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Immunogenicity of N-(2-Hydroxypropyl)-methacrylamide Copolymers — Potential Hapten or Drug Carriers

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ABSTRACT. After repeated i.p. immunizations of mice with 10 μg of homopolymer poly (HPMA) no antibodies were detected by the ELISA test.

Immunization with copolymer P-Acap-Leu-HMDA leads to a weak antibody response, while immunization with a copolymer with some side chains modified with ARS or FITC groups (P-Acap-Leu-HMDA-ARS or P-Acap-Leu-HMDA-FITC) leads to a significant antibody response detectable by PFC, ELISA and haemagglutination tests. Most of these antibodies are aimed against the modifying haptenic group, a smaller amount against side oligopeptide sequences of the carrier.

Intensity of the antibody response depends on: 1) the antigen dose — the optimal dose was 10 μg: both the higher (100 μg) and the lower doses (1 and 0.1 μg) induced considerably lower antibody responses; 2) molar mass of the immunizing fractions—fractions of high molar mass induced up to five times higher responses than those of a low molar mass; 3) the bound haptenic group — the ARS-copolymers induced ten times lower response than the FITC-copolymers.

We detected no difference between capacities of the H-2^a, H-2^b and H-2^d haplotypes to react with anti-ARS antibodies after immunization with P-Acap-Leu-HMDA-ARS.

Copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA) are used in an attempt to produce an effective drug carrier system (Kopeček *et al.* 1981*a*; Kopeček 1981; Duncan *et al.* 1980; Duncan *et al.* 1981).

For the practical applicability of the polymer-bound drugs it is necessary to know more about the immunogenicity of this group of compounds.

As a drug model we used two defined small organic compounds —haptens arsanilic acid and fluorescein isothiocyanate (ARS, FITC) attached to P-Acap-Leu-HMDA (*for abbreviations see* Table I). The primary and secondary antibody responses were studied in inbred strains of mice. The antibody response was determined by the number of plaque-forming cells (PFC) in spleen, or as a serum antibody level by passive haemagglutination with ARS-SRBC or FITC-SRBC indicator cells, or by ELISA test.

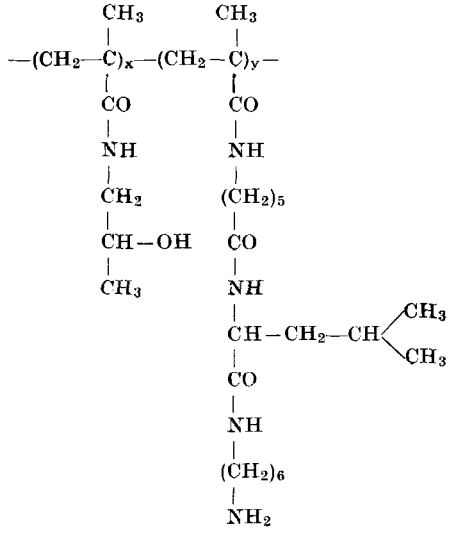
MATERIALS AND METHODS

Antigens. Copolymers of N-(2-hydroxypropyl)methacrylamide were used (Table I).

N-(2-Hydroxypropyl)methacrylamide (HPMA), m.p. 67—68 °C, was prepared as described earlier (Kopeček and Bažilová 1973; Strohalm and Kopeček 1978).

Preparation of hapten carrier (P-Acap-Leu-HMDA). N-Methacryloyl 5-aminocaproylleucine 4-nitrophenyl ester (MA-Acap-Leu-ONp), m.p. 84 to 87 °C, was prepared according to Kopeček (1977).

The polymer precursor was prepared with the structure of P-Acap-Leu-ONp (Kopeček 1977) by a radical heterogeneous copolymerization of HPMA with MA-Acap-Leu-ONp. This polymer was dissolved in dimethyl sulphoxide and reacted with a 100-fold excess of hexamethylenediamine for 10 h at 25 °C. The reaction mixture was vacuum-concentrated and the polymer was precipitated in an excess of acetone, filtered off, washed with acetone and vacuum-dried. The polymer contained 3.25% of NH_2 groups. The polymer had the following structure:



