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BIOAFFINITY THERAPY WITH ANTIBODIES AND DRUGS BOUND TO SOLUBLE SYNTHETIC POLYMERS

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SUMMARY

Soluble synthetic copolymers based on N-(2-hydroxypropyl)methacrylamide (HPMA) containing different oligopeptide side-sequences were tested as transport molecules for drugs and anti-Thy 1.2 antibodies in affinity therapy. As target cells, T lymphocytes were studied. (1) HPMA copolymers containing targeting anti-Thy 1.2 antibodies are 70 times more cytotoxic against T lymphocytes than HPMA copolymers with non-specific immunoglobulin. (2) Daunomycin conjugated to a biodegradable side-sequence (Gly-Phe-Leu-Gly) is effective in a concentration 100 times lower than daunomycin conjugated to a non-cleavable sequence (Gly-Gly). (3) HPMA copolymers containing drug and targeting antibodies are effective both in vitro and in vivo.

INTRODUCTION

There are many serious diseases caused by extensive proliferation or hyperreactivity of some cell subpopulations. Among them certainly the most common are different types of cancer, but autoimmune diseases that irreversibly damage self-tissue are no less dangerous. These pathological situations are most often treated with cytostatic drugs. Unfortunately, most of them are highly

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toxic and substantially damage a number of other tissues. For this reason, at the beginning of this century Ehrlich suggested attaching drugs to carriers capable of targeting them specifically to the affected tissues.

Effectiveness of such affinity therapy is predominantly a question of specificity and effectiveness of the targeting moiety of the drug complex. Many different targeting structures such as lectins [1], liposomes [2—4], hormones [5], polypeptides [6], dextrans [7] and carbohydrates [8—9] are now studied as molecules with affinities for specific cells or organs. The most effective targeting structures with the ability to specify precisely the slightest cell differences are antibodies.

There are many attempts to bind drugs directly to antibody molecules [10—18]; however, such direct binding has a number of disadvantages. In addition to the fact that antibody and/or drug often loose their original activity, the greatest problem is that from such a complex the drug is not released in a controlled way. For a successful application it is absolutely necessary to be sure that the drug is firmly attached to the carrier, is inactive during transport in body fluids and is released in an active form by the action of intracellular enzymes only at the place of the pathological process. If the drug in itself acts upon the cell surface, i.e. is surface-active, the problem of biodegradability is lessened. However, the requirement of inactivity during transport applies to such types of drugs too.

Synthetic polymers based on N-(2-hydropropyl)methacrylamide (HPMA) are studied as possible transport molecules for drugs and targeting moieties in affinity therapy [19—30]. They contain oligopeptidic side-sequences that are terminated by reactive *p*-nitrophenylester groups, which allow binding by aminolysis to biologically active compounds containing amine groups. It is possible to synthesize such oligopeptide side-chains, which guarantee the stability of the bond between drug and polymer in the blood stream but are susceptible to biodegradation by intracellular lysosomal enzymes [31] and thus allow the release of active drug only intracellularly.

The aim of this study was: (a) to compare the effectiveness of drug—polymer complexes with either specific antibody or non-specific γ-globulin; (b) to compare the activity of drug bound to copolymers with degradable or non-degradable oligopeptide side-chains; (c) to test whether such complexes are not only active in vitro but also find the target in the intact organism; (d) to test the validity of the assumption that, for an efficient affinity therapy, substantially lower quantities of cytostatic drugs are needed than in conventional therapy.

T Lymphocytes, important for all types of immune reaction, were used as target cells. Antibodies against their surface Thy 1.2 alloantigen (anti-Thy 1.2; ATS) served as the targeting structure and were bound by aminolysis either to HPMA copolymers with daunomycin (DNM) or to pharmacologically active HPMA copolymer with quaternary ammonium groups. The same coupling procedure was used for daunomycin.

Two types of HPMA copolymers, differing in oligopeptide side-chains, were compared. The sequences Gly-Gly and Gly-Phe-Leu-Gly are stable in plasma during transport [31] but only the tetrapeptide is susceptible to cleavage by lysosomal enzymes [35]. As drugs, daunomycin or pharmacologically active copolymer of HPMA with methacryloyloxyethyltrimethylammonium chloride were used.

