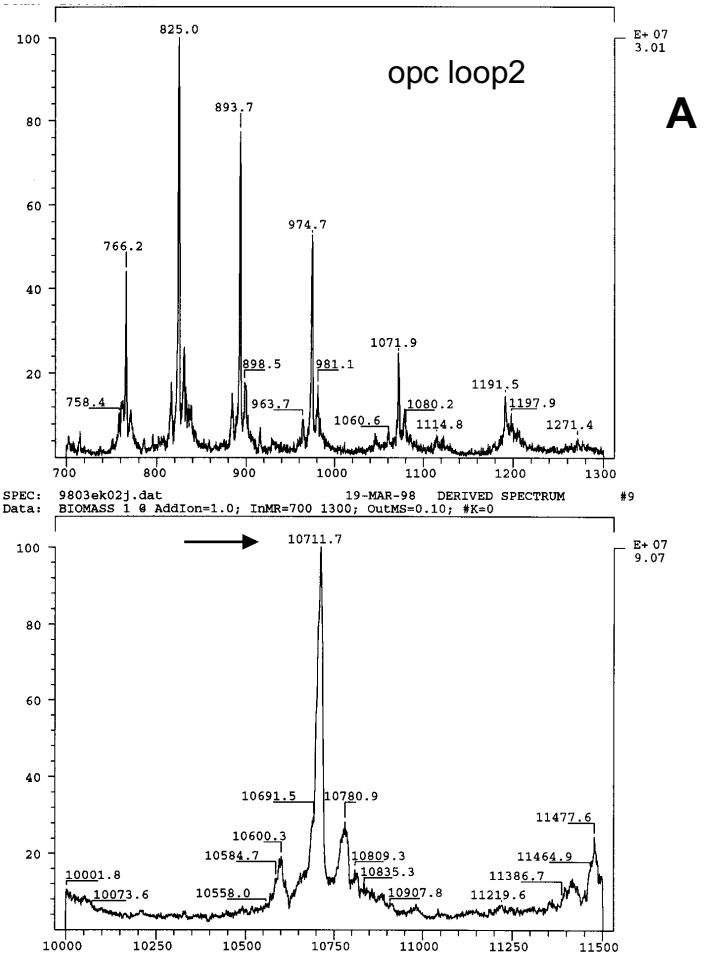
As it was not possible to positively confirm the IgA1-PA50 tetra-oxime by means of MALDI mass spectrometry, alternative methods were sought. Mass spectrometry output is not a quantitative measure. Rather it displays molecules with a sufficient ionisation capability, i.e. those sample compounds and compound fragments which could be accelerated by the field of force. If IgA1-PA50 tetra-oxime ionizes sluggishly it would be difficult to detect. If it is fragile it may decay upon desorption.

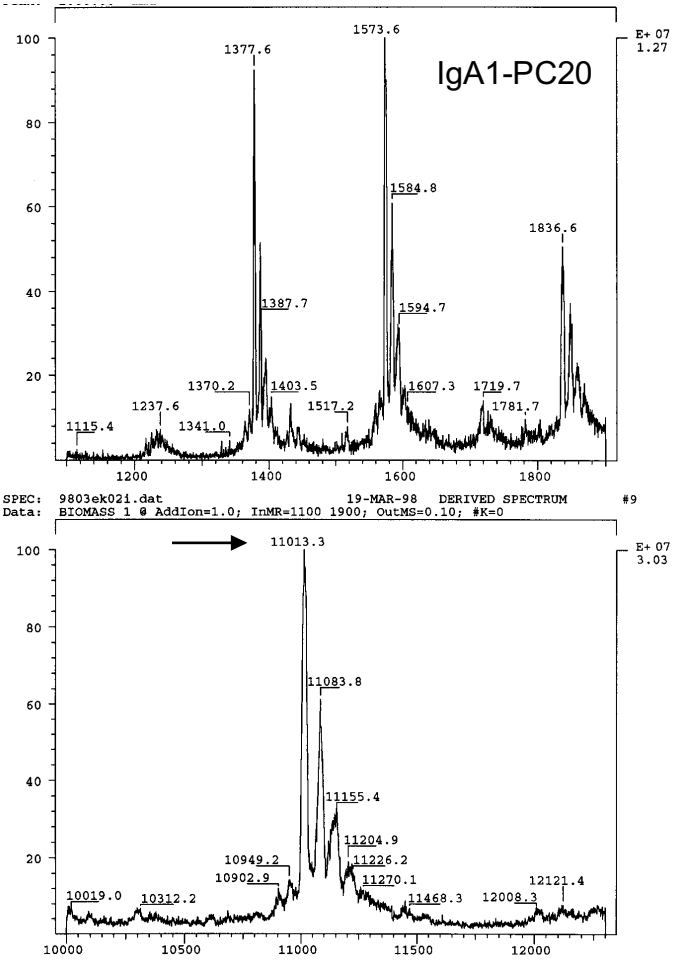
ESI-mass spectrometry analysis

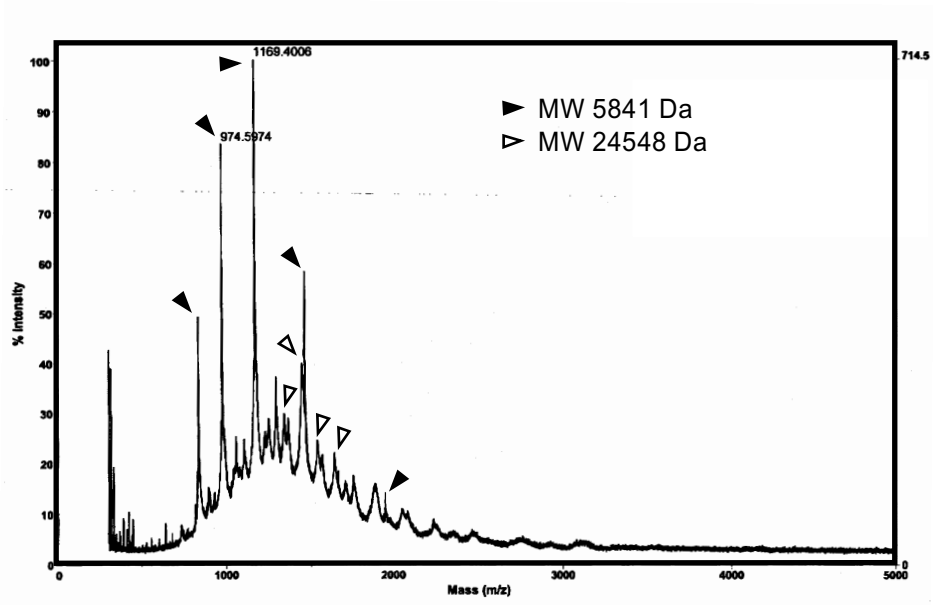
One possible alternative method to circumvent laser-related obstructions was electrospray ionisation mass spectrometry (ESI-MS). ESI-MS electrostatically evaporates the sample rather than desorbing it with a laser beam. ESI-MS also has limitations. The ESI-MS quadrupole has a very narrow m/z window, i.e. molecules of unfavorable ionisation behavior (high molecular weight m, low ionisation value z) might not be detected. Again, decay of fragile molecules has also been observed. The putative IgA1-PA50 50-mer was tested on a ESI-MS quadrupole (Figure 10).

Again, the two smaller tetra-oximes unambiguously appeared to be relatively homogenous (A, B). For the IgA1-PA50 tetra-oxime, however, no positive confirmation could be obtained (data not shown).

Figure 10: Electrospray ionisation mass spectrometry of tetra-oximes, original spectra (upper panels in A, B, C) and deconvoluted spectra (bottom panels in A, B, C; true mass scale). (A) Opc loop 2 tetra-oxime, experimentally determined molecular mass 10711.7 Da (calculated, unprotonated 10711.2 Da) and (B) IgA1-PC20 tetra-oxime, experimentally determined 11013.3 Da (calculated 11015.8 Da). The 11083.8 Da signal corresponds to the sodium/potassium salt of the main component (B, bottom). (C): Time-of-flight electrospray ionisation (ESI-TOF) of IgA1-PA50 tetra-oxime, experimentally determined 24548.5 Da (calculated 24548 Da). Solid triangles indicate signals from which the 5841 Da molecule was iterated, open triangles indicate the 24548 Da compound.

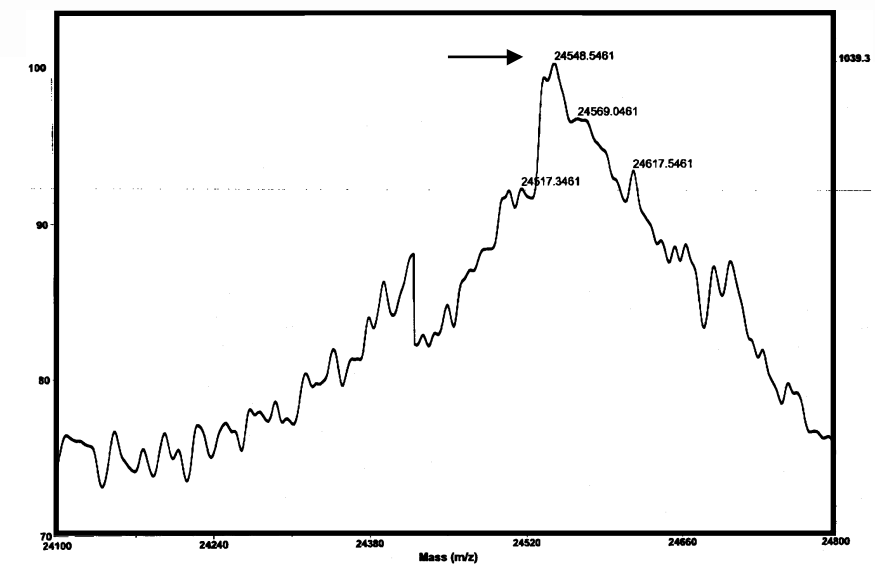

opc loop2
A
825.0
893.7
974.7
766.2
898.5
981.1
1071.9
758.4
963.7
1060.6
1080.2
1114.8
1191.5
1197.9
1271.4
E+ 07
3.01
SPEC: 9803ek02j.dat 19-MAR-98 DERIVED SPECTRUM #9
Data: BIOMASS 1 @ AddIon=1.0; InMR=700 1300; OutMS=0.10; #K=0
10711.7
10691.5
10780.9
10600.3
10584.7
10809.3
10835.3
10001.8
10073.6
10558.0
10907.8
11386.7
11219.6
11477.6
11464.9
E+ 07
9.07


IgA1-PC20
B
1573.6
1377.6
1584.8
1836.6
1387.7
1594.7
1370.2
1403.5
1607.3
1719.7
1237.6
1517.2
1781.7
1115.4
1341.0
E+ 07
1.27
SPEC: 9803ek02i.dat 19-MAR-98 DERIVED SPECTRUM #9
Data: BIOMASS 1 @ AddIon=1.0; InMR=1100 1900; OutMS=0.10; #K=0
11013.3
11083.8
11155.4
11204.9
10949.2
11226.2
10902.9
11270.1
12121.4
10019.0
10312.2
11468.3
12008.3
E+ 07
3.03


1169.4006
714.5
974.5974
MW 5841 Da
MW 24548 Da
% Intensity
Mass (m/z)

C

IgA1-PA50


24548.5461
1639.3
24569.0461
24617.5461
24517.3461
Mass (m/z)

Therefore another sample of the large oxime IgA1-PA50 was analyzed by PerSeptive Biosystems on a newly introduced ESI-TOF mass spectrometer (ESI-TOF = ESI **T**ime **o**f **f**light mass spectrometry). Basically, ESI-TOF has a wider m/z detection window than the ESI quadrupole [187].

By ESI-TOF MS two major compounds were identified with MWs of 5841 Da and 24548 Da. Again, it was difficult to quantify the measurements since the homo-tetramer produced ambiguous m/z values that hampered the iteration of the parent masses. The most appealing explanation for the small 5841 Da compound is parent molecule decay (rather than incomplete turnover) since the precursor structure D92X has a MW of 5828.5 Da and had been shown to be pure. It could not be said to what extent different mobilization characteristics of the two detected compounds contribute to the MS output. As outlined above, a MS signal provides information about the successful mobilization of a sample compound and only limited validity on its concentration in the sample.

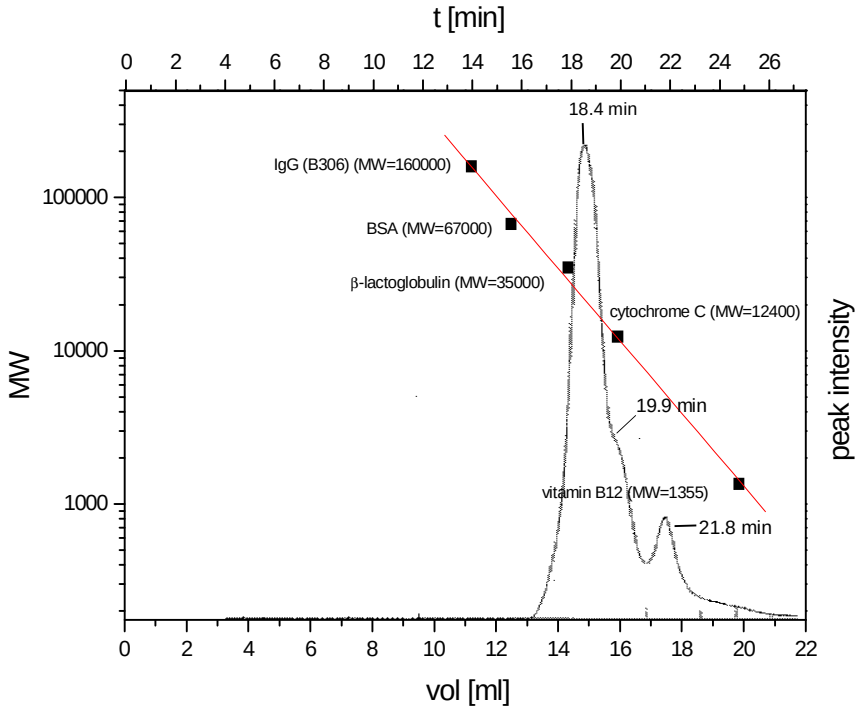


Figure 11: Gel filtration of tetra-oxime IgA1-PA50. The main compound has an interpolated size of 23305 Da and is probably the expected target molecule of 24.5 kDa (interpolated to). A shoulder at 19.9 min. was calculated to be of 12122 Da in weight and the small peak at 21.8 min. corresponds to a 5297 Da contaminant (The molecular weight was estimated by calibrating with five standard proteins (log (y [Da])=A+B*X[ml]; regression parameters: A=7.84962,B=-0.2365; Buffer: PBS; Flow rate: 0.8 ml min^{-1}).