$x = 96.75\ \%$

$y = 3.25\ \%$

Reaction of P-Acap-Leu-HMDA with 4-aminophenylarsonic acid. The reaction was carried out according to Tabachnik and Sobotka (1959). 0.284 g (2 mmol) of 4-aminophenylarsonic acid was diazotized with 2 mmol $NaNO_2$ and 5 mmol HCl in the presence of 0.4 mM KBr. The conjugation of diazonium salt with 0.5 g (0.11 mM of NH_2 groups) of the polymer (P-Acap-Leu-HMDA) was carried out at pH 10.5 at 2—5 °C. The polymer was dialyzed in a Visking dialysis tubing (Serva) against water and isolated by lyophilization. The content of As was determined by atomic absorption spectrometry. Using the described method each modified side chain carriers two arsanilic groups. Experiments with P-Acap-Leu-Gly-ARS copolymers in which the side oligopeptide chains are substituted with one molecule of arsanilic acid via a stable amide bond are in progress.

Reaction of P-Acap-Leu-HMDA with fluorescein isothiocyanate. P-Acap-Leu-HMDA-FITC was prepared according to The and Feltkamp (1970): 10 mL of P-Acap-Leu-HMDA (25 mg/mL saline) was mixed with 10 mL 0.5 M carbonate buffer (pH 9.0) followed with 5 mg of isomer I fluorescein isothiocyanate (Baltimore Biological Laboratories, USA) in 1 mL carbonate

TABLE I. Characterization of polymer-bound haptens

Structure of polymer (copolymer)[a]	Content of side chains molar %	Content of hapten molar %	Molar mass[b] kg/mol
Poly(HPMA)	—	—	30
P-Acap-Leu-HMDA[c]	3.25	—	23
P-Acap-Leu-HMDA-ARS[c]	3.25	0.12	23
P-Acap-Leu-HMDA-FITC[c]	3.25	0.31	23
P-Acap-Leu-HMDA-ARS[d]	3.25	0.03	150 200[e]
P-Acap-Leu-HMDA-ARS[d]	3.25	0.03	47
P-Acap-Leu-HMDA-ARS[d]	3.25	0.03	25
P-Acap-Leu-HMDA-ARS[d]	3.25	0.09	5

[a] Poly(HPMA) homopolymer of N-(2-hydroxypropyl)methacrylamide, P copolymer of N-(2-hydroxypropymethacrylamide), Acap-Leu-HMDA side chain, Acap 5-aminocyproyl, HMDA hexamethylenediamine, ARS 4-azophenylarsonate group, FITC fluorescein isothiocyanate; each side chain contains two residues of arsanilic acid (Tabachnik and Sobotka 1959), *i.e.* the number of modified side chains is twice lower.

[b] Determined by gel filtration.

[c] Initial, unfractionated copolymer.

[d] Fractions of the initial copolymer.

[e] Assessed from elution volume of a fraction on GPC curve of the original copolymer (due to a short supply of the sample we did not determine the molar mass).

buffer (pH 9.0). Conjugation proceeded for 16 h at 4 °C with constant stirring. The nonbound FITC was separated by filtration on Sephadex G-25. Content of FITC in the conjugate was assayed spectrophotometrically at 495 nm.

Fractionation of the haptenated polymer. The polymer was fractionated on GPC column (30 × 1000 mm) packed with Sepharose 6B. The column was equilibrated and eluted with 50 mM CH_3COONH_4 (flow rate 25 mL/h). Individual fractions were lyophilized and their molar mass estimated using GPC columns calibrated with fractions of poly(HPMA) (Rejmanová *et al.* 1977; Bohdanecký *et al.* 1974). A lower content of ARS groups in individual fractions with respect to the original nonfractionated copolymer can probably be ascribed to a partial hydrolysis of the hapten-polymer bond during fractionation and isolation.

Inbred strains of mice. All experiments were performed on 10—12 weeks old females of inbred strains A/J, Balb/c, C57BL/10ScSn and C57L/J (Institute of Physiology, Czechoslovak Academy of Sciences, Prague). During experiments mice were kept under standard conditions.

Immunization. Mice were immunized by intraperitoneal injection of antigens as an alum precipitate or in a complete Freund's adjuvant (CFA). The doses of antigens used are given in Results and Discussion. After immunization mice were bled from retroorbital plexus or exsanquinated, spleens were removed and used for the PFC assay.