The toxicity of the conjugates to T lymphocytes was tested either in vitro by a cytotoxic test or in vivo where the suppression of the immune response was determined by enumeration of antibody-releasing (plaque-forming) cells.

EXPERIMENTAL

Cytotoxic agents

Daunomycin (Rhône-Poulenc, France, Batch 87). The HPMA copolymer containing quaternary ammonium groups (6), i.e., copolymer of HPMA, N-methacryloylglycylglycine *p*-nitrophenyl ester and methacryloyloxyethyltrimethylammonium chloride, was prepared as described previously [32,39].

The copolymer contained 10.6 mol.% of reactive *p*-nitrophenoxy groups and 23.0 mol.% of charged groups. Binding of antibodies was performed by aminolysis. Before determining the biological properties of the carrier itself, the reactive *p*-nitrophenoxy groups were deactivated by aminolysis with 1-aminopropan-2-ol (16), weight-average molecular weight ($\overline{M}_w$) = 13 000.

Preparation of anti-Thy 1.2 antibodies

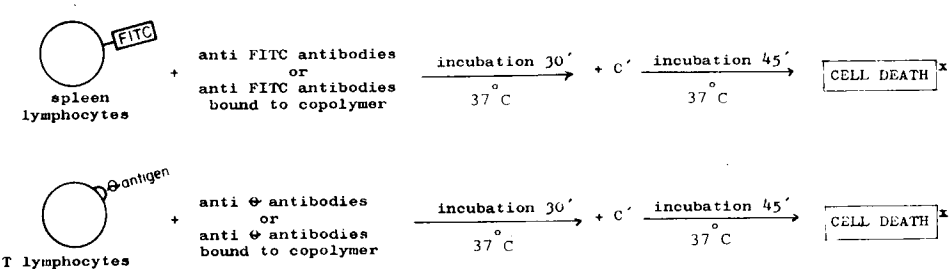
Anti-Thy 1.2 serum was prepared according to the method of Levey and Medawar [33]. The immunoglobulin fraction was prepared by precipitation of sera with ammonium sulphate (40% saturated).

Monoclonal antibodies anti-Thy 1.2 (1aG4/C5) were provided by the Production Unit of the Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague, Czechoslovakia [34].

Rabbit γ-globulin (RGG) was prepared by DEAE cellulose column chromatography.

Preparation of anti-Thy 1.2 antibody—polymer—daunomycin conjugates

These polymers were prepared in three steps as described earlier [30]: (a) preparation of polymer precursors by copolymerization of HPMA with N-methacryloylglycylglycine *p*-nitrophenyl ester (polymer precursor 4) or N-methacryloylglycylphenylalanylleucylglycine *p*-nitrophenyl ester [35] (polymer precursor 5); (b) binding of daunomycin by partial aminolysis to the polymer precursor (polymer precursors 7 and 8); (c) binding of anti-Thy 1.2 antibody to the drug polymer conjugate (conjugates 17 and 18).



x Detected in vitro by trypan blue exclusion test.

Fig. 1. Schematic diagram of cytotoxicity test. Θantigen = Thy 1.2 antigen.

Inbred strains of mice

The plaque assay was performed on eight-week-old females of strain A/J (Institute of Physiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). During experiments, the mice were kept under standard conditions.

Cytotoxicity assay

This was performed with spleen target cells of three-month-old A/J mice. The two-stage dye-exclusion microcytotoxic test was carried out as described in ref. 36. A schematic diagram of the cytotoxic test is presented in Fig. 1. The cytotoxicity index (CI) was calculated as

$$\frac{\text{percentage of dead cells with antiserum} - \text{percentage of dead cells in control}}{100 - \text{percentage of dead cells in control}} \times 100$$