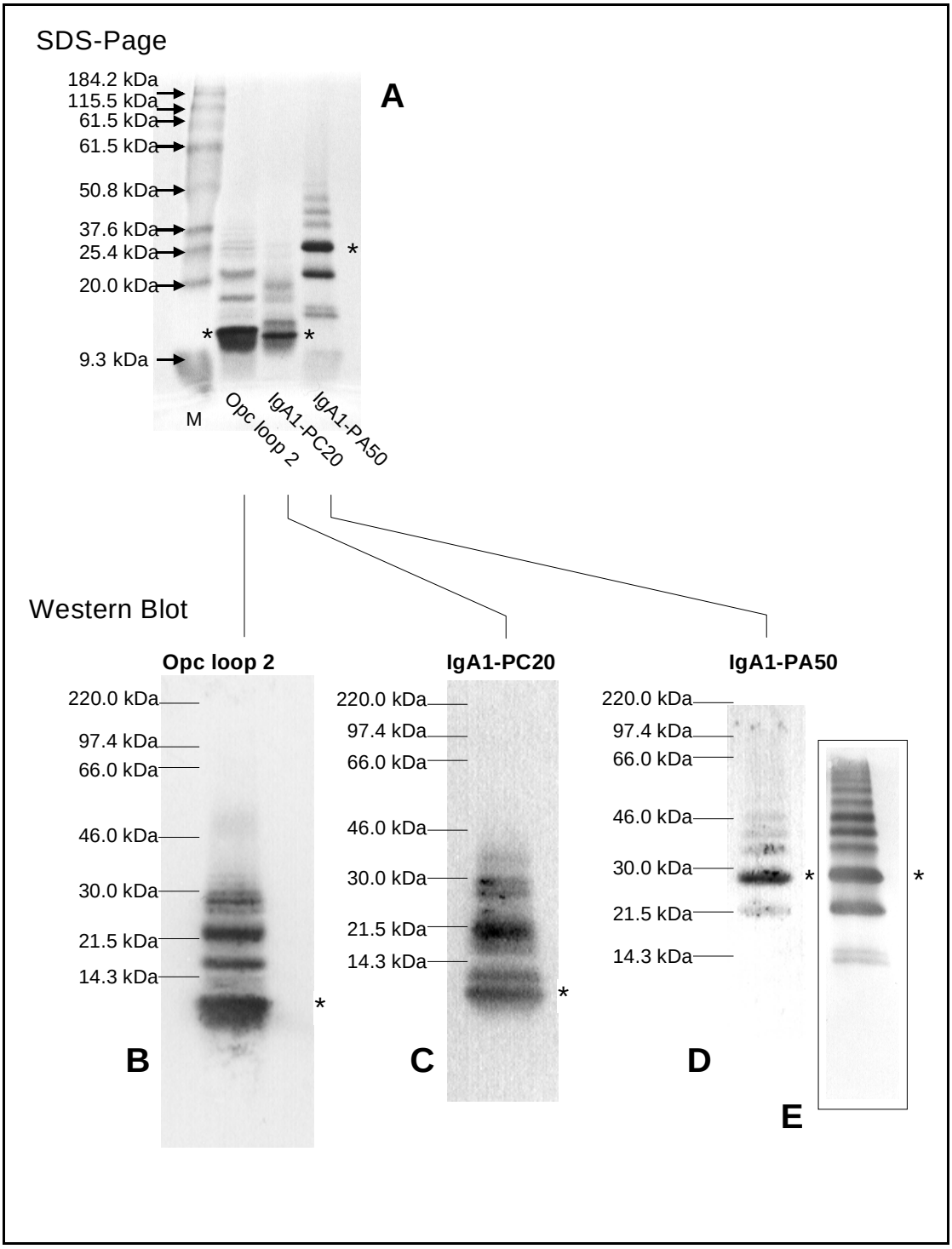


Figure 12: SDS-PAGE/Coomassie and Western Blot of Opc loop 2, IgA1-PC20 and IgA1-PA50 tetra-oximes. Tetra-oximes were electrophoresed on 11-20% gradient (A-D) or 11% (E) polyacrylamide gels (without β-mercapto-ethanol) and Coomassie stained (A) or analyzed by Western Blot (B-E). Bands corresponding to the expected molecular weights are marked with an asterisk (*). OX-Opc loop 2: 10711 Da; OX-IgA1-PC20: 11015 Da; OX-IgA1-PA50: 24530 Da. Western Blot antibodies: B: murine MAb B306 α Opc loop 2; C: pooled murine polyclonal serum stimulated against IgA1-PC20 conjugates; D: murine MAb AH623 against serogroup A IgA1-protease epitope 4; E: affinity purified polyclonal rabbit serum α*IgA1-PA50* (S3939).

Gel-filtration

Since MS was not satisfactory, another approach to characterize the IgA1-PA50 tetra-oxime preparation was tried, gel-filtration (Figure 11). The chromatogram exhibits a major peak at 18.4 min. This compound was estimated to 23305 Da corresponding to the expected IgA1-PA50 tetra-oxime. A shoulder at 19.9 min. and a minor peak at 21.8 min. were also visible.

The 19.9 min. structure is likely to be an incompletely substituted or fragmented oxime dimer (having 2 instead of 4 antigenic peptide ligands) and the 21.8 min. peak is due to free ligands. It was not clear whether these two compounds were the result of incomplete turnover (non-reactive precursors) or decomposition, e.g. hydrolysis. It is likely that the main peak also "hides" oxime trimers between 18.4 and 19.9 min

Gel filtration was followed by SDS-PAGE and Western Blot analysis. The results are depicted in Figure 12. Bands of the expected molecular weight (OX-Opc loop 2: 10711 Da; OX-IgA1-PC20: 11015 Da; OX-IgA1-PA50: 24530 Da) are indicated by an asterisk. β-Mercapto-ethanol was not used in the polyacrylamide gels because the oxime bonds are sensitive to reduction. Coomassie staining (A) shows the Opc loop 2 and IgA1-PC20 oxime preparations, which had been previously confirmed by ESI-MS. The tetra-oximes gave a rather dispersed band on the gel and aggregate to form multimers that also run non-uniformly. As evident from gel filtration, the IgA1-PA50 preparation contains the expected 24.5 kDa tetramer as the main compound. A putative trimer of MW≈20 kDa can be seen which was not detected in gel filtration. As in gel filtration, a dimeric variant is visible as a double band at roughly 13 kDa and monomeric ligands or fragments at MW≈5kDa.

The IgA1-PA50 tetra-oxime is reactive with the corresponding "conformational" monoclonal antibody AH623 (D) as are the two other oximes with corresponding antibodies or sera: the murine monoclonal MAb B306 recognized OX-Opc loop 2 and a pooled polyclonal murine serum stimulated against IgA1-PC20 was reactive with OX-IgA1-PC20 (B, C). Because of the low resolution of the MAb AH 623 (D) affinity purified rabbit polyclonal serum (α*IgA1-PA50*) specific for the N-terminal 30 amino acids (= peptide D35) of IgA1-PA50 was also tested and shown to react with the IgA1-PA50 tetra-oxime (boxed inset E, without MW marker).

These results show that the IgA1-PA50 oxime preparation contains the anticipated 24.5 kDa target tetramer as the main component. To various extents compounds of lower molecular weight were also detected for which it could not be fully elucidated whether they are due to side reactions, incomplete reactions, or fragmentation/hydrolysis.

3.3. Molecular characterization of peptide immunogens

Prior to immunization, the immunogens underwent molecular characterization (Figure 13). Synthetic peptides, tetra-oximes and their intermediates were characterized by mass spectrometry or chromatographic methods, as described before. Protein conjugated and liposomal immunogens were subjected to amino acid analysis. The rational for this characterization was to determine the antigenic peptide concentration in the various immunogen formulations, in order to allow their standardized application in immunization. A summary of the molecular characteristics of the immunogens is given in Table 17, p. 90.

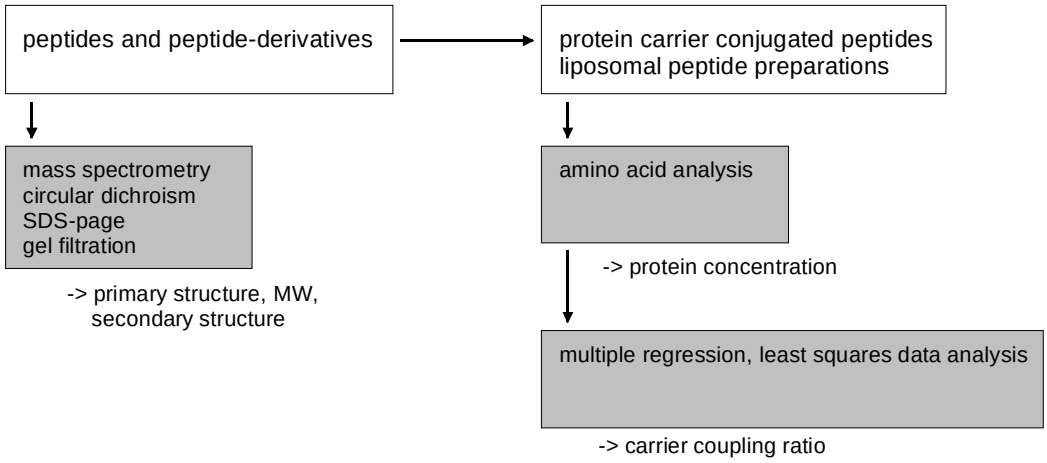


Figure 13: Molecular characterization of immunogens. Peptides and peptide derivatives were analyzed by mass spectrometry (tetra-oximes additionally by SDS-PAGE and gel-filtration, IgA1-PA50 additionally by CD). Carrier conjugated peptides were analyzed by amino acid analysis in order to determine protein concentration and to calculate the coupling ratio.

3.3.1. Mass-spectrometric characterization of synthetic peptides

The results of the mass-spectrometric characterization of the synthetic peptide antigens were discussed in Chapters 3.2.1 (Synthetic peptide antigens) and 3.2.5 (Tetra-oximes).

3.3.2. Structural characterization of peptide IgA1-PA50

The conformation of the IgA1-protease 50-mer was analyzed by circular dichroism (CD). The structure and stability of the peptide were investigated in aqueous solutions with variable pH, concentration and temperature. Secondary structure was estimated

from the CD spectra by the methods of Manavalan and Johnson (Variable Selection [164]), and Provencher and Glöckner (CONTIN [163]).

α-helix	β-sheet			β-turn	remainder	Σ	conc. (mg/ml)	
	parallel	anti parallel	Σ					
0.07	0.01	0.34	0.35	0.21	0.35	0.98	0.1	VS
0.00			0.52	0.15	0.33	1.00		CONTIN
0.00			0.51	0.17	0.33	1.01	1.0	CONTIN
0.10	0.00	0.33	0.33	0.22	0.34	0.99	5.0	VS
0.02			0.49	0.17	0.32	1.00		CONTIN

Table 15: Conformation of peptide IgA1-PA50 in H_2O at three different peptide concentrations, 0.1, 1.0 and 5 mg/ml. Comparison of secondary structure estimates calculated by the methods of Manavalan and Johnson (Variable Selection, [164]), and Provencher and Glöckner (CONTIN, [163]).

CD-spectrum of IgA1-PA50

The CD spectrum of the IgA1-P 50-mer in H_2O is shown in Figure 14. The spectrum is characterized by a positive maximum in the 186 nm band and negative values in the peptide bond region (190-240nm) indicative of secondary structure formation. Secondary structure prediction by Variable Selection and CONTIN are in Table 15. While Variable Selection and CONTIN generally disagreed in estimating the proportion of β-turns and -sheets, their predictions corresponded well with respect to random coil ("remainder") structures.

Sequence based structure prediction

Secondary structure predictions based on the amino acid sequence using the algorithms developed by Chou-Fasman [161] and Garnier-Osguthorpe-Robson [162] are shown in Table 16.

α-helix	β-sheet	β-turn	remainder	Σ	
0,18	0,22	0,32	0,28	1,00	Chou-Fasman
0,26	0,12	0,24	0,38	1,00	Garnier-Osguthorpe-Robson

Table 16: Sequence based secondary structure prediction for peptide IgA1-PA50 by Chou-Fasman [161] and Garnier-Osguthorpe-Robson [162].

Buffer and concentration effects on the conformation of the peptide

The specific environment seen by an amino acid is important in determining secondary structure [188]. Buffer, pH and concentration effects contribute to the CD spectrum.

