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Structure, design, and synthesis of immunoactive peptides

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Abstract - The active principle of commercial antitumour bacterial preparation blastolysin was isolated and subjected to the chemical and spectroscopic structural study. The structure was determined as a tetrasaccharide moiety, (N-acetylglucosamine-β-1-4-N-acetylmuramyl)$_2$ to which Ala-, Lys-, D-Gln, D-Asp, and D-Ala containing peptides and teichoic acid residues are linked. The latter do not contribute to the antitumour activity of the preparation. Synthetic approaches are developed to di- and tetrasaccharide containing glycopeptides. A series of such compounds is obtained, and their antitumour and immunoadjuvant properties are characterized. Conformation and calcium binding properties of glycopeptides are studied by CD and NMR spectroscopy. Glycopeptide derivatives are incorporated into a variety of presumably immunogenic constructions containing a synthetic peptide (from the C-terminal part of the foot and mouth disease virus VP_1 surface protein) and a high molecular carrier.

INTRODUCTION

Bacterial preparations such as BCG (ref. 1), *Corynebacterium parvum* (refs 2, 3), prodygiozans (ref. 4), etc. are broadly used as immunomodulators or antitumour agents. To such preparations also belongs a mixture of glycopeptides called blastolysin, isolated from the lysozyme digest of the *Lactobacillus bulgaricus* cell wall by the bulgarian group headed by I. Bogdanov and used by him in cancer therapy (refs 5, 6). Early attempts to establish the structure of one of the active components of blastolysin resulted in a formula with a unique cyclopeptide moiety (ref. 7). However, that structure had to be abandoned since total synthesis of respective compound afforded a weakly active product with quite different physical properties relative to the active fraction (ref. 8). In order to clarify the resultant uncertainty we continued the structural study of blastolysin. Results of the study served as a starting point of further investigation into synthesis, biology, and physicochemical properties of immunoactive peptides.

STRUCTURE OF BLASTOLYSIN

Successive fractionation of blastolysin on Sephadex G-50 and DE-32 (Cl-) allowed separation of a number of inactive fractions (B-3 - B-9) (Figs 1, 2). In HPLC analysis and SDS electrophoresis B-10 fraction behaved as an individual product, accordingly it had only one N-terminal amino acid (Asp), as determined by the standard phenylthiohydantoin procedure (ref. 9).

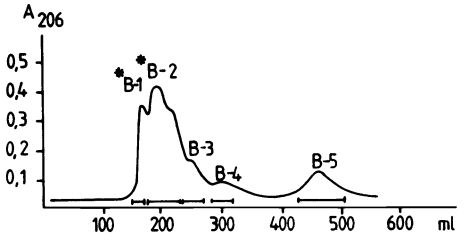

Fig. 1. Chromatography of blastolysin (100 mg) on Sephadex G-50 superfine column (2.6x80 cm) in 0.1 M AcOH. Flow rate 16 ml/min. UV (206 nm) detection. * Fractions displaying antitumour activity.

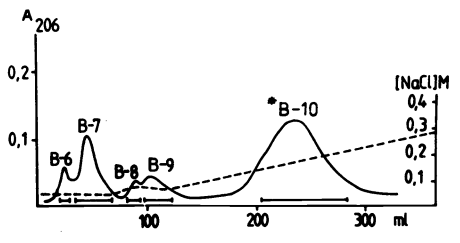

Fig. 2. Chromatography of fraction B-12 (40 mg) on DE-32 (Cl^-) cellulose column (0.9x30 cm). Flow rate 1.67 ml/min, pH 6.0. The NaCl gradient is shown by dotted line. * Fraction displaying antitumour activity.