Plaque assay

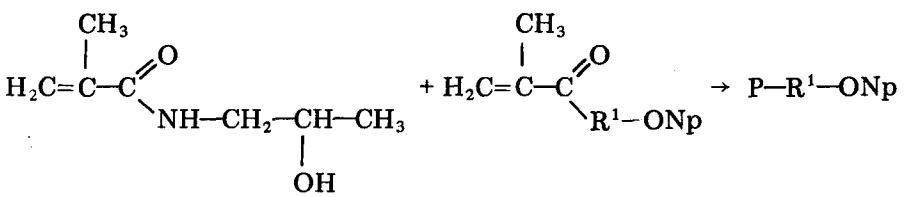
Mice were injected intraperitoneally (i.p.) on day 4, 3, 2 and 1 before antigen stimulation with the different samples tested. On day 0, they were immunized with 0.5 ml of $1 \cdot 10^8$ sheep red blood cells (SRBC) i.p. On day 5 after immunization, their spleens were removed and homogenized in a tissue homogenizer in medium 199 (Sevac, Prague, Czechoslovakia). The cells were washed three times in an ice-cold medium 199 and their viability was estimated by the trypan blue exclusion test. The number of plaque-forming cells (PFC) was estimated by the method of Šterzl and Mandel [37].

RESULTS

Characterization of polymers, polymer–drug and polymer–drug–antibody conjugates

For the preparation of polymer-modified antibodies, two types of polymer precursors were used.

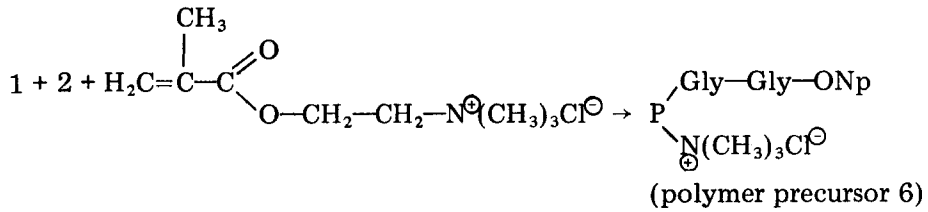
(a) Copolymers of N-(2-hydroxypropyl)methacrylamide (comonomer 1) with *p*-nitrophenylesters of N-methacryloylated oligopeptides (comonomers 2 and 3).



(1) (comonomers 2 and 3) (polymer precursors 4 and 5)

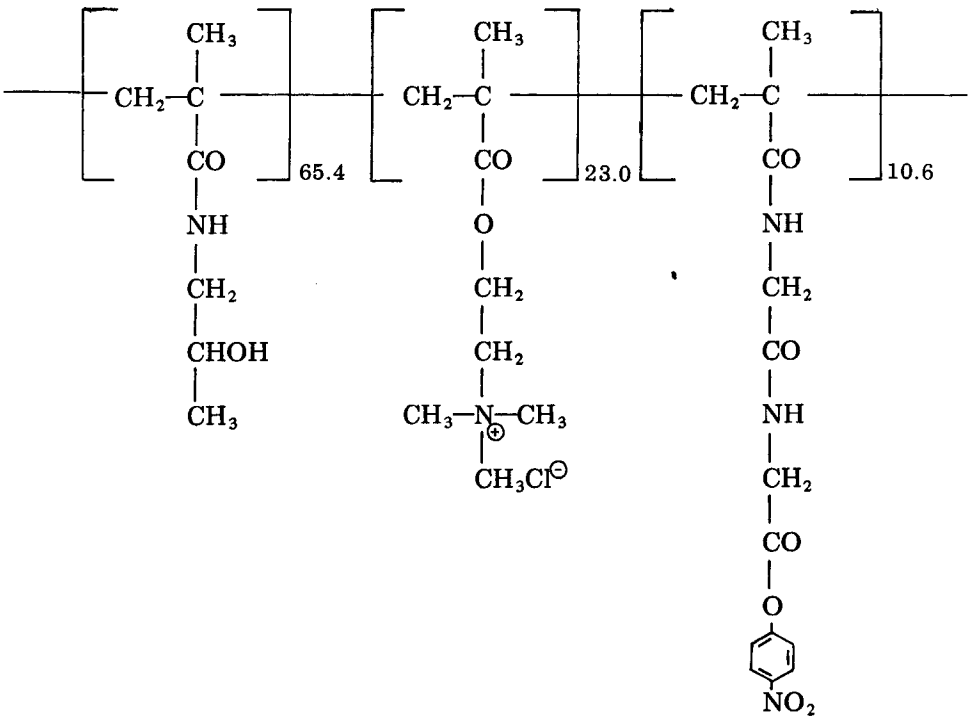
P = Polymer backbone, ONp = *p*-nitrophenoxy. For comonomer 2 and polymer precursor 4, R^1 = Gly-Gly; for comonomer 3 and polymer precursor 5, R^1 = Gly-Phe-Leu-Gly.