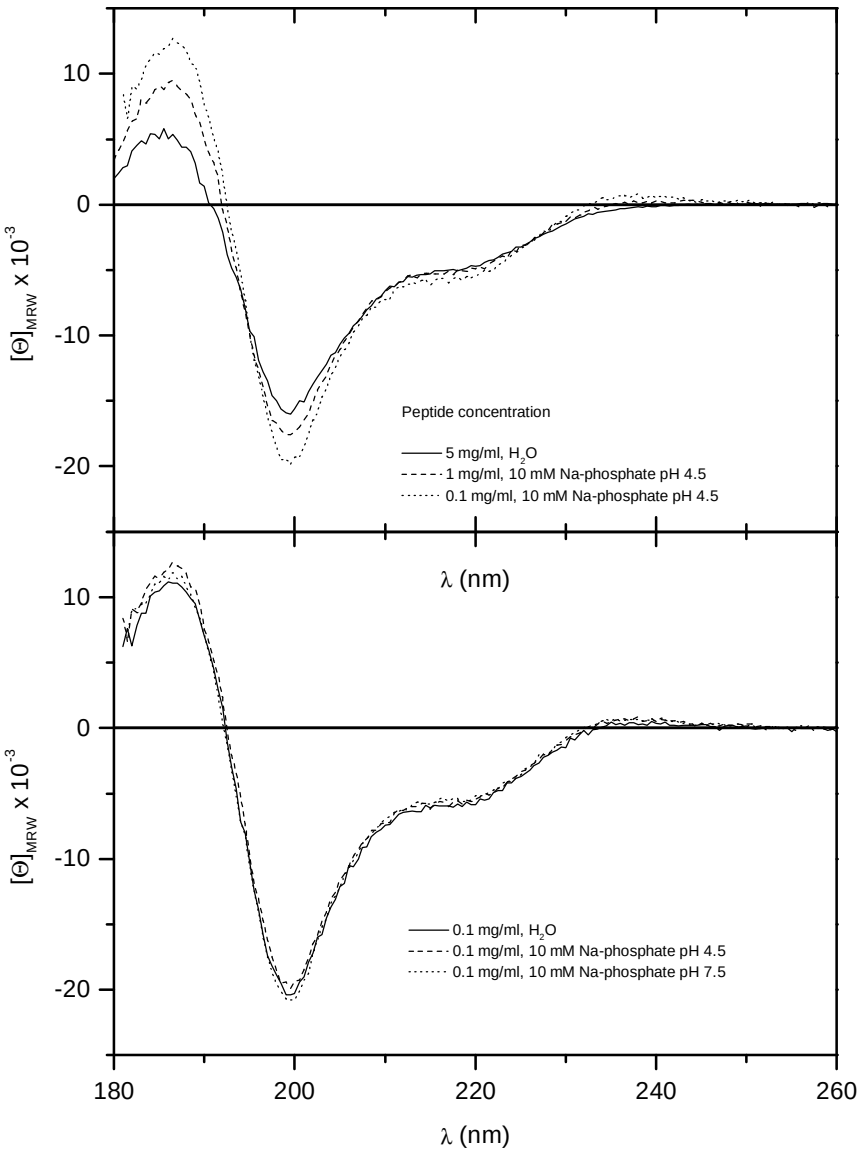


Figure 14: Circular dichroism spectra of peptide IgA1-PA50. Buffer and pH dependency , 0.1 mg/ml in H_2O (—), in 10 mM Na-phosphate pH 4.5 (- -), in 10 mM Na-phosphate pH 7.5 (···). $[\Theta]_{MRW}$, the mean residue ellipticity, is based on the concentration of the sum of the amino acids in the peptide solution
(Θ: ellipticity; MW: molecular weight; c: peptide concentration [mg/ml]; d: path length [cm]; N_A: number of amino acids per peptide):

$$[\Theta]_{MRW} = \frac{\Theta \times 100 \times MW}{c \times d \times N_A}$$

The spectroscopic change provides insight into the peptides response to environmental conditions or the stability of the observed structure [189,190]. Waterhous and Johnson invented the term *well behaved* peptide sequence for sequences that have a strong propensity for only a single secondary structure. They also showed that certain environments, such as high concentrations of TFE or nonmicellar SDS, can override the propensity for a secondary structure due to sequence [188,191].

Peptide IgA1-PA50 was analyzed in H_2O and in a buffered environment at pH 7.0. As the results were intended to being compared with NMR work carried out at pH 4.5, additional CD-spectra were obtained under these conditions, too. pH 4.5 is also a physiologic pH used to simulate endosomal environments in immunological experiments. A pH shift from pH 7 to pH 4.5 may change the CD-spectrum significantly [189]. Finally CD-spectra were obtained at high peptide concentrations of 5 mg/ml.

The CD spectrum and hence the calculated secondary structure content remained unchanged regardless whether water or mild buffers at pH 7.5 or 4.5 were used (Figure 14).

With increasing peptide concentration the peak intensities at 186 nm and 199 nm decreased by 60% and 20%, repectively. This was not accompanied by a qualitative change of the spectrum since structural parameters did not change significantly (Table 15). Both secondary structure estimation methods calculated a slight increase of the α-helical contents at the expense of β-turn/-sheet forming elements. Thus, the peptide did not show cooperative effects -such as inter-molecular structure stabilization- or aggregation.

Thermal stability of the peptide

The temperature dependent conformation of peptide IgA1-PA50 was analyzed by monitoring changes in the CD spectrum while increasing the temperature at a rate of 50°C/h (Figure 15).

Full scans were carried out at the beginning at 25°C, at the temperature maximum of 80°C and after re-cooling the molten peptide down to 25°C again.

Secondary structure analysis was conducted based on the two 25°C spectra pre and post melting. These results show that the peptide melts and refolds in a fully reversible manner. Secondary structure estimation revealed identical structural parameters for the pre and post melting spectra (refer to Table 15: 0.1 mg/ml).

The thermal stability of the peptide was characterized by monitoring the fractional change at λ=186 nm (Figure 15, small inset). The melting curve plot exhibits a rather flat performance (i.e. little conformational change) at the beginning between 25°C and 42°C followed by a linear transitional phase between 42°C and 58°C. The slope maximum lies well above 45°C. The fractional change endpoint is reached at 73°C. T_m, the thermal stability midpoint at 50% fractional change, was determined to be 53°C.

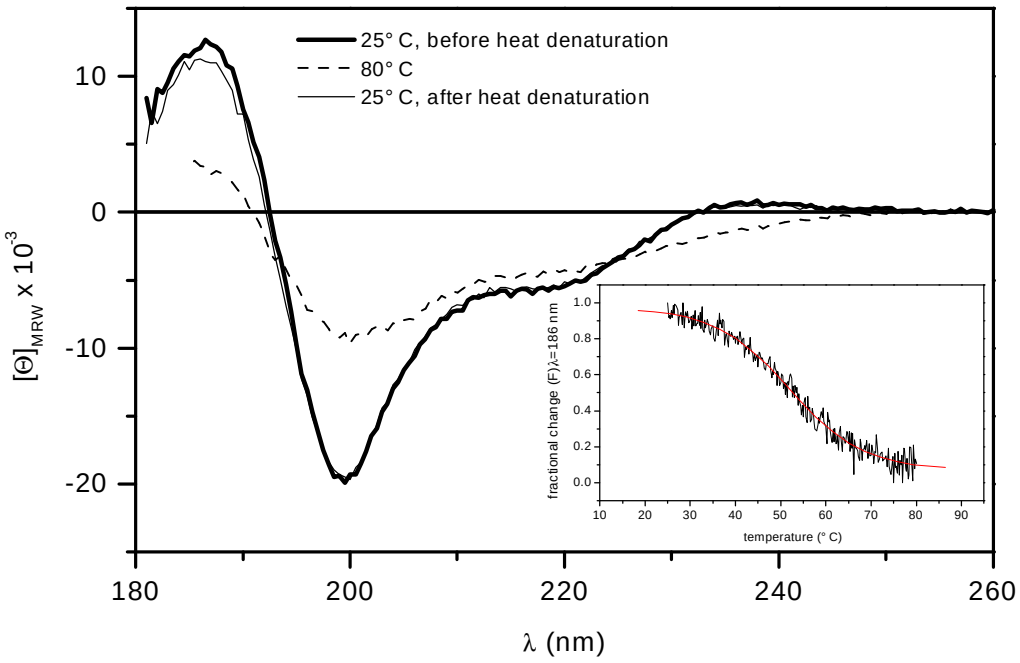


Figure 15: Thermal stability of synthetic peptide IgA1-PA50. Circular dichroism (CD) spectra of the molten peptide at 80°C (- -), pre- and post-melting at 25°C (solid lines). The small inset shows the thermal transition curve monitoring ellipticity $[\Theta]_{MRW}$ at λ=186 nm vs. temperature. The fractional change (F) was determined from $F=([\Theta]_{MRW} - [\Theta]_{MRW,\ 80°C})/([\Theta]_{MRW,25°C} - [\Theta]_{MRW,\ 80°C})$, where $[\Theta]$ is the residue ellipticity measured at a given temperature, and $[\Theta]_{MRW,25°C}$ and $[\Theta]_{MRW,\ 80°C}$ are the residue ellipticities at 25°C and 80°C respectively.

3.3.3. Determination of the coupling ratio of protein-conjugates

Multiple regression, least squares analysis of AAA data

Accurate assessment of the molar concentration of peptide antigens is important for comparing different immunogen preparations, conducting dose-response immunization studies and for characterizing dose-dependent effects of peptides on cellular functions [192]. While the composition of peptides and tetra-oximes could directly be assessed by mass spectrometry and their concentrations standardized gravimetrically, the analysis of liposomes and protein-conjugates was more difficult.

A number of methods have been utilized for the determination of the peptide-protein conjugate composition. Radioactively labeled peptides can be used to provide conjugate ratio information [21], but the accuracy is dependent on the extent of labeling, the possibility of double labeling, and the instability of commonly used radiolabeled derivatives, such as ^{125}[I] iodotyrosine. If a crosslinker such as *N*-

succinimidyl 3-(2-pyridil-dithio) propionate (SPDP) is used, the conjugate ratio can be determined by monitoring the absorbance of released by-products during peptide coupling [77,193]. However this limits the choice of crosslinkers to those which can release a chromophore with an extinction coefficient large enough to allow accurate monitoring of the coupling. The measured absorbance also reflects any degradation of the crosslinker, like hydrolysis, oxidation etc. and, thus, does not always accurately indicate the degree of peptide substitution. Although the conjugate ratio may be estimated by gel electrophoresis [194], large molecular weight proteins such as KLH and its conjugates are not easily resolved on gels.

A more direct approach for determining the conjugate ratio is provided by the comparison of the amino acid composition of the carrier protein with that of the peptide-protein conjugate [195,196]. First, the carrier protein and the peptide-protein conjugate are analyzed by amino acid analysis. Then the number of moles of peptide per mole of carrier is calculated for one or more amino acid residues present in the peptide. The residue average is taken as the coupling ratio. In some cases, the basis for establishing the peptide protein molar ratio in the conjugate requires one or more amino acid residues to be uniquely present in one component. Alternatively, modified amino acids can be introduced to the peptide N- or C-terminally resulting in a quantitative “fingerprint” in amino acid analysis. As a spin-off of various coupling methods, the inclusion of a non-natural amino acid or a non-amino acid crosslinker in the peptides permits an easy quantitation of the conjugation number [84,85].

However, basing a coupling analysis on a single parameter can severely skew the outcome and affect the correct analysis [196]. Inaccuracies hamper the approximation to a varying degree depending on the amino acid composition. Some amino acids may be modified during sample hydrolysis, such as cysteine, threonine, serine, methionine, tyrosine or tryptophan.

In a report by Antoni and co-workers [197], the molar ratios of two different proteins within a conjugate were calculated using a least squares minimization method, based on the composition of each conjugate member individually. The results obtained were independent of the types of residues found in either component, but the method requires prior determination of the amino acid composition of each protein.

In this work a multiple regression, least squares analysis method -a derivative method of the method of Antoni *et al.* developed by Shuler and co-workers [146]- was applied for conjugate analysis.

Coupling ratios of protein conjugates

The *coupling ratio* is defined as the number of antigenic peptide molecules coupled to one carrier molecule. Peptides, carrier proteins and conjugates were determined by amino acid analysis and the data combined in a multiple regression, least squares analysis. The molecular weight of the carrier molecule (*MW carrier*) was based on the sequence of the mature protein.

aa carrier indicates the number of amino acids of the mature carrier protein. For the very heterogeneous reagent KLH, the molecular weight was set to 3 MDa for calculations. From that *aa carrier* was calculated assuming an average MW of 112 g/mol/aa.

A

Opc loop 2 20-mer		non-protein carrier			protein carrier				
immunogen	**Pep**	**L**	**OX**	**MAP**	**BSA**	**KLH**	**OA**	**TG**	**TT**
MW peptide antigen	2444	2444			2444	2444	2444	2444	2444
MW carrier					66433	3000000	42744	301215	150700
aa carrier					583	26786	385	2750	1315
MW carrier conjugate			10711	12683	77483	3854795	50410	329986	193074
coupling ratio					**4.52**	**349.77**	**3.14**	**11.77**	**17.34**
carrier aa / conj. peptide					129	77	123	234	76

B

IgA1-PC20 20-mer		non-protein carrier			protein carrier				
immunogen	**Pep**	**L**	**OX**	**MAP**	**BSA**	**KLH**	**OA**	**TG**	**TT**
MW peptide antigen	2520	2520			2520	2520	2520	2520	2520
MW carrier					66433	3000000	42744	301215	150700
aa carrier					583	26786	385	2750	1315
MW carrier conjugate			11015	12987	77060	3626059	49337	360934	187244
coupling ratio					**4.22**	**248.46**	**2.62**	**23.70**	**14.50**
carrier aa / conj. peptide					138	108	147	116	91

C

IgA1-PA50 50-mer		non-protein carrier			protein carrier				
immunogen	**Pep**	**L**	**OX**	**MAP**	**BSA**	**KLH**	**OA**	**TG**	**TT**
MW peptide antigen	5798	5901			5901	5901	5901	5901	5901
MW carrier					66433	3000000	42744	301215	150700
aa carrier					583	26786	385	2750	1315
MW carrier conjugate			24538	(not used)	86453	4298563	50907	397989	231588
coupling ratio					**3.39**	**220.07**	**1.38**	**16.40**	**13.71**
carrier aa / conj. peptide					172	122	278	168	96

Table 17: Molecular characterization of peptide immunogens. Opc loop 2 immunogens are listed in panel **A**, IgA1-PC20 immunogens in **B** and IgA1-PA50 immunogens in **C**. *Coupling ratio* is the number of peptide ligands coupled to one carrier molecule. *MW* indicates the molecular weight of the synthetic *peptide antigens*, the mature protein *carriers* and the resulting *carrier conjugates*. *aa carrier*: number of amino acids of the mature carrier protein. *Carrier aa/conj. peptide*: frequency of peptide ligands based on the number of carrier amino acids.