TABLE 1. Antitumour activity of blastolysin, the B-10 component and its derivatives (S-180 tumour mice, dose 50 mg/kg, testing procedure given in ref. 11)

Sample / Activity	Blasto-lysin	B-10	B-10 treated with 0.05 M NH_4OH (20°C, 12 hr)	B-10 treated with 5% CCl_3CO_2H (40°C, 100 hr)	B-10 treated with 0.05% HJO_4 (+4°C, 260 hr)
	Antitumour activity				
Necrosis vs total number of mice, %	70	85	70	74	0
Inhibition of tumour growth, %	47	50	50	50	0

Judging from size exclusion chromatographic data (calibrated Sephadex G-50 and TSK-SV-3000 columns) molecular mass of B-10 is ca. 10,000. The B-10 hydrolyzate contains glucosamine, muramic acid, Ala, Glu, Lys, and ammonia in the approximate 1:1:2:1:1:1 ratio characteristic of *Lactobacilli* peptidoglycan (ref. 10) (excepting ammonia which was partially lost during acidic treatments used for preparation of blastolysin). GLC and elemental analysis also revealed glucose, galactose, glycerol, and phosphorus indicating the presence of teichoic acid attached to the glycopeptide core. However, its presence is not essential for biological activity since partial splitting of galactose, glucose, and glycerophosphate by mild acidic or alkaline treatment resulted in fully active preparation (Table 1). On the contrary, HJO_4 oxidation (which in addition to glucose and galactose also destructs the muramic acid residues) gives rise to a totally inactive product.

The next question to be answered was the structure of the polysaccharide chain built of GlcNAc and MurNAc residues. Since the process of blastolysin production includes lysozyme treatment of the bacterial cell wall B-10 is expected to contain $(GlcNAc\text{-}MurNAc)_n$ segments. The known resistance of similar tetrasaccharide to lysozyme (ref. 12) allows us to suggest that n=2. This suggestion was confirmed by $NaBH_4$ treatment of B-10 which as expected resulted in reduction of the muramic acid residues (i.e. the terminal) to muramitol. ^{13}C-NMR spectra of the above mentioned product obtained by mild alkaline treatment of B-10 and a series of model spectra provided additional evidence of this conclusion (refs 13, 14). The partial hydrolyzate of B-10 (6N HCl, 30 min, 100°C) was subjected to trifluoroacetylation and methylation followed by mass-spectral analysis which afforded important structural information on the peptide portion. GLC separation of individual components allowed detection of short peptide sequences, viz. Ala-Glu, Glu-Ala, Ala-Asp, Lys-Ala, Asp⌐Lys, Glu⌐Lys, Asp⌐Lys-Ala. A few longer peptides (Glu, Lys, Ala), (Glu, Asp, Lys), (Ala, Glu, Lys, Asp), (Ala, Glu, Lys, Asp, Ala) were detected by direct chemical ionization mass-spectrometry of the partial hydrolyzate (ref. 15). The total hydrolyzate of B-10 (6N HCl, 16 hr., 90°C) was trifluoroacetylated, esterified with isopropyl alcohol and subjected to enantiomeric analysis by GLC on lauroyl-L-valyl-tert-buthylamine as described in ref. 16. Glu and Asp residues were shown to belong to D-family, while both, L and D enantiomers of Ala were found in the hydrolyzate.

The data above and those on structural principles of the cell wall of *Lactobacilli* lead to the formula presented in Fig. 3. The structure contains 2 tetrasaccharide segments to which teichoic acids and peptide moieties are attached. The segments are linked to D-Ala and D-Asp containing bridges. Some uncertainty remains with respect of the position of D-Ala residues; this is reflected in placing that residue within brackets.

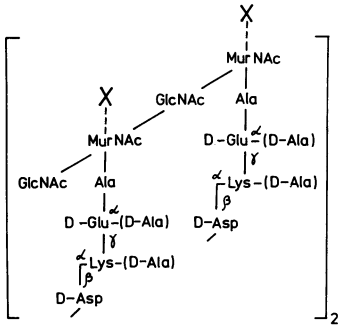

Fig. 3. Structure of the active principle of blastolysin (component B-10).