(b) Copolymer of HPMA with N-methacryloylglycylglycine *p*-nitrophenyl ester and methacryloyloxyethyltriethylammonium chloride.

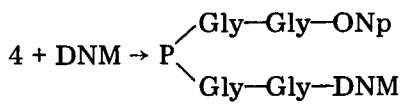


(polymer precursor 6)

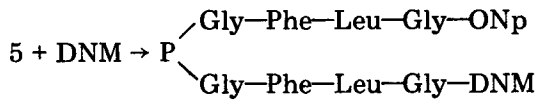
The structure of polymer precursor 6 was as follows:



Daunomycin (DNM) was bound to polymer precursors 4 and 5.

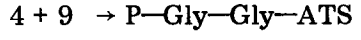


(polymer precursor 7)

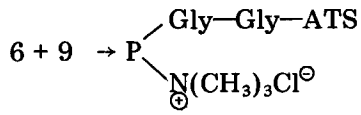


(polymer precursor 8)

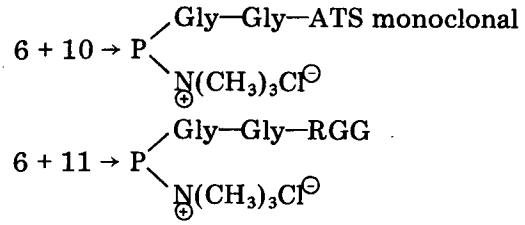
Anti-Thy 1.2 antibodies, ATS (9), monoclonal anti-Thy 1.2 antibodies, ATS monoclonal (10) or non-specific RGG (11) were bound to these polymer precursors (4–8).



(polymer 12)

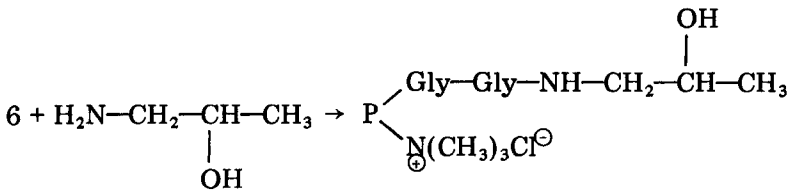


(polymer 13)

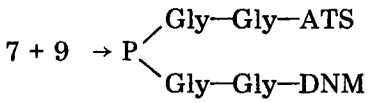


(polymer 14)

(polymer 15)