Protein conjugate coupling ratios (Table 17) varied from 1.38 (IgA1-PA50 coupled to OA) to as much as 349.77 antigenic peptides per protein carrier (Opc loop 2 coupled to KLH). For liposomes, the parameters *coupling ratio* and *MW carrier* could not be determined. For MAP and tetra-oxime variants, only the total molecular weight of the final constructs are indicated.

Carrier aa/conj. peptide is a direct derivation of the parameters *coupling ratio* and *aa carrier*. A value of *carrier aa/conj. peptide* = 100 indicates that peptide is coupled to the carrier on average once every 100 carrier amino acids.

Opc loop 2 **A**
20-mer

		non-protein carrier				protein carrier				
	immunogen	**Pep**	**L**	**OX**	**MAP**	**BSA**	**KLH**	**OA**	**TG**	**TT**
antigenic peptide/inj.	**pmol/inj.**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**
protein conc	µM					9.3	0.1	10.7	0.8	3.9
antigenic peptide conc	µM	409.2	155.2	373.4	315.4	42.0	42.6	33.6	9.6	68.3
total protein, peptide/ injection	µg/inj.	0.5	0.5	0.5	0.5	3.4	2.2	3.2	5.6	2.2

IgA1-PC20 **B**
20-mer

		non-protein carrier				protein carrier				
	immunogen	**Pep**	**L**	**OX**	**MAP**	**BSA**	**KLH**	**OA**	**TG**	**TT**
antigenic peptide/inj.	**pmol/inj.**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**
protein conc	µM					14.8	0.2	21.7	1.8	4.5
antigenic peptide conc	µM	396.9	181.1	363.1	308.0	62.4	41.8	56.7	42.0	65.8
total protein or peptide/ injection	µg/inj.	0.5	0.5	0.6	0.5	3.7	2.9	3.8	3.0	2.6

IgA1-PA50 **C**
50-mer

		non-protein carrier				protein carrier				
	immunogen	**Pep**	**L**	**OX**	**MAP**	**BSA**	**KLH**	**OA**	**TG**	**TT**
antigenic peptide/inj.	**pmol/inj.**	**200**	**200**	**200**		**200**	**200**	**200**	**200**	**200**
protein conc	µM					6.9	0.2	18.5	0.3	4.1
antigenic peptide conc	µM	172.5	91.5	163.0		23.5	38.9	25.5	5.4	55.6
total protein or peptide/ injection	µg/inj.	1.2	1.2	1.2		5.1	3.9	7.4	4.9	3.4

Table 18: Molar normalization of immunogens.

3.3.4. Molar normalization of immunogens

Roman *et al.* found as little as 4 pmol of a 24 amino acid protein-attached peptide to be immunogenic in BALB/c mice [81]. In the course of the *Immunogenicity Study* (Chapter 3.5.2, p. 101), antigenic peptides were injected into mice in equimolar quantities of 200 pmol/injection (Table 18). Based on the amino acid analysis above, molar antigen concentrations could be derived from the coupling ratio of the protein conjugates. Antigen concentrations in liposomal immunogens were also based on AAA data. For free peptides, MAPs and tetra-oximes, the immunogen formulations were standardized gravimetrically.

Due to the fact that peptides were coupled to protein carriers while others were not, the protein concentration considerably varied up to 7.4 µg protein/injection.

3.4. Enzyme-linked immunospot assay (ELISPOT)

One of the main parameters of immunogenicity assessed in this work is the frequency of antigen-specific immunoglobulin secreting spleen cells, or splenocytes. An enzyme-linked immunospot assay (ELISPOT) was set up to directly enumerate antigen-specific antibody-secreting B cells (ASC) in the spleen.

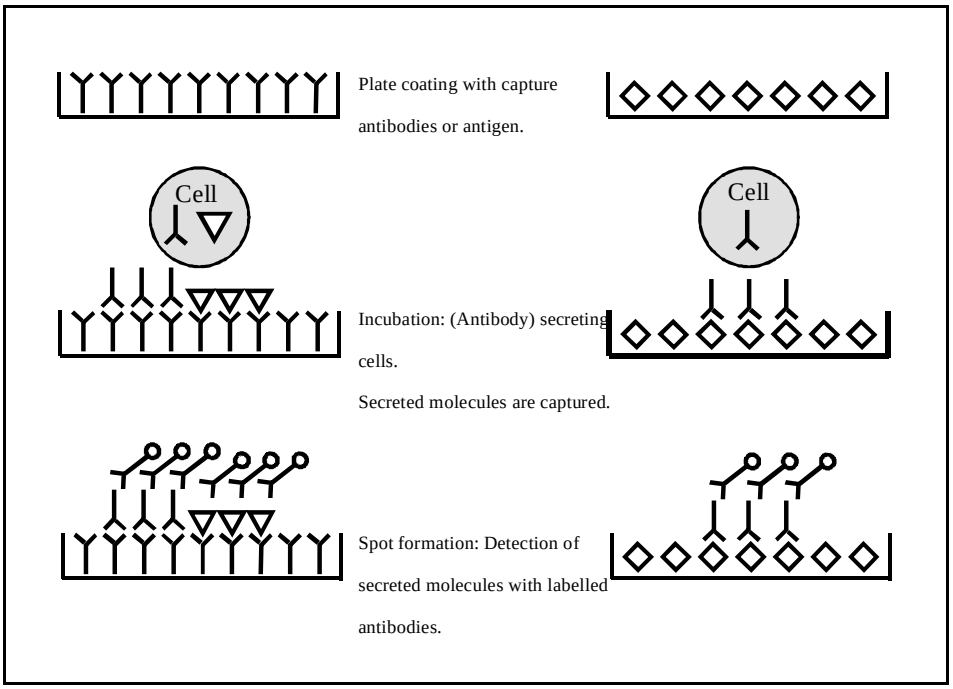


Figure 16: ELISPOT. Detection, quantification and identification of protein secreting cells.

The ELISPOT generates spots based on a colorimetric reaction that detects proteins secreted by a single cell. The spot represents a "footprint" of the original protein-producing cell. Since the detection parameter is binary (spot/no spot), the ELISPOT is less prone to "noise" that can cause an elevated background in ELISA.

This method was originally developed in 1983 by Czerkinsky *et al.* and Sedgewick & Holt [198,199] as an alternative to Jerne's hemolytic plaque forming cell (PFC) assay [200]. Czerkinsky and co-workers incubated B-lymphocytes in large antigen-coated polystyrene plates. After removal of the cells, bound antibodies were visualized by means of an immunoenzyme procedure in which enzyme-substrate reactions were performed in an agarose layer resulting in colored circular spots.

Since then ELISPOTs have been repeatedly modified. The ELISPOT functions without gel-layers, in 24 well titer plates [201,202], in 96 well microtiter plates [203-205] and on surfaces such as polystyrene, PVC and various membrane types [203,206,207]. The ELISPOT has been adapted to other applications such as the simultaneous detection of more than one immunoglobulin isotype, or to detect soluble antigens like cytokines, polysaccharides, haptens and nucleic acids [208-210]. It has

been combined with other techniques such as cell sorting [211]. An excellent review is provided by [208].

The ELISPOT technique is up to 20- to 200-fold more sensitive than ELISA [212]. Cells producing as few as 100 molecules per second of a specific protein can be detected. It takes advantage of the relatively high concentration of a given protein in the environment immediately surrounding the protein-secreting cell. Besides its sensitivity, this technique can provide an estimate of the amount of immunoglobulins produced on a per-cell basis. Unlike current assays using ELISA to identify specific antibodies in serum, the ELISPOT-based method identifies specific antibody-secreting cells directly. ELISA measures the accumulated amount of specific antibody circulating. In contrast the ELISPOT-assay provides a "flashlight" impression of the immune response providing information on the concentration of the antibody-secreting cell at a particular instant in time and at a particular location.

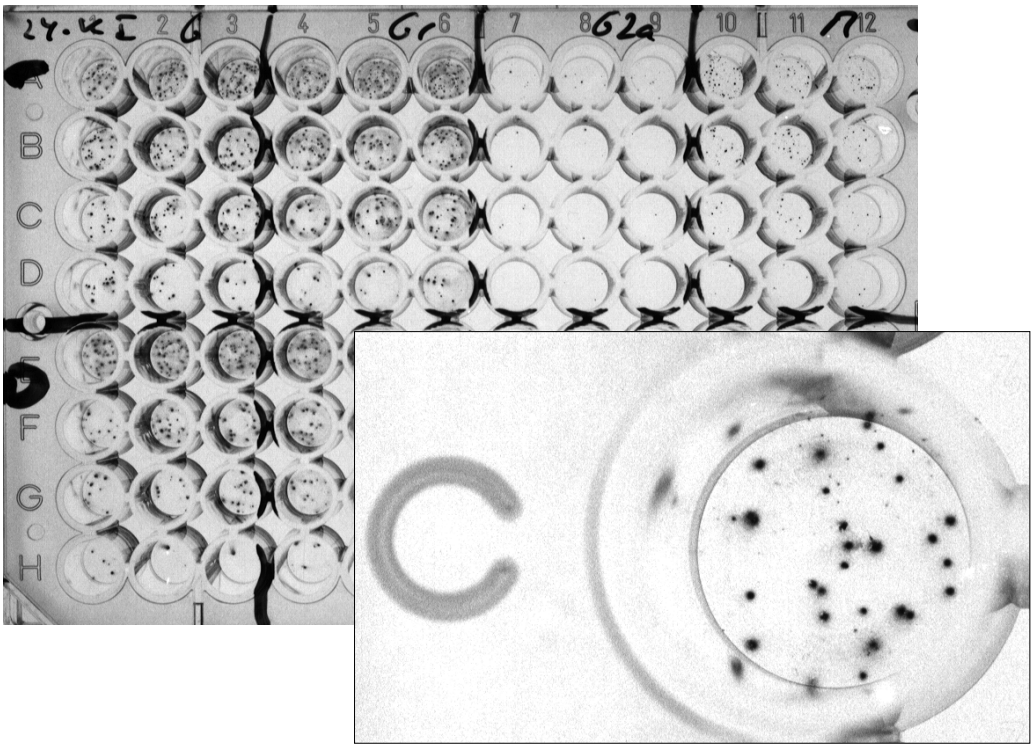


Figure 17: 96 well microtiter plate ELISPOT. The framed inset shows well C1 at 6x magnification.

Here a very efficient, but less sensitive, modification adapted to 96-well microtiter plates [203,204] was used. 5 spots/well were regarded as significant, i.e. the detection limit of the ELISPOT assay was 1 ASC per 10^5 splenocytes.

The ELISPOT assay involves three specific steps: (1) coating the plate with antigen, or capture antibody and blocking the plate to prevent non-specific absorption of any other proteins; (2) incubating with protein-secreting cells; (3) spot formation followed by detection with labeled antibodies.

The procedure is outlined in Figure 16. A photo of a developed ELISPOT microtiter plate ready for enumeration is seen in Figure 17.

3.5. Immunogenicity of peptide immunogens

In order to characterize the immune response to the various peptide immunogens, mice were sacrificed and their spleens removed. Blood samples were also collected after the final immunization. However, a variable which needed to be assessed initially, was the optimal time for blood sample collection and spleen removal.

After stimulation, splenocytes remain active for a limited period of time only. The number of active B-lymphocytes found in the spleen increases for a period before decreasing again, as more and more ASC leave the spleen, circulating or migrating to other lymphoid tissues or organs such as the bone marrow [213]. As the number of spleen resident antibody-secreting cells (ASC) decreases, the antibody titer continues to increase. Therefore, attempting to simultaneously measure activity of ASC and serum antibody titer yields complementary results.

The normal murine immune response time course appears to peak at a few days up to one week after the final boost [214]. Vadolas *et al.* investigated the immune response time course of antigen-specific B-lymphocytes from murine lungs. After intranasal challenge (secondary immunization) and response analysis at days 3, 6, 9 and 18 maximal ELISPOT numbers were detected at day 6 [215]. Zigterman and co-workers observed IgG secreting murine spleen cells 3 days after the first boost (i. p.) in a study with *S. pneumonia* antigens [203]. In a response study with LCM virus in mice, IgM and IgG secreting splenocytes appeared on days 4 and 5 post infection (secondary infection, intravenous) [202]. The same authors, Moskophidis *et al.*, made another important observation: the response profile of IgM and IgG secreting splenocytes is not necessarily synchronized. In a *primary* infection experiment IgM ASCs showed up two days before their IgG counterparts. Nevertheless, simultaneous secretion patterns were observed after secondary infection.

In order to set up a reasonable time frame first, the immunogen analysis was divided into two parts (Figure 18):

I. *Response Peak Study*

II. *Immunogenicity Study*

The *Response Peak Study*, a pilot study to the subsequent *Immunogenicity Study*, was addressing three questions:

1. What is the time course of the murine B-Lymphocyte activity?
2. What is the time course of the murine serum titer ?
3. How to combine the above observations in a practical experimental set-up?

Therefore, mice were immunized in parallel and analyzed on five consecutive days post-immunization. Based on these data, the *Immunogenicity Study* was carried out: A comparative analysis of the various immunogens, analyzing multiple animals on one day.