SYNTHESIS AND PROPERTIES OF DISACCHARIDE AND TETRASACCHARIDE CONTAINING GLYCOPEPTIDES

The structure obtained resembles the water soluble muramyl peptides which already in the early 70s were shown to imitate the mycobacterial component in the classic immunoadjuvant, complete Freund's adjuvant. The simplest representative of these peptides, N-acetylmuramyl-alanyl-D-isoglutamine (muramyldipeptide, MDP) was synthesized in 1974 (ref. 17). That was followed by preparation of many novel MDP analogues and intensive biological study of the entire group (refs 18, 19). An important difference of these glycopeptides from the structure in Fig. 3 is the presence of additional N-acetylglucosamine residues in the latter. Bearing in mind the remarkable biological activity of blastolysin (refs. 6, 20, 21) we attempted the synthesis of a series of GlcNAc-β-1-4-MurNAc and $(GlcNAc-β-1-4-MurNAc)_2$ containing glycopeptides. Two principal schemes were developed. The first, shown in Fig. 4, implies direct condensation of the unprotected sugar with the peptide component and the second (Fig. 5) is based on dimethoxytrityl protection widely used in current oligonucleotide synthesis. The former approach proved very practical with the disaccharide (n=1) while the latter gave better results with the tetrasaccharide.

Both carbohydrate components were obtained by treating the crude *Micrococcus lysodeiktikus* preparation with lysozyme followed by simple ion exchange and size exclusion fractionations. The major product of that process (recently adapted to industrial scale) is the disaccharide, the dimer being obtained in much smaller quantities. The methods presented in Figs 4 and 5 are much faster and cheaper than the alternative procedures (refs 24, 25) leading to glycopeptide (2) (GMDP, see below the Table 2). A series of novel glycopeptides was prepared with the help of these methods. A fraction of these derivatives together with the results of their preliminary biological testing is presented in Table 2. MDP (3)also prepared by direct coupling (ref. 11) and blastolysin were used as reference samples.

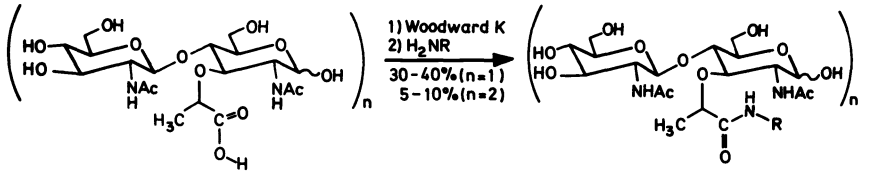

Fig. 4. Direct coupling of sugar moiety with the peptide (refs 11, 22) n=1, R=Ala, Ala-D-Glu(OBzl)NH_2, D-Ala-D-Glu(OBzl)NH_2, Ala-Glu(OBzl)NH_2, Ala-D-Glu(OBzl)$_2$, D-Ala-D-Glu(OBzl)$_2$, Ala-D-Asp(OBzl)NH_2, Ala-D-Glu(OBu), Ala-D-Glu(OBu)NH_2, Val-D-Glu(OBzl)NH_2, Val-D-Glu(OBzl)$_2$, Gly-D-Glu(OBzl)NH_2, Ser(Bzl)-D-Glu(OBzl)$_2$, Ala-D-Glu(Lys)NH_2, Ala-D-Glu(Ala-D-Glu(OBzl)NH_2)NH_2 or Ala-D-Glu(Ala-D-Glu(OBzl)NH_2)$_2NH_2$; n=2, R=Ala-D-Glu(OBzl)NH_2.