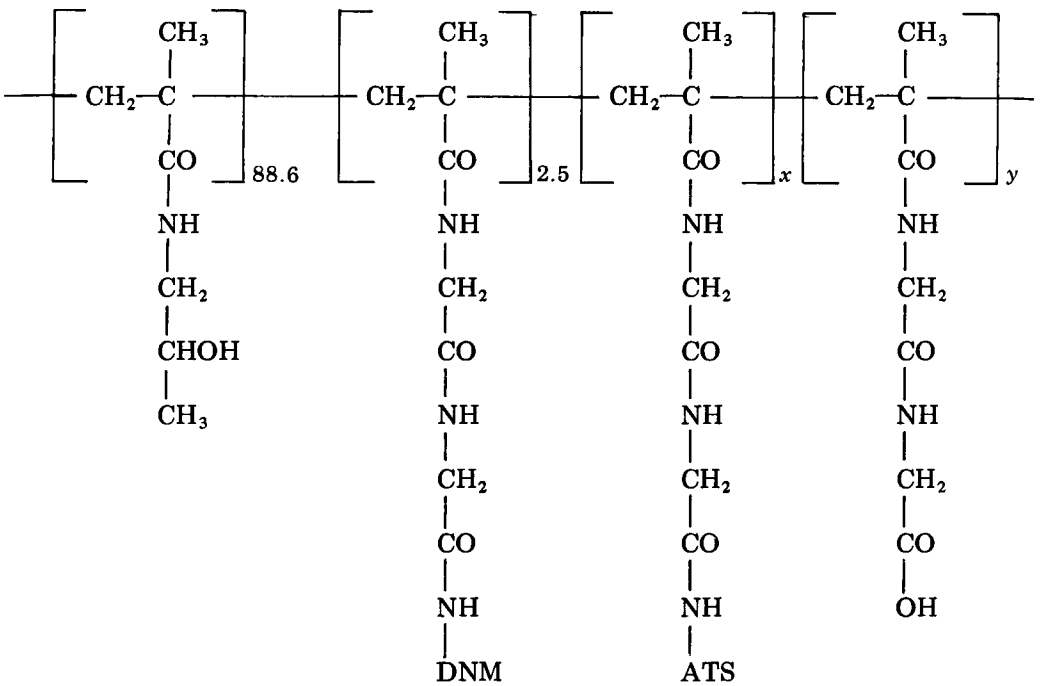


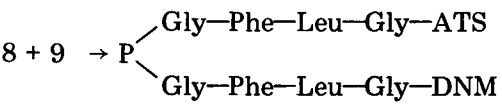
(polymer 16)



(polymer 17)

The structure of polymer 17 with Gly—Gly side-chains was as follows:





(polymer 18)

Comparison of in vitro cytotoxicity of pharmacologically active HPMA copolymers containing either specific anti-Thy 1.2 antibodies or non-specific γ-globulin

Conventional or monoclonal antibodies, i.e. rabbit antibodies against mouse

TABLE I

IN VITRO CYTOTOXICITY OF PHARMACOLOGICALLY ACTIVE HPMA COPOLYMERS CONTAINING EITHER TARGETING ANTIBODIES OR NON-SPECIFIC γ-GLOBULIN

P = Polymer backbone; ATS = anti-Thy 1.2 serum; C′ = complement.

Sample No.	Structure	Concentration (μg/ml)		Cytotoxicity index	
		Copolymer	ATS (RGG)	With C′	Without C′
12	P—Gly—Gly—ATS	5000	2500	61	5
		2500	1250	54	3
		1000	500	46	3
		500	250	39	3
		100	50	21	3
		50	25	13	3
		25	5	3	3
13	P(Gly—Gly—ATS)(N⊕(CH₃)₃Cl⊖)	7500	4500	96	43
		3750	2250	79	33
		1500	900	67	29
		750	450	50	17
		150	90	28	5
		75	45	20	7
		25	15	15	5
14	P(Gly—Gly—ATS (monoclonal))(N⊕(CH₃)₃Cl⊖)	3750	2250	73	32
		1875	1125	65	27
		750	450	57	20
		375	225	46	10
		75	45	34	4
		37	22	22	2
		10	6	17	5
15	P(Gly—Gly—RGG)(N⊕(CH₃)₃Cl⊖)	3600	2250	22	28
		1800	1125	11	14
		720	450	12	15
		360	225	8	5
		72	45	6	8
		36	22	7	7
16	P(Gly—Gly—aminopropanol)(N⊕(CH₃)₃Cl⊖)	5000	—	—	70
		2500	—	—	68
		1000	—	—	60
		500	—	—	45
		250	—	—	40
		200	—	—	32
		150	—	—	20
		100	—	—	0
		50	—	—	0
		25	—	—	0

Thy 1.2 alloantigen or non-specific γ-globulin of the same origin, were attached to the pharmacologically active copolymer of HPMA.

The results are summarized in Table I, where toxicity of the pharmacologically active HPMA copolymer (16), the same copolymer conjugated to rabbit anti-Thy 1.2 immunoglobulin (13) or to monoclonal anti-Thy 1.2 antibody (14) or to non-specific rabbit γ-globulin (15) is compared. As a control, non-toxic HPMA copolymer modified with anti-Thy 1.2 antibody (12) was used.