Isolation of spleen cells. Spleens were homogenized in a tissue homogenizer in medium 199 (Sevac, Prague). The cells were washed three times in an ice-cold medium and viability was estimated by the trypan blue exclusion test.

TABLE II. Serum antibody level in A/J mice immunized with poly(HPMA), P-Acap-Leu-HMDA or P-Acap-Leu-HMDA-ARS, detected by ELISA test

Antigen used for			
immunization	detection	immunization No.	$\log_2$ of serum dilution[a]
P-Acap-Leu-HMDA	P-Acap-Leu-HMDA	2	6
		3	6
P-Acap-Leu-HMDA-ARS	P-Acap-Leu-HMDA	2	4
		3	4
P-Acap-Leu-HMDA-ARS	ARS-BGG	2	10
		3	9
poly(HPMA)	poly(HPMA)	2	—[b]
		3	—[b]

[a] Antibody concentration was measured six days after 2nd or 3rd immunization. The numbers represent arithmetic means of 10 mice per group.
[b] No antibodies were detected.

Plaque technique. Number of plaque-forming cells (PFC) was estimated by the method of Šterzl and Mandel (1964). Indirect PFC were counted by the method of Šterzl and Říha (1965).

Antisera. Antiserum used for development of indirect PFC was a serum against mouse immunoglobulins prepared by immunization of sheep with mouse IgG in complete Freund's adjuvant (CFA).

Sensitization of erythrocytes for the PFC assay. Sheep red blood cells were sensitized with arsanilic acid according to Říhová *et al.* (1980).

Detection of an antibodies by haemagglutination. Antibody content in sera was determined by passive microhaemagglutination in plastic plates using sheep red blood cells sensitized with arsanilic acid. The sensitization was carried out by the method of Ingraham (1952).

Detection of antibodies by the ELISA test. Detection was performed according to Engvall and Perlmann (1972). Adsorption of antigen to microplates (Koh-i-Noor, Dalečín, Czechoslovakia) proceeded overnight at 4 °C. Wells were filled with aliquots of 100 μg of antigen (10 mg/mL). Next day the microplates were rinsed with PBS and incubated for 1 h in PBS containing 0.02 % gelatine and 1 % BSA. After five more rinsings with PBS and PBS with 0.2 % Tween 20 the wells were filled with 100 μL of different dilutions of the tested sera and the plates were kept overnight at 4 °C. On the next day the microplates were rinsed and horseradish peroxidase-conjugated porcine anti-mouse IgG diluted 1 : 500 was added (Engvall 1980). The tested sera and the conjugate were diluted in PBS with 1 % BSA. After 1 h of incubation the conjugate was removed, microplates rinsed and 0.015 % H_2O_2 with 1,2-phenylenediamine (5 mg/10 μL substrate) was added. The reaction was stopped after 10 min with 20 μL 2 M H_2SO_4 and absorbance was determined using the ELISA-reader (Minireader MR 590, Dynatech) at 492nm.

Statistical analysis. Wilcoxon's statistics (Quenouille 1959) was used to test the differences in antibody formation between different inbred strains. Significant differences were not found at the level of 0.05 probability.

RESULTS AND DISCUSSION

Serum antibody level after repeated immunizations of the A/J mice with poly(HPMA), P-Acap-Leu-HMDA and P-Acap-Leu-HMDA-ARS

Mice were immunized three times in two-week intervals by an i.p. injection of 10 μg of alum-precipitated poly(HPMA) or nonfractioned copolymers.

After immunization with homopolymer poly(HPMA) there were no detectable antibodies in any sample taken during the experiment (Table II). Immunogenicity of poly(HPMA) was studied earlier by monitoring the activation of lymphatic cells in draining lymph nodes. Using this method Korčáková *et al.* (1976) detected no increased production of RNA in nucleoli of lymphocytes after administration of poly(HPMA). All the above results indicate that the poly(HPMA) polymer alone, with no attached side oligopeptide chains, is not recognized as a foreign macromolecule, and after its introduction into the organism it induces no immune response.