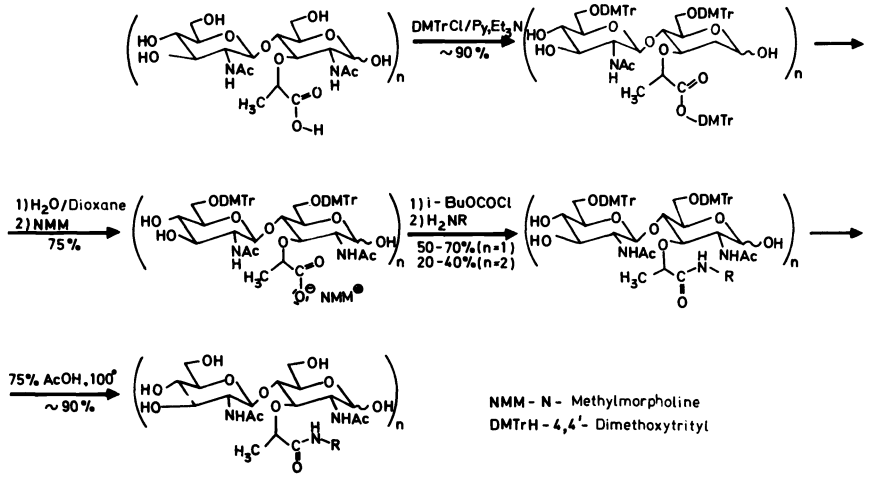

Fig. 5. Synthesis of glycopeptides with intermediate dimethoxytrityl protection of sugar hydroxyls (ref. 23). n=1, R=Ala-D-Glu(OBzl)NH_2; n=2, R=AlaOBzl, Ala-D-Glu(OBzl)$_2$, Ala-D-Glu(OBzl)NH_2, Gly-D-Glu(OBzl)$_2$, Val-D-Glu(OBzl)$_2$, Leu-D-Glu(OBzl)$_2$ or Ser(Bzl)-D-Glu(OBzl)$_2$.

TABLE 2. Antitumour and immunostimulating activity of glycopeptides

No	Compound	Antitimour activity[a] Dose mg/kg	Necrosis %	Tumour growth inhibition, %	Antibody titers against bovine serum albumin (Exp./Contr.)[b] Dose mg/kg	Effect
1.	Blastolysin	50	70	47	5	19.0
		5	40	20	0.5	2.0
2.	GlcNAc-MurNAc-Ala-D-Glu-NH_2 (GMDP)	50	51	40	5	10.2
		5	56	36	0.5	5.3
		0.5	53	29	0.05	2.2
3.	MurNAc-Ala-D-Glu-NH_2 (MDP)	50	45	38	5	2.0
		5	45	29	0.5	5.2
		0.5	30	13	0.05	1.0
4.	GlcNAc-MurNAc-Ala-D-Gln-OBu	50	40	9	5	3.2
		5	20	0	0.5	1.9
5.	GlcNAc-MurNAc-Ala-D-Glu-NH_2	50	40	0	5	16.2
		5	60	38	0.5	1.1
6.	GlcNAc-MurNAc-Val-D-Glu-NH_2	50	50	11	5	11.1
		5	0	0	0.5	8.9
					0.05	1.0
7.	GlcNAc-MurNAc-Gly-D-Glu-NH_2	-	-	-	5	1.0
					0.5	0.9
					0.05	1.0
8.	(GlcNAc-MurNAc)$_2$ Ala-D-Glu-NH_2	50	63	46	5	7.5
		5	40	12		
		0.5	20	12		
9.	(GlcNAc-MurNAc)$_2$ Ala-D-Glu(OBzl)NH_2	50	50	44	5	9.8
		5	50	48		
		0.5	50	28		
10.	(GlcNAc-MurNAc)$_2$ Ala-D-Glu	50	50	59	5	47.0
		5	45	58	0.5	16.6
11.	(GlcNAc-MurNAc)$_2$ Ala-D-Glu(OBzl)$_2$	50	50	54	5	2.2
		5	40	50	0.5	1.5
					0.05	1.0
12.	(GlcNAc-MurNAc)$_2$ D-Ala-D-Glu(OBzl)$_2$	50	16	46	5	4.0
		5	15	0	0.5	1.0
					0.05	1.0
13.	(GlcNAc-MurNAc)$_2$ Val-D-Glu	50	0	27	-	-
		5	0	0		
14.	(GlcNAc-MurNAc)$_2$ Val-D-Glu(OBzl)$_2$	50	0	22	5	30.0
		5	0	22	0.5	4.1
					0.05	1.0
15.	(GlcNAc-MurNAc)$_2$ Gly-D-Glu(OBzl)$_2$	50	0	0	-	-
		5	0	0		