The cytotoxic test was performed in the presence and in the absence of complement (C′) to discriminate the cytotoxicity due solely to the antibodies from the effect of the pharmacologically active groups or drug.

The cytotoxicity of pharmacologically active copolymer containing the targeting structure is 2.5 times higher in a system without C′ and 70 times higher in a system with C′ than the cytotoxicity of a similar copolymer containing only normal rabbit γ-globulin.

The toxicity of the original copolymer with quaternary ammonium groups decreases after binding to the immunoglobulin molecule, obviously due to a limited interaction with the cell surface.

Effect of degradability or non-degradability of oligopeptidic side-sequences on the cytotoxicity of HPMA copolymers containing daunomycin

In Table II, in vitro activity of daunomycin bound to the copolymer containing sequences either degradable (18) or non-degradable (17) by lysosomal enzymes is compared. The copolymer with cleavable tetrapeptide (Gly-Phe-Leu-Gly) is still cytotoxic at a dose of 3 μg of antibodies and 0.4 μg of daunomycin, while for the same toxicity more than 100 times more DNM on the

TABLE II

IN VITRO CYTOTOXICITY OF HPMA COPOLYMERS CONTAINING DEGRADABLE OR NON-DEGRADABLE SIDE-CHAIN SEQUENCES CONJUGATED TO ANTIBODIES AND DAUNOMYCIN

P = Polymer backbone; ATS = anti-Thy 1.2 serum.

Sample No.	Structure	Concentration (μg/ml)			Cytotoxicity index	
		Polymer	ATS	DNM	With C′	Without C′
17	P—Gly—Gly—DNM / P—Gly—Gly—ATS	6500	2500	500	43	0
		3250	1250	250	40	0
		1300	500	100	7	0
		650	250	50	5	0
		130	50	10	6	0
		65	25	5	2	0
18	P—Gly—Phe—Leu—Gly—DNM / P—Gly—Phe—Leu—Gly—ATS	4800	2500	355	99	99
		2400	1250	177	99	72
		960	500	71	99	43
		480	250	35.5	99	30
		96	50	7.1	70	11
		48	25	3.5	56	0
		24	12	1.8	39	0
		12	6	0.9	30	0
		6	3	0.4	23	0

polymer with non-degradable sequences is needed. No cytotoxicity of the polymer with Gly-Gly sequences in the system without complement can be taken as proof that DNM on non-degradable sequences is inactive and the cytotoxicity of the sample is due to toxic effect of targeting antibodies on T lymphocytes.

In vivo suppression of antibody response against sheep red blood cells by copolymers containing daunomycin and targeting antibody

A schematic diagram of the experiment is presented in Fig. 2. After immunization with SRBC in the spleen of the immunized organism (in our case, A/J mice), lymphatic cells can be detected which release antibodies against this antigen. These cells can be enumerated by a technique which detects the individual anti-SRBC antibody producing PFC. By injection of anti-Thy 1.2 antibodies, either alone or bound to the copolymer with drug attached, those immunocompetent cells that carry the surface Thy 1.2 alloantigen are eliminated. The decrease in the number of Thy 1.2^+ lymphocytes (T lymphocytes) impairs the immune capacity of the organism and reduces the number of PFC. In Tables III and IV, the effect of degradable or non-degradable sequences in the daunomycin-containing polymers with targeting anti-Thy 1.2 antibody on the immune response is compared. In addition, some groups of mice were injected only with anti-Thy 1.2 antiserum or free DNM alone or a mixture of DNM and anti-Thy 1.2 or a polymer to which only anti-Thy 1.2 without DNM was attached. Mice that received a corresponding quantity of normal rabbit serum served as controls and the number of PFC was taken as 100% of the immune response (on average, 50 000 IgM PFC per 10^8 spleen cells).