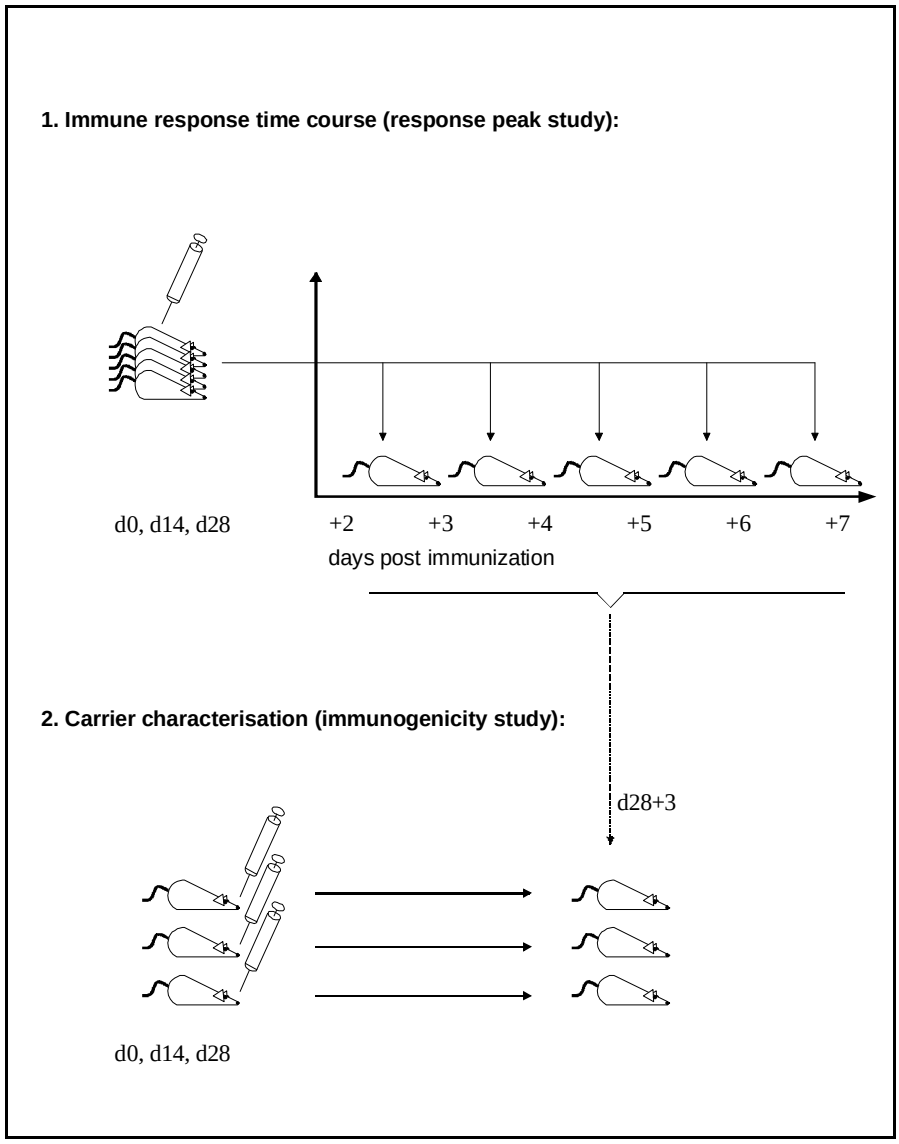


Figure 18: Immunogenicity of peptide immunogens, experimental outline. **1.** Analysis of the time course of the murine response post-immunization (*peak response study*). **2.** Comparative analysis of the various immunogens by analyzing multiple animals on one day (*Immunogenicity Study*).

3.5.1. Time course of the immune response (response peak study)

The *Response Peak Study* was limited to two peptide antigens (Opc loop 2 and IgA1-PA50). They were each tested in 4 formulations: free peptide and conjugated to protein carriers BSA, ovalbumin and thyroglobulin.

All mice were immunized simultaneously: A primary immunization (caudal) on day 0, was followed by two boost immunizations (i.p.) on days 14 and 28. The immune response was analyzed pair-wise on five consecutive days post-immunization. Mice receiving Opc loop 2 immunogens were analyzed on days 3 to 7 post-immunization.

Mice receiving IgA1-PA50 immunogens were analyzed on days 2 to 6 post-immunization. Two main parameters were measured: The number of ASC specific to peptide antigen using an ELISPOT assay, and peptide specific serum antibody titers (ELISA).

Antibody-secreting spleen cells

Antibody-secreting cells specific to the synthetic peptide antigens were detected in splenocyte preparations after the second boost immunization, as seen in Figure 19 and Figure 20. Both, IgG- and IgM-isotype secreting cells could be detected.

Isotype specific differences were observed. While protein conjugated peptides (BSA, OA, TG) were able to induce both IgG- and IgM-ASC, free peptide (Pep) preparations failed to stimulate a detectable IgG response (Figure 19 and Figure 20).

Serum antibody titers

Both for Opc loop 2 and IgA1-PA50 immunogens, BSA-, OA- or TG-conjugated peptides induced peptide specific antibody responses (Figure 21 and Figure 22). Free peptide stimulated poor antibody responses.

Within the experimental time frame (days 2 to 7 post-immunization), no significant change of antibody titers could be observed.

Concluding remarks

The observed ASC response from these results suggested it would be preferable to select one of the days 2-6 for splenocyte analysis in the subsequent *Immunogenicity Study*. To be on the safe side day 3 was selected.

The antibody response presented a very continuous picture. When ELISA analysis was carried out on days 2-6 and 3-7, after the third immunization antibodies had been accumulating for more than 28 days and boosted twice already. Vijayakrishnan and co-workers have shown that after a single primary challenge murine Ab may keep accumulating as long as 20 to 30 days until threadmill consumption outweighing secretion will lead to a downswing in antibody titers again [216]. These “developed” antibody titers did not increase significantly further during the 5 day “experimental window”. The kinetic profiles of type IgG and IgM antibodies did not show conspicuous differences as expected for a secondary response [202].

Thus, the dynamic parameters lymphocyte proliferation, kinetics of antibody secretion and isotype modulation interfered little with the regime of a 3-fold immunization schedule followed by simultaneous spleen and serum titer analysis on day 3 post-immunization. A comparative immunogenicity analysis appeared to be feasible under the experimental conditions selected.

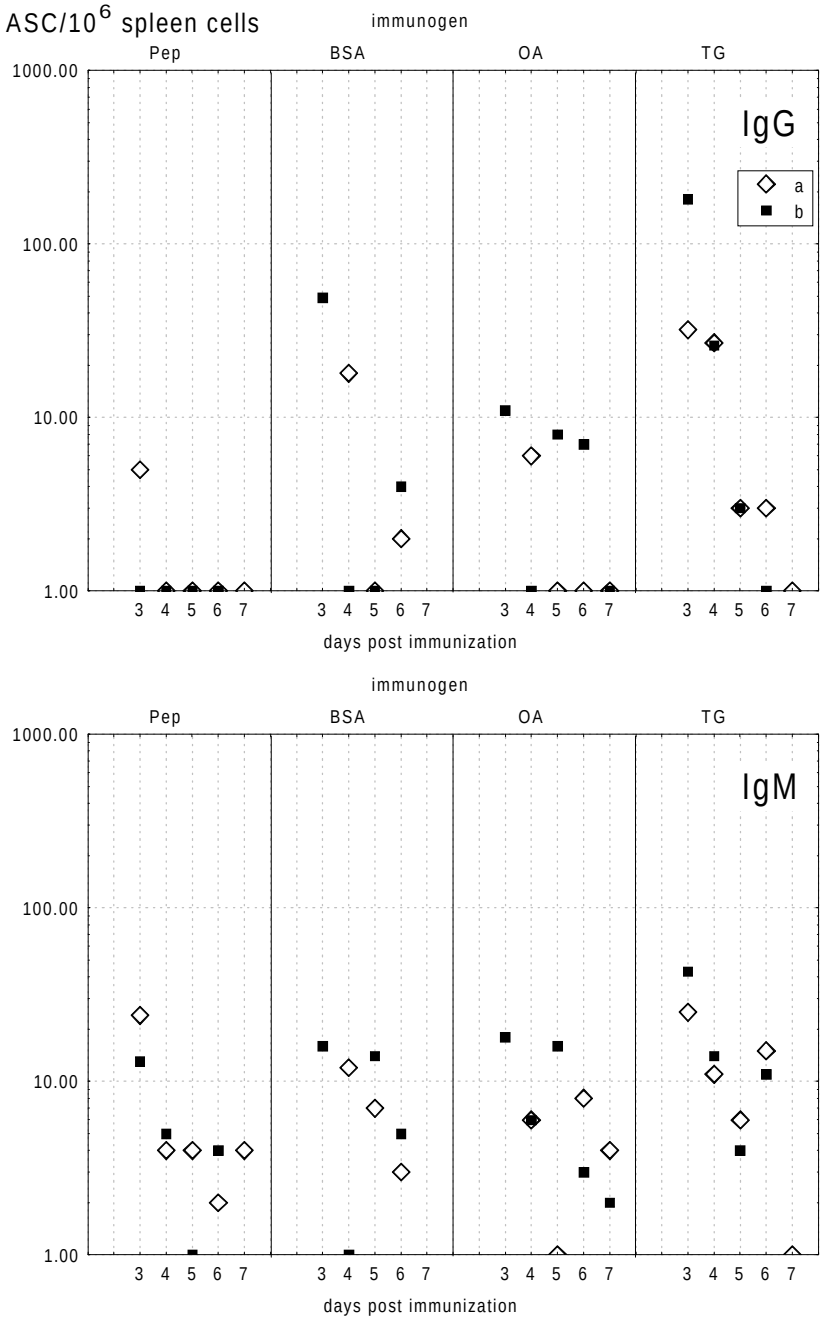


Figure 19: *Response Peak Study*. Opc loop 2-antigen-specific ASC counts per 10^6 spleen cells. Mice were challenged simultaneously with four different immunogen formulations on day 0 (caudal, s.c.) and boosted twice on days 14 and 28 (i.p.), followed by pair-wise analysis on days 3-7 post-immunization. Numbers of IgM-/IgG-secreting peptide specific splenocytes were determined for each of the two animals (mouse a: open rhombi, mouse b: solid squares). Mice BSA-3a and OA-3a died during the study. Mice BSA-7a/b, Pep-7b and TG-7b produced implausible ELISPOT results. Immunogen formulations: free synthetic peptides (Pep), peptides coupled to BSA, ovalbumin (OA) and thyroglobulin (TG). 50% Freund's Complete (day 0) and Incomplete (days 14, 28) Adjuvants were co-administered in PBS.

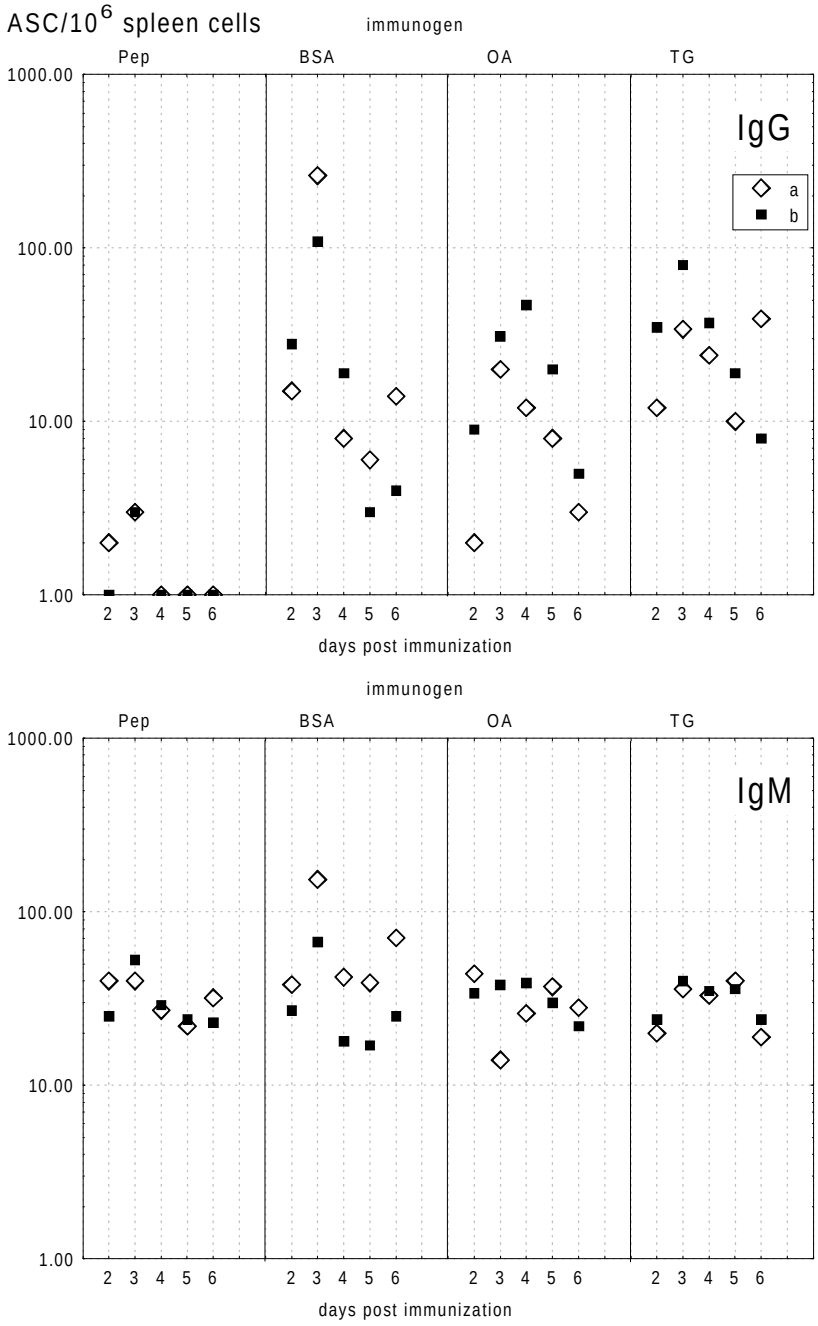


Figure 20: *Response Peak Study*. IgA1-PA50-antigen-specific ASC counts per 10^6 spleen cells. Details as in Figure 19.