[a] mice, A-180, testing procedure given in (ref. 11)
[b] mice (CBA x C57 Bl/6)F_1. The sera assayed on day 34 (passive hemagglutination).

The results indicate that biological activity in the glycopeptide family is both carbohydrate- and peptide-dependent. Elongation of the glycopeptide chain (MDP → GMDP) is accompanied with an increase in adjuvancy and retention of high antitumour potency. On the other hand, replacement of Ala for Gly (analogue 7) lowers the activity of GMDP; elongation of the latter with Lys residue has little effect on the activity (analogue 5). Esterification of the carboxyls of the most powerful adjuvant (10) almost totally abolishes this type of activity, with practically no effect on the antitumour activity (analogue 11). Retention of antitumour action in weak adjuvants (11 and 12) allows a suggestion that this action is mediated by cellular immunity, i.e. T-killers, NK-cells or macrophages. On the contrary Val-derivatives (6 and 14) show low antitumour activity being at the same time powerful adjuvants. On the whole the tetrasaccharide derivatives are more active than respective mono- and disaccharide derivatives. The same trend holds for the protective effect of synthetic glycopeptides in experimental microbial infections (refs 13, 26).

The molecular mechanism of action of muramyl peptides is not elucidated yet. The NMR spectroscopic studies of MDP in dimethyl sulfoxide (refs 27, 28) and semiempirical calculations of GMDP conformation (ref. 29) did not reveal any specific structural features of the glycopep-

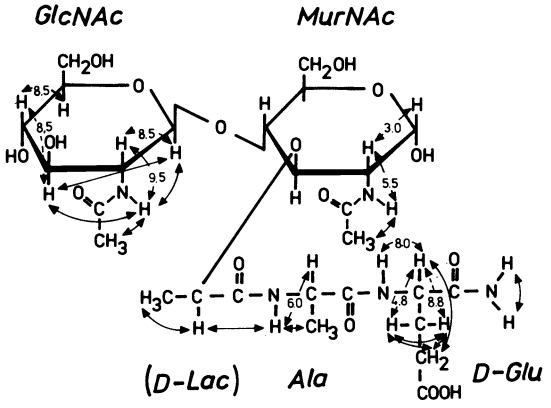

Fig. 6. NOE deduced intramolecular contacts (solid arrows) and selected spin-spin coupling constants (figures and broken arrows) of GMDP in ethanol.

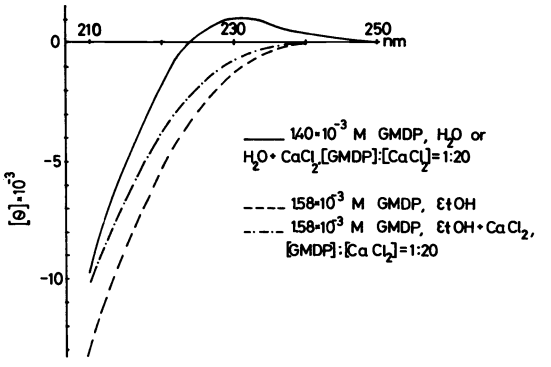

Fig. 7. CD spectra of GMDP.