Immunization with a copolymer with side oligopeptide sequences induces a poor antibody response (Table II). The most marked antibody reaction was found after immunization with a copolymer with substituted hapten, such as arsanilic acid. Small molecules, such as aromatic haptens, coenzymes and drugs may, after binding to poorly immunogenic carriers, substantially increase their immunogenicity (Sela 1969). Table II shows that the induced antibodies after immunization with copolymer with bound hapten are aimed particularly against the arsanilic acid group, while the antibody response to the proper carrier is very weak, lower than after immunization with a copolymer with no hapten. We explain this lower antibody response as an antigenic competition which takes place between the poorly immunogenic side oligopeptide sequences and the highly immunogenic arsanilic acid.

Dynamics of the primary PFC response to P-Acap-Leu-HMDA-ARS in the A/J mice

The primary reaction was followed after immunization with fractions of molar mass 150—200, 47, 25 and 5 kg/mol. The animals were immunized with doses of 100 μg or 10 μg administered i.p. as an alum precipitate.

Results of the experiment are given in Table III. PFC could be detected in spleen already three days after the i.p. immunization. On the following days the intensity of the antibody response increased, reaching a maximum after 6 or 7 d. After that the immune response sharply decreased. Table III also shows that the reaction intensity depends on the antigen dose and its molar mass. After immunization with fractions of the highest molar mass (150—200 and 47 kg/mol) or with the nonfractionated copolymer the highest dose of antigen (100 μg) induces an immune reaction that is lower than that induced by 10 μg. For a polymer with molar mass of 25 kg/mol the responses to these two doses of antigen were identical, while for a polymer of the lowest molar mass (5 kg/mol) the response was more intense after immunization with 100 μg. Indirect PFC were not found throughout the whole experiment.

The tolerogenicity of the high molar mass polymers injected in high doses can be explained by the nonbiological character of the studied copolymer. Even though the side oligopeptide sequences are cleaved *in vivo* (Kopeček *et al.* 1981*b*), the antigen could be detected in blood circulator (in dependence on molar mass) up to 10 days after administration. Despite the fact

TABLE III. Dynamics of the primary PFC response to 4-azophenylarsonate group in A/J mice after immunization with P-Acap-Leu-HMDA-ARS

Molar mass of antigen fraction kg/mol	Dose of antigen μg	IgM PFC per 10^8 spleen cells ± SE[a], days after immunization				
		3	5	6	7	10
150–200	100	145 ± 29.5	170 ± 29.3	205 ± 27.7	155 ± 20.9	94 ± 15.9
	10	178 ± 27.8	220 ± 53.5	305 ± 42.3	300 ± 42.3	205 ± 30.1
47	100	165 ± 31.7	185 ± 36.2	216 ± 37.2	207 ± 39.0	84 ± 11.6
	10	156 ± 27.3	197 ± 36.4	265 ± 40.7	260 ± 44.8	
25	100	137 ± 19.2	158 ± 30.3	224 ± 28.8	245 ± 35.5	165 ± 24.6
	10	135 ± 23.6	186 ± 34.4	227 ± 32.8	248 ± 42.7	124 ± 17.9
5	100	156 ± 22.9	160 ± 32.6	200 ± 35.7	205 ± 30.5	104 ± 15.6
	10	104 ± 13.8	115 ± 22.5	176 ± 30.3	165 ± 24.2	143 ± 24.2
Initial copolymer	100	132 ± 22.3	189 ± 33.7	206 ± 35.5	195 ± 39.7	110 ± 18.9
	10	159 ± 20.1	199 ± 36.8	245 ± 31.0	265 ± 55.2	168 ± 22.4

[a] The numbers represent arithmetic means of 15 mice per group.

TABLE IV. Effect of molecular size of P-Acap-Leu-HMDA-ARS on PFC response of A/J and C57BL/10ScSn mice to 4-azophenylarsonate group