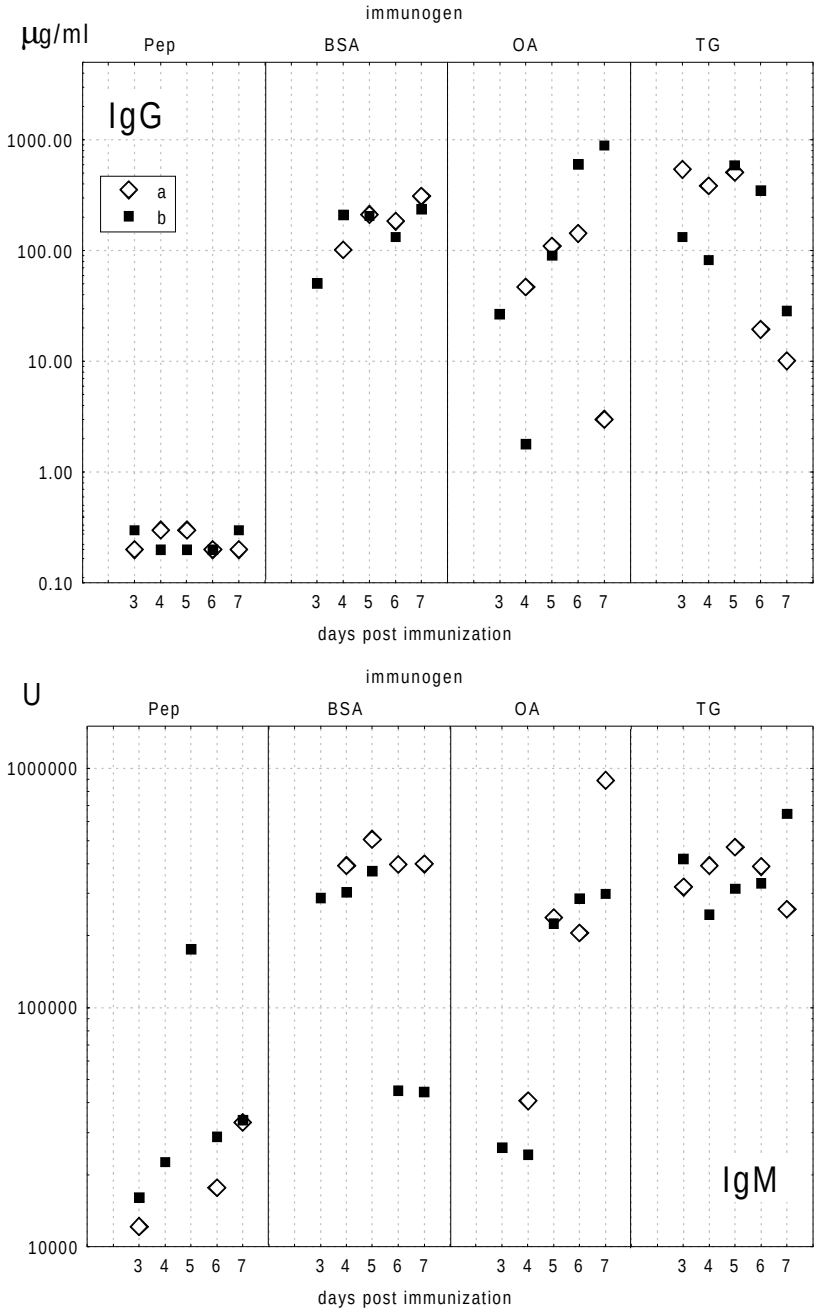


Figure 21: *Response Peak Study*. Serum antibody titers against peptide Opc loop 2. IgM in arbitrary units, U. Details as in Figure 19.

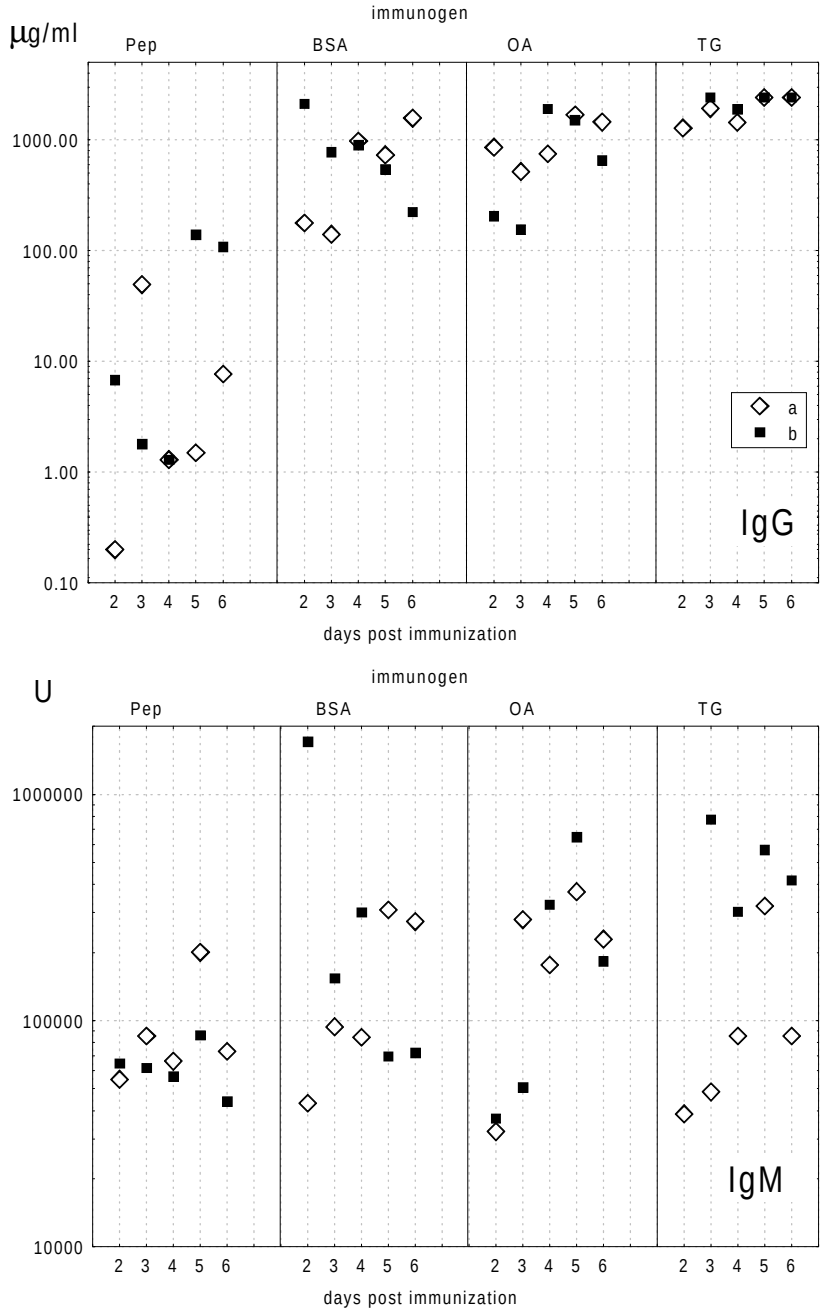


Figure 22: *Response Peak Study*. Serum antibody titers against peptide IgA1-PA50. IgM in arbitrary units, U. Details as in Figure 19.

3.5.2. Immunogenicity study

Separate groups of five mice were immunized with 3 doses of either peptide antigen Opc loop 2, IgA1-PC20 or IgA1-PA50. The three sets of model antigens were applied in 10 different immunogen formulations, as shown in Table 12 on p. 62 ("Summary of immunogens").

Free peptides of Opc loop 2 and IgA1-PA50, and the tetra-oxime of IgA1-PA50 were also co-administered with 2 cytokines, IL-4 and GM-CSF (abbreviations: CP and COX respectively). For a systematic listing, please refer to the in Table 12

The murine anti-peptide responses were assessed at day 3 post-immunization, i.e. day 31 after the first immunization. Blood samples were collected, the spleens of 5 mice per group were removed and their cells harvested. Antigen-specific antibody-secreting spleen cells were analyzed by ELISPOT (Figure 23 to Figure 25) and sera were analyzed by ELISA (Figure 26 to Figure 29). One control group consisting of 5 mice was immunised with PBS plus adjuvants; these mice showed no reactivity with any of the antigens investigated.

Antibody-secreting spleen cells

The immune response was investigated both to the immunizing peptide and to the parent protein. ASC were differentiated using isotype-specific, conjugated antibody to IgA, IgE, IgG1, IgG2a and IgM. None of the 29 antigen formulations stimulated a detectable IgE response. IgA or IgG2 responses were also not detected or were negligible. IgM and IgG1 were the predominant antibody isotypes.

Opc loop 2 immunogens

The frequencies of peptide-specific B cells from spleen are shown in Figure 23, upper panel. The lower panel depicts the reactivity with the parent protein. The frequency of IgG1 secreting splenocytes ranged from 0 to 361 and for IgM from 0 to 105 ASC per 10^6 cells (cells reacting with the parent protein were less frequent: 0 to 150 and 0 to 83 per 10^6 cells respectively). In protein-free immunogen formulations (Pep, M, OX, L), the numbers of antibody-secreting cells were below the detection limit of 10 ASC/10^6 spleen cells except for CP, which induced 16.5 ±4.3 IgM secreting cells to peptide and 27.5 ±14.0 to protein.

Peptide Opc loop 2 was immunogenic when conjugated to KLH, TG or TT. In case peptide Opc loop 2 was coupled to TT the highest number of antigen-specific B-cells was induced.

IgA1-PC20 immunogens

As seen in Figure 24 (upper panel), the protein carrier immunogens (B, K, OA, TG, TT) induced detectable peptide-specific IgG1 secreting B-cells with up to 225 ASC per 10^6 cells. IgM-secreting ASC ranged from 49 to 206/10^6. The highest anti-IgA1-PC20 response was induced by a KLH conjugate. Unlike Opc loop 2, ASC induced by non-protein carriers (Pep, M, OX, L) were also detected. Their response remained restricted to IgM isotypes and varied from 28 to 63 ASC per 10^6 cells. On

average more ASC were induced by liposomes than by tetra oximes followed by MAPs and free peptide.

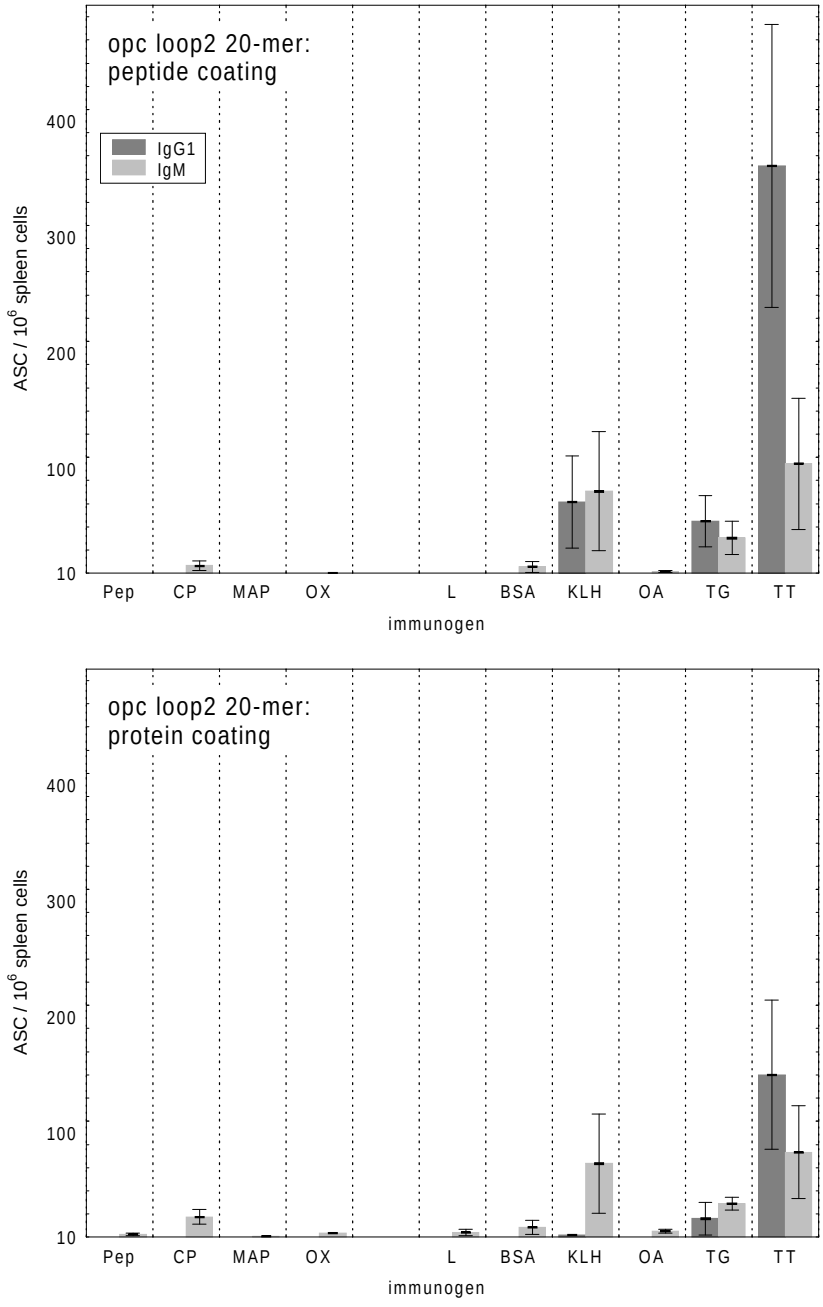


Figure 23: *Immunogenicity Study*. Opc loop 2 immunogens: Concentration of antigen-specific ASC in spleen on day 3 post-immunization. Plates were coated with peptide IgA1-PC20 (peptide coating) or the parent protein serogroup C IgA1-protease (protein coating). Bars indicate mean values of 5 mice/group, each of which was titrated in triplicate. Whiskers indicate +/- standard error. Antibody isotypes: IgG1 (dark gray) and IgM (light gray). Mice were challenged with different immunogen formulations on day 0 and boosted twice on days 14 and 28, followed by spleen excision on day 3 post-immunization. Immunogen formulations: Free synthetic peptides (Pep), peptides co-administered with cytokines (CP), MAPs, tetra-oximes (OX), peptides associated with liposomes (L), coupled to BSA, KLH, ovalbumin (OA), thyroglobulin (TG) and tetanus toxoid (TT). 50% Freund's complete (day 0) and incomplete (days 14, 28) adjuvant were co-administered in PBS.