tides which could be related to their biological function. We have studied the 500 MHz PMR spectra of GMDP in ethanol. Fig. 6 presents the intramolecular contacts deduced from respective NOE interactions in the α-anomeric form which dominates the equilibrium (ca. 70%). Some spin-spin couplings are also shown. These data impose some restrictions on the structure of the peptide and the sugar moieties, providing at the same time practically no information on their relative arrangement. In all probability, there are no important peptide-sugar interactions in that class of immunomodulators. Adam and Lederer (ref. 3) suggested that perhaps some aspects of glycopeptide behavior are explained if they are ascribed the ability to stimulate calcium transport across the cellular membrane, i.e. if they act as calcium ionophores. In order to probe that hypothesis we studied calcium binding of GMDP in solution. CD spectra of GMDP were calcium-independent in water and calcium dependent in dry ethanol in the $0\text{-}10^{-2}$M $CaCl_2$ range (Fig. 7). Calcium binding in the latter solvent was confirmed by NMR followed titration of GMDP by $CaCl_2$. In particular, considerable shifts occurred in the region of NH signals (7.0-8.7 ppm). As seen from Fig. 8, the initial slope of the titration curves most sensitive to the presence of Ca^{2+} (amide NH_2 protons as well as the peptide NH of Ala and D-GluNH2) points to the presence of rather a stable 2:1 GMDP:Ca^{2+} complex in equilibrium with the usual equimolar complex. Analogous experiment with the inactive LL-analogue also showed considerable Ca dependence of the NH shifts. However, there was no indication of 2:1 complex formation in this case. Further studies are required to find whether this fact is relevant to sharp difference in biological properties of these diastereomers (ref. 11).

DESIGN AND SYNTHESIS OF IMMUNOGENIC PEPTIDE COMPLEXES

The muramyl, disaccharide and tetrasaccharide containing glycopeptides certainly deserve further study as potential antitumour, protective antibacterial and antiviral drugs, synergistic agents for various lymphokines etc. However, the most obvious field of their potential application is construction of synthetic vaccines. The general principles of that approach are formulated in the pioneering works of M.Sela, L.Chedid, F.Audibert and their collaborators (refs 30-34). According to these principles the peptide epitope, usually a fragment of a

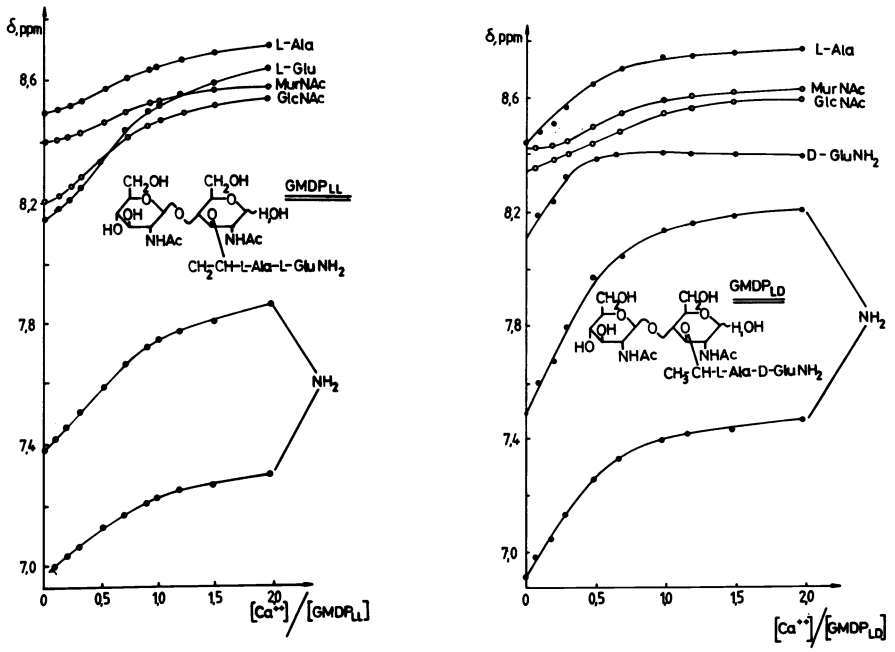

Fig. 8. PMR-monitored titration of GMDP and its LL-diastereomer. Starting concentration of the glycopeptide is 1.2×10^{-2} M.