Molar mass of antigen fraction kg/mol	Dose of antigen μg	IgM PFC per 10^6 spleen cells ± SE[a]	
		A/J	C57BL/10ScSn
150–200	100	241 ± 53.5	83 ± 21.0
	10	465 ± 136.9	218 ± 30.7
	1	182 ± 28.0	110 ± 17.4
	0.1	92 ± 12.3	21 ± 3.6
47	100	191 ± 83.1	49 ± 8.7
	10	382 ± 55.2	125 ± 21.5
	1	75 ± 14.7	99 ± 17.3
	0.1	77 ± 10.8	18 ± 3.3
25	100	177 ± 24.5	32 ± 4.7
	10	281 ± 49.2	103 ± 21.0
	1	66 ± 12.6	73 ± 14.8
	0.1	27 ± 4.1	6 ± 0.8
5	100	110 ± 17.4	25 ± 4.1
	10	249 ± 51.8	92 ± 18.0
	1	37 ± 5.1	28 ± 5.1
	0.1	20 ± 3.4	7 ± 1.0
Initial copolymer	100	185 ± 40.2	69 ± 12.7
	10	256 ± 65.6	119 ± 31.3
	1	84 ± 14.4	65 ± 12.0
	0.1	23 ± 3.5	21 ± 3.4

[a] The numbers represent arithmetic means of 15 mice per group; 1st and 2nd immunization with 0.1 – 100 μg of alum-precipitated antigen i.p.

that after this period most of the antigen is eliminated from the blood circulation, fractions with molar mass higher than 20 kg/mol could be detected in kidneys and organs with phagocytic and pinocitic activities, such as liver and spleen (Šprinel *et al.* 1976). The antigens, particularly the synthetic ones, that are only slowly cleaved and consequently survive longer in blood circulation or in lymphatic tissues, easily induce immunological paralysis in doses which are fully immunogenic for other natural antigens (Andersson 1969).

Secondary response of the C57BL/10ScSn and A/J mice to P-Acap-Leu-HMDA-ARS and P-Acap-Leu-HMDA-FITC

Our preliminary experiments showed that the highest response after the second immunization can be detected on the fourth day. Antigens were administered i.p. as an alum precipitate and the interval between the first and the second immunization was 14 d.

The highest immune response (Table IV) was induced by a dose of 10 μg, while higher (100 μg) and lower (1 and 0.1 μg) doses led to considerably lower immune responses. This finding holds not only for fractions of high molar mass such as for those used in the primary response but for fractions with low molar mass (around 5 kg/mol) as well, for which we found in the primary response after a higher dose a higher antibody response.

TABLE V. PFC response of A/J and C57BL/10ScSn mice to fluorescein after immunization with P-Acap-Leu-HMDA-FITC

Dose of antigen μg	IgM PFC per 10^8 spleen cells ± SE[a]	
	A/J	C57BL/10ScSn
100	5 401 ± 794	2 409 ± 339
10	5 211 ± 766	2 305 ± 324
1	1 680 ± 401	1 010 ± 350

[a] Numbers represent the arithmetic mean of 15 mice per group; 1st and 2nd immunization with 1 — 100 μg of alum-precipitated antigen i.p.

The direct relation between the intensity of the antibody response and the molar mass of copolymers that has been hinted at already by the effect of the primary immunization, was confirmed by the secondary reaction. As compared with fractions of low molar mass (5 kg/mol), fractions with M between 150—200 kg/mol bring about a 2—5 fold increase in the level of PFC in spleen. The direct dependence between the immunogenicity and molar mass of synthetic antigens was described by Brown and Glynn (1969) for poly-L-proline preparations. Synthetic antigens with low M can be removed from blood circulation and tissues more easily and hence, more rapidly than the heavy ones. This decreases the chance that the antigenic determinants of the synthetic antigen come into contact with the appropriate immunocompetent cells.

To analyze the effect of the haptenic group on the antibody response we followed the response to copolymers substituted with two qualitatively different types of haptens. One was arsanilic acid — representative of a small hapten consisting of one benzene ring, the second was fluorescein isothiocyanate — representative of a large hapten consisting of four benzene rings which behaves, from the immunochemical point of view as a bifunctional molecule.

For immunization three different doses (100, 10 and 1 μg) of a non-fractionated copolymer of M = 23 kg/mol with a bound FITC group were used. The antigen was administered i.p. as an alum precipitate, similarly as the ARS-copolymers. Table V shows that in comparison with the response to the ARS-copolymers the response to the FITC-copolymer was ten times higher. The tolerogenicity which was observed for the ARS-copolymers after high immunization doses was not detected with the FITC-copolymers. Antibody responses to the two doses tested (100 and 10 μg) were comparable, only after the dose 1 μg spleen exhibited a lower level of PFC. Most likely the higher immunogenicity of the FITC-copolymers is responsible for this discrepancy as it requires higher doses for tolerance induction than 100 μg. Throughout the experiments we were unable to find presence of IgG PFC.