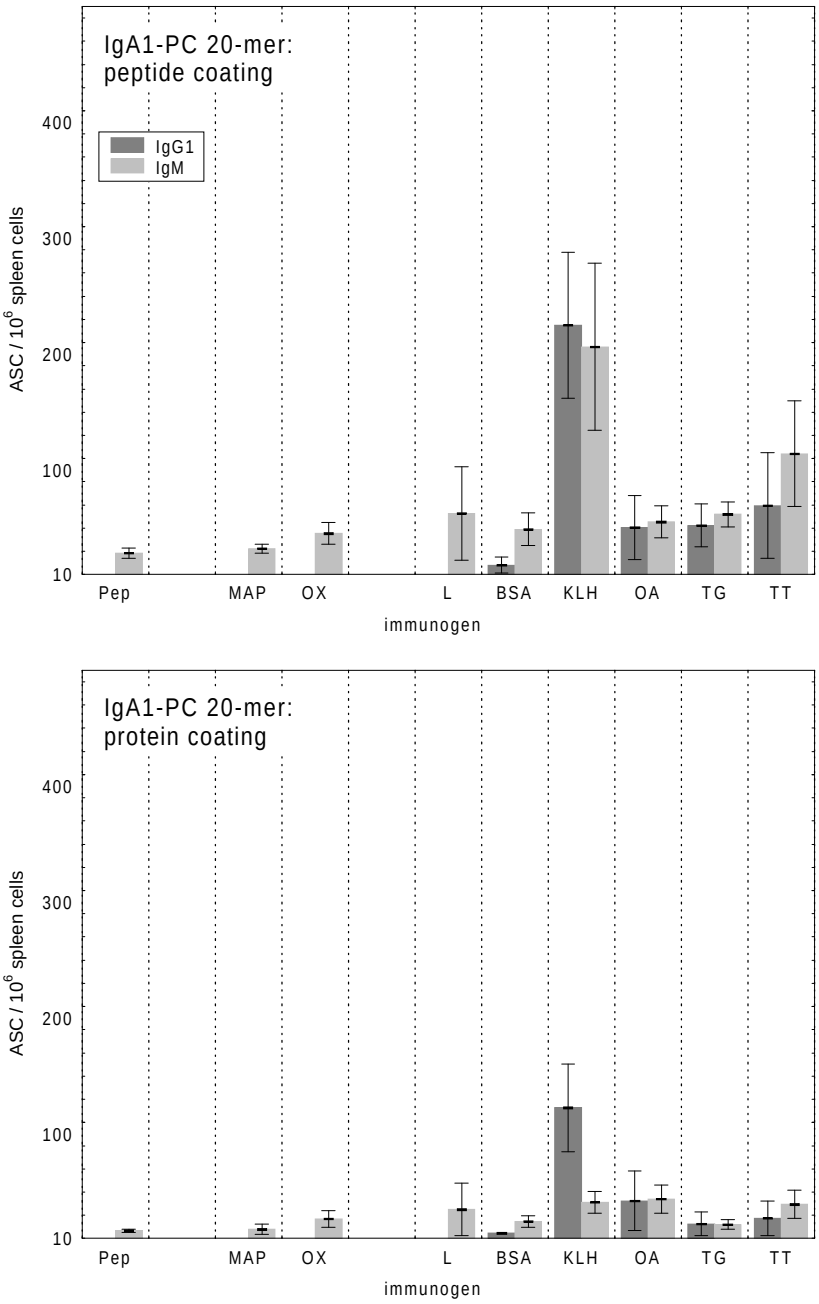


Figure 24: *Immunogenicity Study*. IgA1-PC20 immunogens: Concentration of antigen-specific ASC in spleen on day 3 post-immunization. Plates were coated with peptide IgA1-PC20 (peptide coating) or the parent protein serogroup C IgA1-protease (protein coating). Details as in Figure 23.

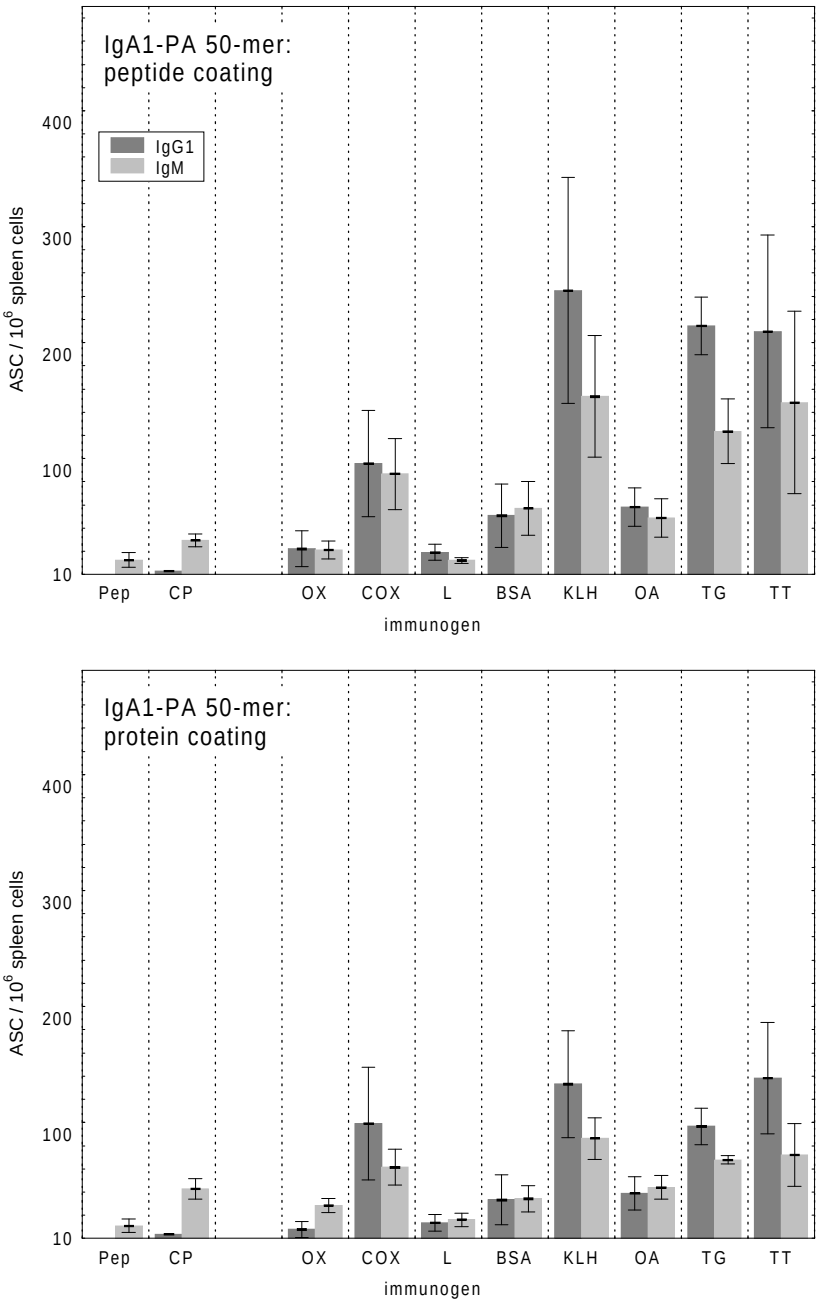


Figure 25: *Immunogenicity Study*. IgA1-PA50 immunogens: Concentration of antigen-specific ASC in spleen on day 3 post-immunization. Plates were coated with peptide IgA1-PC50 (peptide coating) or the parent protein serogroup A IgA1-protease (protein coating). Details as in Figure 23.

As with the Opc 20-mer immunogens above, splenocytes specific to the parent protein (serogroup C IgA1-protease) were also detected (Figure 24, lower panel).

IgA1-PA50 immunogens

With the exception of free peptide, all immunogen formulations induced detectable numbers of both IgG1- and IgM-secreting spleen cells specific to peptide IgA1-PA50 (Figure 25, upper panel). The frequency varied from 13 to 255 ASC per 10^6 spleen cells for IgG1, and from 22 to 164 for IgM.

The hierarchy of responses remained unchanged, with protein carrier immunogens being most stimulatory impact. KLH stimulated the highest numbers of ASC, closely followed by thyroglobulin and tetanus toxoid. Ovalbumin and BSA were less immunogenic. K, TG and TT also stimulated detectable numbers of IgA- and IgG1-secreting lymphocytes. In contrast to Opc loop 2 and IgA1-PC20, this 50 amino acid peptide also stimulated detectable IgG1-ASC in non-protein formulations (CP, OX, COX, L). If free peptide was co-administered with cytokines (CP), IgG1-ASC and IgM-ASC were stimulated slightly. Likewise cytokines increased IgG1- and IgM-ASC more than 3-fold to 106 and 97 ASC/10^6 spleen cells, respectively, when co-administered with tetra-oxime (COX). Splenocytes reactive with the parent protein IgA1-protease ("protein coating") were again detected in all immunogen formulations, although at lower levels than to synthetic peptide antigen.

Serum antibody titers

Serum antibody titers were measured against the immunizing antigenic peptides Opc loop 2, IgA1-PC20 and IgA1-PA50 (peptide coating), and their cognate parent proteins (protein coating). Titers were determined for antigen-specific IgG (µg/ml) and IgM (arbitrary units, U, that cannot be compared between different antigens). Antibody concentrations are represented as box and whisker plots. The boxes contain the second and third quartiles separated by a horizontal line indicating the median value of 5 samples per group. The first and fourth quartiles are indicated by whiskers. The horizontal threshold indicates non-specific background reactivity of pre-immune sera for both IgG and IgM.

Opc loop 2 immunogens

The immunizing antigens cluster in two groups with respect to response intensity: Protein carrier-conjugated peptide and non-protein formulations. The two groupings generally show marked differences in IgG response intensity. For IgM titers the differences were smaller.

In peptide ELISA (Figure 26 + Figure 27, upper panel), IgG responses to free peptide, peptide + cytokines, MAPs, tetra-oximes and liposomes were negligible. All non-protein immunogens showed slightly elevated IgM levels of about 0.2 U. More vigorous IgM responses were seen after Opc loop 2 was coupled to protein-carriers. In these cases, median IgG titers varied from 32 to 458 µg/ml with tetanus toxoid showing the highest response and very low response variation. Median IgM titers ranged between 0.4 and 2.7 U (TT).In protein ELISA (Figure 28, Figure 29, upper panel), IgG titers to Opc protein could not be determined due to cross-reactivity of conjugated detection antibodies.

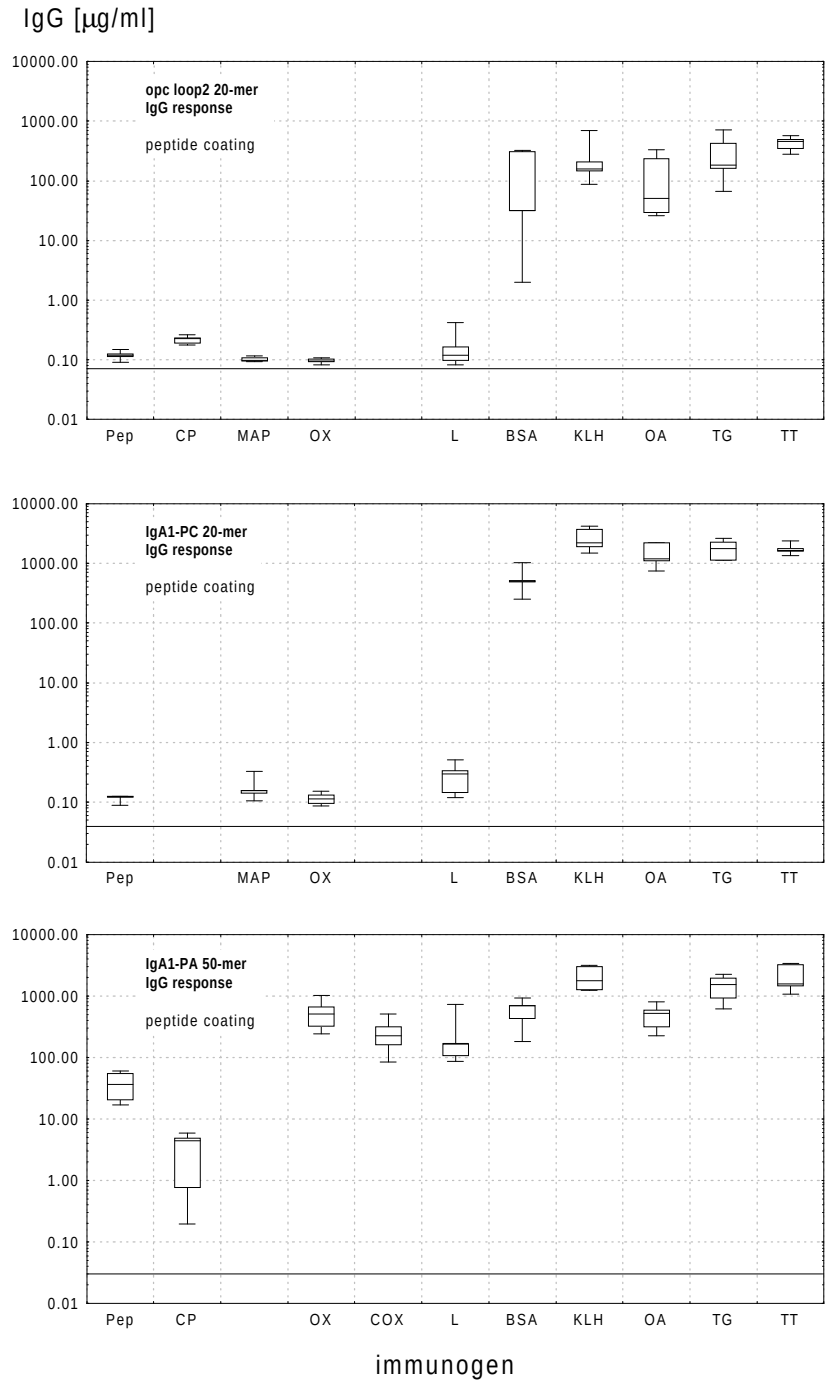


Figure 26: *Immunogenicity Study*. IgG serum titers of peptide-specific immunoglobulins. ELISA plates were coated with the immunizing synthetic peptides Opc loop 2 (upper panel), IgA1-PC20 (central) or IgA1-PA50 (lower). Box and whisker plots indicate concentrations of IgG (µg/ml). The boxes contain the second and third quartiles separated by a horizontal line indicating the median value of five samples. The first and fourth quartiles are indicated by whiskers. The horizontal baseline indicates non-specific background reactivity of pre-immune sera. Mice were challenged with different immunogen formulations on day 0 and boosted twice on days 14 and 28. Serum samples were collected on day 3 post-immunization. Immunogen formulations: 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 BSA, KLH, ovalbumin (OA), thyroglobulin (TG) and tetanus toxoid (TT). 50% Freund's Complete (day 0) and Incomplete (days 14, 28) Adjuvants were co-administered in PBS.