All samples tested were active and have affected cellular cooperation of the immune system, as reflected in the reduced number of PFC. The DNM bound to the copolymer containing a degradable sequence is active, but compared with free daunomycin for the same suppression a higher quantity of conjugated DNM is needed. Daunomycin bound to the copolymer with a non-degradable sequence appears to be inactive, similar to the situation in vitro,

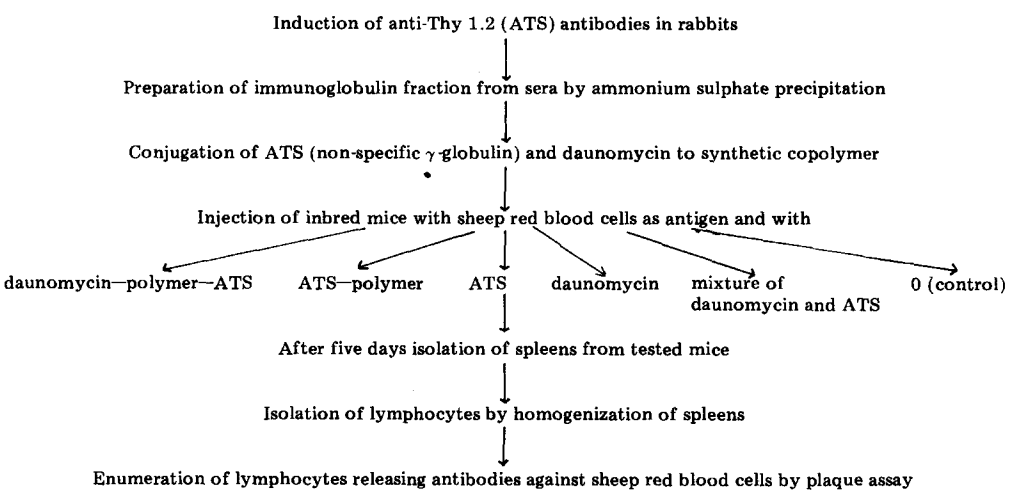


Fig. 2. Schematic diagram of plaque assay.

TABLE III

IN VIVO SUPPRESSION OF ANTI-SRBC ANTIBODY RESPONSE BY DNM AND TARGETING OF Thy 1.2 ANTIBODIES BOUND TO HPMA COPOLYMERS CONTAINING EITHER DEGRADABLE (Gly—Phe—Leu—Gly) OR NON-DEGRADABLE (Gly—Gly) SEQUENCES

P = Polymer backbone; ATS = anti-Thy 1.2 serum.

Sample (structure)	Total dose per mouse (mg)		Suppression (% of control)
	Immunoglobulin	DNM	
P Gly—Gly—DNM Gly—Gly—ATS	12.0 3.0	2.5 0.6	69 0
P Gly—Phe—Leu—Gly—DNM Gly—Phe—Leu—Gly—ATS	3.0	0.4	97.9
ATS	12.0	—	87
ATS	3.0	—	59
P—Gly—Gly—ATS	12.0	—	67
	3.0	—	32

TABLE IV

IN VIVO SUPPRESSION OF ANTI-SRBC ANTIBODY RESPONSE BY FREE DNM OR A MIXTURE OF DNM AND ATS

ATS = anti-Thy 1.2 serum.

Sample (structure)	Total dose per mouse (mg)		Suppression (% of control)
	Immunoglobulin	DNM	
DNM	—	150	99.4
	—	30	44.0
	—	7.5	0
	—	1.5	0
	—	0.5	0
DNM + ATS	12.0	150	99.4
	12.0	30	99.1
	12.0	7.5	95.2
	12.0	1.5	93.5
	12.0	0.5	92.1
DNM + ATS	3.0	150	96.8
	3.0	30	75.0
	3.0	7.5	33.3
	3.0	1.5	24.0
	3.0	0.5	14.0

because suppression of the immune response is the same as after the copolymer carrying anti-Thy 1.2 only. The mixture of anti-Thy 1.2 and DNM exhibited more suppressive activity than the same amount of DNM and ATS bound to the degradable polymer.