The secondary reaction was studied with two inbred strains differing in their H-2 haplotype, *i.e.* strains A/J and B10. The B10 strain is characterized by its limited production of antibodies against a number of antigens expressed in a lower level of PFC (Říhová *et al.* 1981*a*) and a limited heterogeneity of antibodies, that can be demonstrated by isoelectric focussing (Říhová *et al.*

TABLE VI. Antibody response of different inbred strains of mice to P-Acap-Leu-HMDA-ARS[a,b]

Molar mass of antigen fraction kg/mol	Dose of antigen μg	Inbred strains of mice			
		A/J H-2[a]	C57L/J H-2[b]	C57B10/ScSn H-2[b]	Balb/c H-2[d]
150–200	100	10	7	10	9
		14	14	12	14
	10	7	8	9	8
		10	10	11	10
47	100	10	8	10	10
		12	13	10	12
	10	6	6	9	8
		10	10	12	10
25	100	10	8	11	10
		10	10	8	10
	10	10	7	8	12
		9	10	12	12
5	100	8	8	10	10
		8	10	8	10
	10	10	7	8	12
		10	11	11	13

[a] Numbers represent arithmetic means of 10 mice per group. Antibody titres of passive haemagglutination test are expressed as $\log_2$ of serum dilution.

[b] ***First line*** — second immunization, ***second line*** — third immunization.

1981*b*). Tables IV and V show that the B10 strain responds to immunization by a lower level of PFC, both after the ARS- and the FITC-copolymer immunization. Differences become marked only for higher immunization doses. On the grounds of these results we were not able to conclude whether the reactions against P-Acap-Leu-HMDA-ARS and P-Acap-Leu-HMDA-FITC were or were not dependent on the H-2 haplotype (and consequently on the appropriate Ir genes). We therefore studied the hyperimmune reaction in four different inbred mouse strains after immunization with copolymers administered simultaneously with CFA.

Hyperimmune reactions of inbred mouse strains with haplotypes H-2^a, H-2^b and H-2^d

A study was done on inbred strains of three different haplotypes (Balb/c = = H-2^d, C57BL/10ScSn = H-2^b and A/J = H-2^a). We also studied responses to the same copolymer in two strains of the same hyplotype but differing in the remaining genetic background (strains C57BL/10ScSn and C57L/J). The antibody response was followed in blood by haemagglutination of arsanilic acid conjugated erythrocytes. Antigen was administered three times in two-week intervals in two different doses — 100 and 10 μg in CFA. Antibodies were assayed one week after the last immunization. The results (Table VI) reveal some differences in the antibody response of the immunized strains. These differences, however, are not significant and cannot be taken as proof of the effect of the H-2 haplotype on the course of the immune response to the studied copolymer. Dintzis *et al.* (1976) studying the immunogenicity of a linear polyacrylamide substituted with DNP also found no difference in the immune responses of six inbred strains. We did not find such differences in the immunogenic effect of 100 μg or 10 μg doses of injected antigen on the level of antibody formed as we did in primary and secondary PFC responses. This result might be explained by the fact that in these experiments the antigen was administrated emulsified in CFA which minimized the possible tolerogenic effect of higher antigenic doses.

Homopolymer poly(HPMA) with no side oligopeptide sequences is not recognized by an organism as foreign and does not induce production of detectable antibodies. Immunization with a copolymer linked with the side chain P-Acap-Leu-HMDA, but without a bound haptenic group, leads to a weak immune response that is manifested by a low antibody level in peripheral blood. However, if the end of the side chain is modified with a substance that behaves as a hapten the immune reaction against it could be very intense, depending on the character of the bound compound. This should be kept in mind if copolymers of similar composition are used in the future as carriers of drugs. After binding to copolymers the drugs could behave as hapten and thus induce, according to their chemical composition, undesirable antibody reactions of different intensities. The reactions could be weakened or eliminated not only by the choice of copolymers of suitable molar mass but also by the selection of suitable side oligopeptide sequences.

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