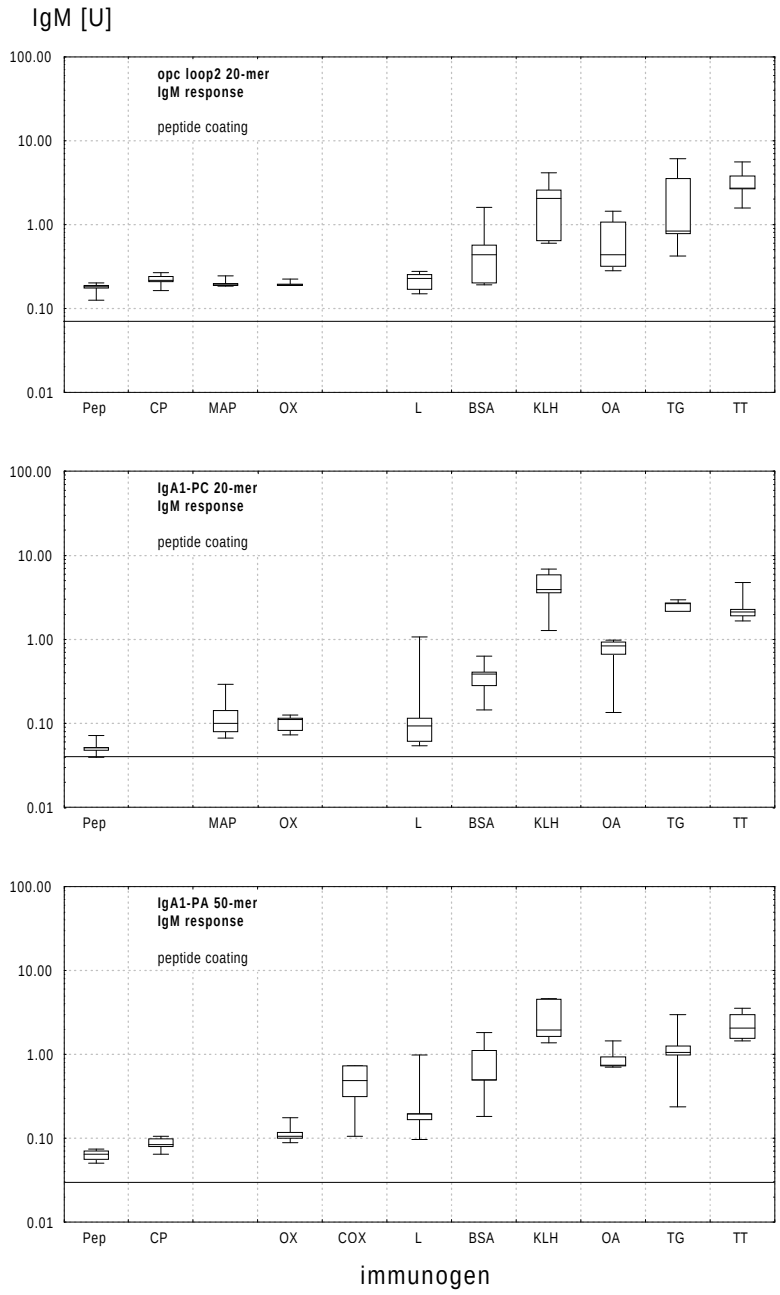


Figure 27: *Immunogenicity Study*. IgM serum titers of peptide-specific immunoglobulins (arbitrary units, U). ELISA plates were coated with the immunizing synthetic peptides Opc loop 2 (upper panel), IgA1-PC20 (central) or IgA1-PA50 (lower). Details as in Figure 26.

IgM reactivity with protein was slightly lower than against peptide. Except for peptide + cytokine (CP) which induced a weak IgM response, non-protein formulations did not stimulate significant IgM against Opc protein. Protein carrier immunogens stimulated antigen-specific IgM and showed the same hierarchy as described for the anti-peptide ELISA. Titers ranged from a maximum of 1.4 U for TT followed by TG, KLH and OA, down to 0.4 U for BSA.

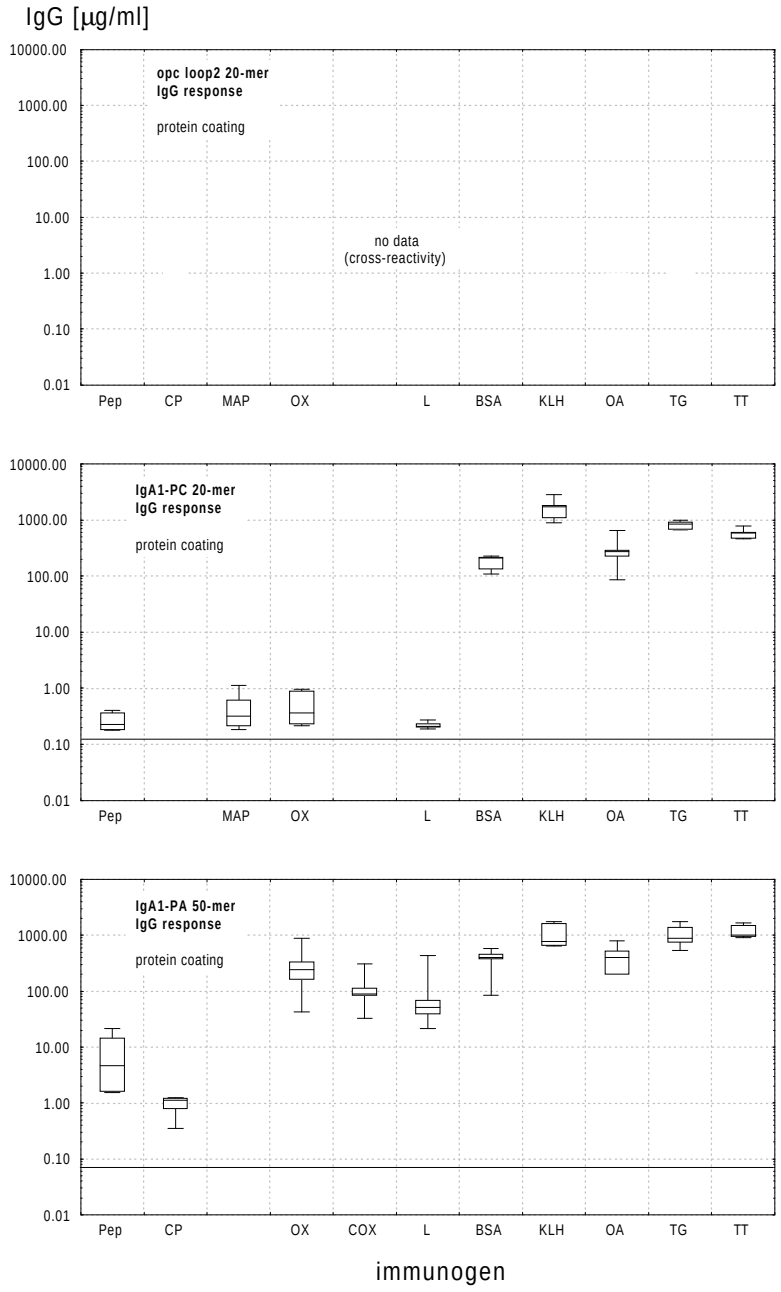


Figure 28: *Immunogenicity Study*. IgG serum titers of parent protein-specific immunoglobulins. ELISA plates were coated with cognate proteins Opc (upper panel), serogroup C IgA1-protease (central) or serogroup A IgA1-protease (lower). Details as in Figure 26.

IgA1-PC20 immunogens

Immune responses to peptide IgA1-PC20 gave a protein-carrier immunogen/non-protein immunogen division as well (Figure 26, Figure 27, central). Non-protein immunogens (Pep, M, OX, L) invariably induced low IgM and IgG responses. Protein carrier conjugated IgA1-PC20 resulted in high IgG titers of 2159 µg/ml for KLH followed by thyroglobulin (1758 µg/ml), tetanus toxoid (1645 µg/ml), ovalbumin (1170 µg/ml) and BSA (491 µg/ml).

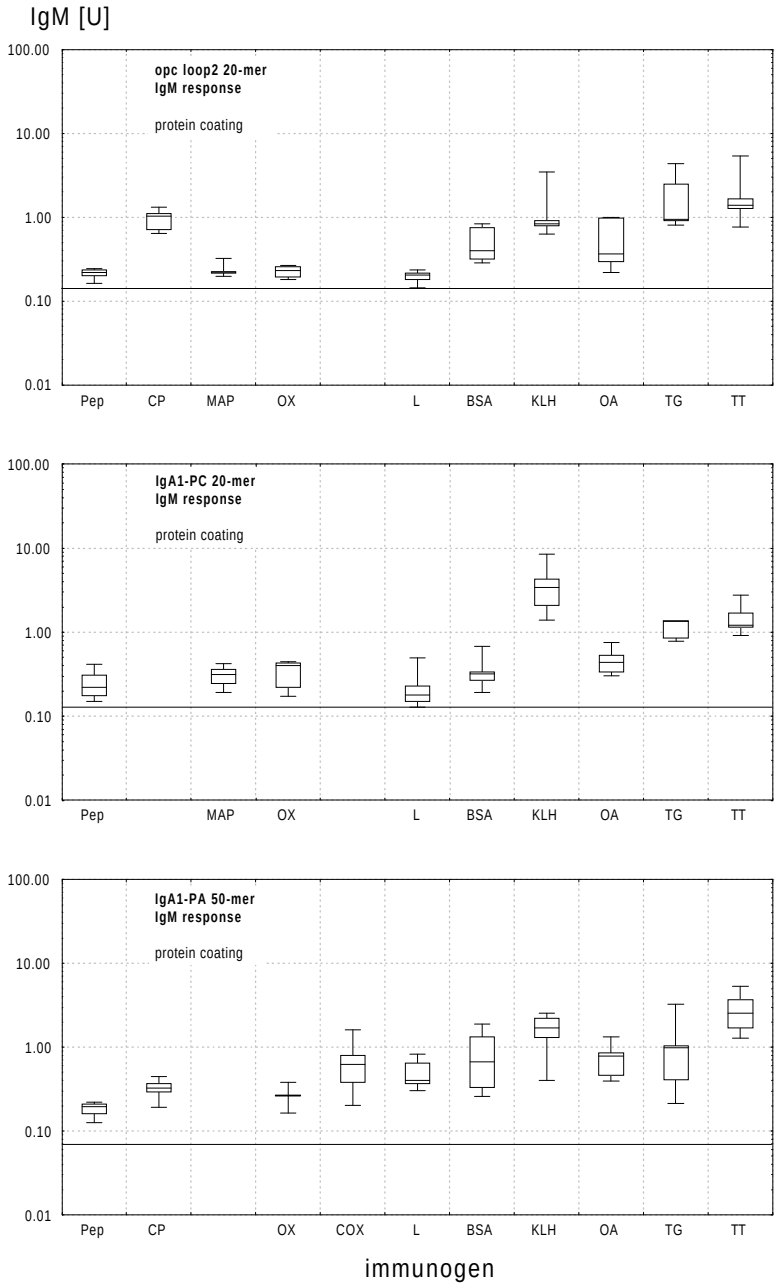


Figure 29: *Immunogenicity Study*. IgM serum titers of parent protein-specific immunoglobulins (arbitrary units, U). ELISA plates were coated with cognate proteins Opc (upper panel), serogroup C IgA1-protease (central) or serogroup A IgA1-protease (lower). Details as in Figure 26.

In protein ELISA (Figure 28, Figure 29, central) the superior immunogenicity of KLH, TG and TT became even more noticeable. Protein-specific IgG titers ranged from 208 µg/ml (B, median) to 1702 µg/ml (KLH) and IgM from 0.3 U (B) to 3.4 U (KLH).

IgA1-PA50 immunogens

In peptide ELISA (Figure 26, Figure 27, lower panel) KLH, thyroglobulin and tetanus toxoid stimulated the highest IgG concentrations with 1766, 1568 and 1546 µg/ml, respectively. BSA induced 691 µg/ml and OA 515 µg/ml, followed by OX (506 µg/ml), COX (226 µg/ml), L (166 µg/ml), Pep(36.4 µg/ml) and CP (4.4 µg/ml). Except for the cytokine formulations, the same hierarchy was observed for IgM titers which ranged from 2.07 (TT) and 1.95 U (KLH) to 0.07 U (Pep). Cytokine co-administration (CP, COX) induced lower IgG, but higher IgM titers than cytokine-free preparations (Pep, OX).

In contrast to the Opc loop 2 and IgA1-PC20 20-mers, the 50 aa peptide IgA1-PA50 appeared highly immunogenic on its own and induced a clear IgG response.

Antibody reactivity with the parent protein serogroup A IgA1-protease (protein ELISA) gave a similar, less intense pattern (Figure 28, Figure 29, lower panel).