DISCUSSION

Soluble synthetic polymers based on N-(2-hydroxypropyl)methacrylamide are studied as transport molecules of drug and targeting moiety [8, 9, 19—30] in affinity therapy. In this study, the anti-Thy 1.2 antibodies specific to the surface Thy 1.2 alloantigen of T lymphocytes were tested as a targeting structure. With the help of such polymer—drug—antibody complexes, tentatively all those pathological situations where high activity of T lymphocytes is unwanted could be treated, e.g. T cell leukaemia, autoimmune disorders and alloimmune reaction in transplanted patients.

One of the problems of affinity therapy lies in the fact that, due to binding, both the antibody and the drug often loose some part of their original activity. Therefore we have tested, except for aminolyses, various procedures and conditions of binding of antibodies to soluble HPMA copolymers, which would guarantee that after binding no free antibodies or drug remain in the system and that their inactivation will be as small as possible [30]. For the binding of antibodies to HPMA copolymers we have tried the following reagents: N-cyclohexyl-N′-(morpholinoethyl)carbodiimide methyl 4-toluenesulphonate, Woodwards reagent K and 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester. However, in all these cases the reaction mixtures contained both free and polymer-bound antibodies even when polymers with high concentrations of carboxyl or amine groups were used. For this reason, we have used samples prepared by aminolysis [32], which gave the best results [30].

For successful application of such polymer—drug—antibody complexes, the most important factor is the specificity and ability of the targeting structure to transport and concentrate a toxic substance at the target place. We have tested these capacities on copolymers to which either specific targeting antibodies or non-specific γ-globulin of the same origin were attached. Instead of a drug, pharmacologically active copolymer containing a quaternary ammonium group was used. The interaction between such a copolymer and membranes both of rat liver and rat visceral yolk sac was proved with limited internalization into the cell [38, 39] and a high in vitro toxicity against spleen T lymphocytes was demonstrated (Table I). Although the toxicity of the original polymer has been decreased by more than half owing to binding to the protein molecule, the targeting anti-Thy 1.2 antibodies have raised the toxicity of the complex by a factor of 70 compared to the complex with non-specific γ-globulin.

If the compound bound to the polymer in itself acts upon the cell surface, i.e. is surface active, or if the copolymer itself exerts such an effect, the problem of degradability of side-chains is lessened. On the other hand, it assumes greater importance if the drug to be bound acts only intracellularly. Such a drug must penetrate into the cells by endocytosis and must be released from the polymer by lysosomal enzymes in order to become active.

The toxicity of polymers containing daunomycin bound at the ends of oligopeptide sequences which are either degradable (polymer 18) or non-degradable (polymer 17) by lysosomal enzymes were compared in vitro and in vivo.

It was shown in vitro that 12 μg of anti-Thy 1.2 antibodies and 1.8 μg of

DNM on polymer with degradable sequences (Gly-Phe-Leu-Gly) has a comparable cytotoxic effect to 1250 μg of anti-Thy 1.2 and 250 μg of DNM on the polymer with non-degradable sequences (Gly-Gly). As was shown in the system without addition of complement, DNM on the latter type of polymer is evidently inactive and cytotoxic action of the whole complex is merely due to the bound anti-Thy 1.2 antibody.

Similar results to those in vitro were also obtained in vivo, where the effect of daunomycin bound to the polymer with degradable or non-degradable sequences on the immune response against SRBC was tested. Daunomycin and anti-Thy 1.2 antibodies bound to a polymer with degradable sequences are more active than anti-Thy 1.2 alone. Daunomycin bound to non-degradable sequence is inactive both in vitro and in vivo.

The results show the importance of the drug being bound to a sequence from which it could be released in a controlled way at the site of the assumed effect. A random and insufficiently defined bond (in our case, non-degradable sequence) between the drug and the carrier may easily lead to the inactivity of the whole complex. Furthermore, it has been demonstrated that targeting antibodies are highly effective if compared with non-specific γ-globulin. Another important finding resulting from the in vivo experiments is that, under conditions of intact organism, the antibodies and the drug bound to soluble HPMA copolymers with a degradable sequence may reach the target tissue and act upon it.

A great advantage of affinity therapy in comparison with conventional therapy is the fact that it limits the toxic action of cytostatic drugs to the site of the pathological process only. Experiments on limited toxicity are in progress.

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