viral protein or of a toxic bacterial protein, is coupled with a high molecular carrier and with a glycopeptide giving rise to a conjugate capable of inducing immune response against the virus or the toxin. The first positive results were obtained with coliphage MS-2 (ref. 31) and diphtheria toxin (ref. 32) but the rules leading to optimal structures of components to be combined or the ways of their conjugation are studied insufficiently.

There are various ways of collecting the components within the triple conjugate, some of which are presented schematically in Fig. 9. In the above mentioned works (refs 31, 32) the upper-left (I) and apparently the lower-left (III) constructions were obtained. In (ref. 26) we also described the preparation and immunogenic properties of the type III conjugate containing the 197-208 segment of the foot mouth disease (FMDV) viral protein VP_1 (ThrGluAlaArgHisLys-GluLysIleValAlaPro). According to (refs 35, 36) the C-terminal sequences of that protein to which our peptide belongs are able to induce virus neutralizing antibodies. Indeed, as seen in Fig. 10 the type III conjugate proved superior to the conventional conjugate with keyhole limpet hemocyanine (KLH).

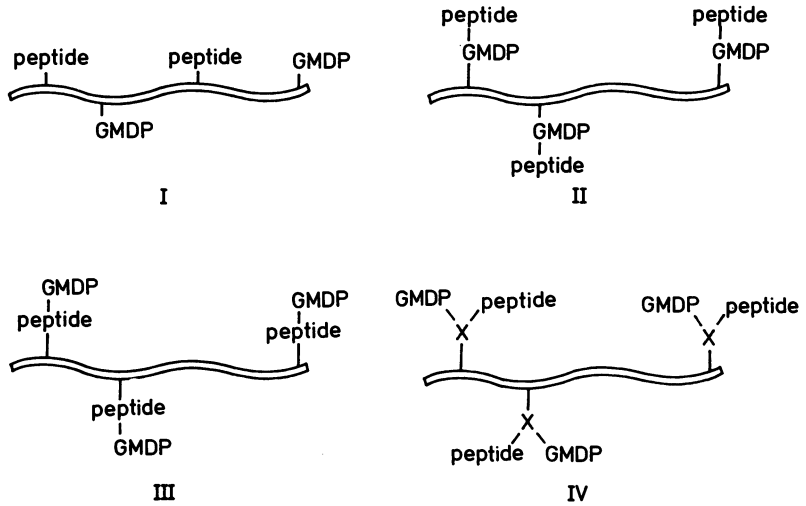

Fig. 9. Relative arrangements of GMDP, the peptide and the polymer in the triple conjugate.

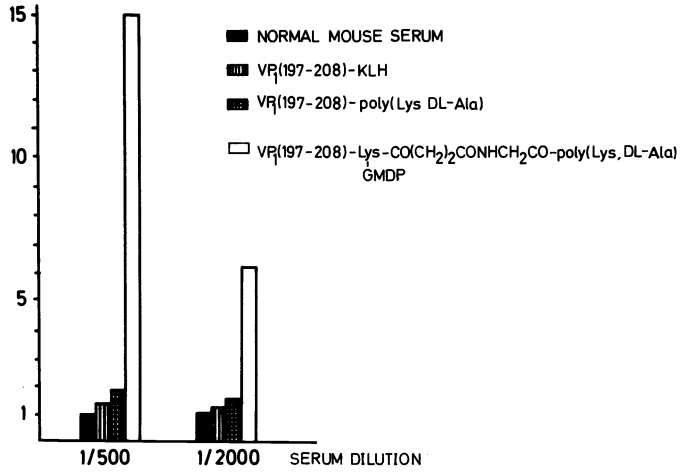

Fig. 10. Binding of inactivated FMDV (O_1K strain) to VP-1 (197-208) sera measured by enzyme linked immunosorbent assay and expressed in relative units. Sera were obtained from BALB/c mice after three immunization with conjugate - saline solutions (30+15+15 μg of the peptide per animal). The weight content of peptide in the conjugate is 10%, the Ala:Lys ratio being 8.9:1. The overall molecular mass of the conjugate is 150,000.

Recently a novel series of peptide conjugates was obtained (Table 3). These include the VP_1(205-213) segment IleValAlaProValLysGluThrLeu, GMDP and the polycationic copolymer poly(Lys,DL-Ala) (AL, see legend to Fig. 10) or the polyanionic copolymer of maleic anhydride and vinylpyrrolidone (MAVP, prepared as described in ref. 37). Samples 4-7 were obtained by 1-ethyl-3(3'-dimethylaminopropyl)carbodiimide hydrochloride (WRCD)/HOBt coupling of variable amounts of GMDP followed by unprotected nonapeptide with AL. Compounds 12-14, also belonging to type I in Fig. 9 were prepared by simultaneous coupling of the MAVP with GlcNac-MurNAc-Ala-D-iGln($NHCH_2CH_2NH_2$) and the nonapeptide. The GMDP ethylendiamine derivative was obtained from GMDP pentafluorophenylate by treatment with a tenfold excess of the diamine. A similar series with the ε-aminocaproyl spacer (compounds 17-20) as well as the respective two component comjugates 1-3, 10, 11, 15 and 16 were also obtained. The above mentioned pentafluorophenylate was used to acylate the aminoterminal and the ε-Lys amino moieties of the nonapeptide, giving rise to $VP_1(GMDP)_2$. The latter was coupled by WSCD/HOBt method with AL to give 8 and 9 (type III conjugates in Fig. 9). Conjugates 1-20 were purified by gel-filtration on Sephadex G-50, characterized by amino acid and carbohydrate analysis and are presently subjected to biological studies. The search for better ways to immunogenic complexes will be continued when results of these studies are available.

TABLE 3. Composition of immunogenic complexes

No	Structure	Content (%, w/w) of VP_1(205-213)	Content (%, w/w) of GMDP
1	VP_1(205-213)~AL	3.5	-
2	VP_1(205-213)~AL	7.0	-
3	VP_1(205-213)~AL	10.2	-
4	VP_1(205-213)~AL~GMDP	5.2	5.2
5	VP_1(205-213)~AL~GMDP	2.1	5.4
6	VP_1(205-213)~AL~GMDP	5.0	12.3
7	VP_1(205-213)~AL~GMDP	2.0	31.0
8	$(GMDP)_2$-VP_1(205-213)~AL	2.0	3.5
9	$(GMDP)_2$-VP_1(205-213)~AL	11.1	16.6
10	MAVP~VP_1(205-213)	3.0	-
11	MAVP~VP_1(205-213)	11.2	-
12	VP_1(205-213)~MAVP~$HNCH_2CH_2NHGMDP$	2.9	1.5
13	VP_1(205-213)~MAVP~$HNCH_2CH_2NHGMDP$	6.1	1.9
14	VP_1(205-213)~MAVP~$HNCH_2CH_2NHGMDP$	13.5	3.8
15	MAVP(Aca)~VP_1(205-213)	6.1	-
16	MAVP(Aca)~VP_1(205-213)	2.9	-
17	VP_1(205-213)~MAVP(Aca)~$HNCH_2CH_2NHGMDP$	1.1	1.0
18	VP_1(205-213)~MAVP(Aca)~$HNCH_2CH_2NHGMDP$	2.1	2.0
19	VP_1(205-213)~MAVP(Aca)~$HNCH_2CH_2NHGMDP$	4.3	1.5
20	VP_1(205-213)~MAVP(Aca)~$HNCH_2CH_2NHGMDP$	1.0	2.5

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