can be absorbed percutaneously although to a considerable smaller extent than by ingestion. The individual differences from rat to rat are considerable. The following conclusions can be drawn from the experiment: a) the 2 N-oxides of the isomers 7-acetyl-lycopsamine and 7-acetyl-intermedine are metabolized to a great extent to the free alkaloids and to the deacetylated forms in orally-treated animals, b) no reduction of N-oxides took place by dermal application of the compounds within the limits of detection, c) the percutaneous absorption of N-oxides is smaller by a factor of 20–50 compared to the gastrointestinal absorption when the excretion of N-oxides and metabolites in the urine is taken as a measure.

In our samples the *Symphytum* alkaloids were present almost completely as N-oxides. The dermally absorbed PA-N-oxides are not or only to a small extent converted to the free alkaloids in the organism. This conversion seems to be an essential step for the toxic action of PA-N-oxides[17,18]. Our data are in agreement with the findings of Powis et al.[19] that the gut flora plays a major role in the metabolic reduction of PA-N-oxides. This difference in the metabolism together with the small degree of dermal absorption makes it likely that the occasional external use of *Symphytum* preparations should not be hazardous.

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Conjugates of adenine 9-α-D-arabinofuranoside monophosphate (ara-AMP) with lactosaminated homologous albumin are not immunogenic in the mouse[1]

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Summary. Conjugates of adenine-9-β-D-arabinofuranoside (ara-A) or of ara-A monophosphate (ara-AMP) with asialofetuin or with heterologous lactosaminated serum albumin (L-SA) are strong antibody inducers. But ara-AMP conjugates prepared with homologous L-SA are not immunogenic, at least in mice.

In the therapy of some diseases (e.g. tumors, infections caused by intracellular microorganisms) drug side effects might be circumvented by coupling the drug to a protein carrier which is selectively taken up by the cells where the pharmacological action is required. If the bond between the drug and the vector is broken down in lysosomes, the drug should be released free and concentrated in the cells into which it was transported[2–5]. With the aim of reducing side effects occurring in the treatment of chronic hepatitis B with adenine-9-β-D-arabinofuranoside (ara-A)[6–8], this drug and its monophosphate derivative (ara-AMP) were coupled to asialofetuin (AF)[9] and to lactosaminated serum albumin (L-SA)[10]. These galactosyl-terminating glycoproteins are internalized only in hepatocytes where they are delivered to lysosomes[11–14].

After injection of AF or L-SA conjugates in mice with hepatitis caused by *Ectromelia* virus, ara-A and ara-AMP are selectively concentrated, in a pharmacologically active form, into hepatocytes[9,10]. L-SA-ara-AMP conjugates do not display acute toxic effects at least in mice (unpublished experiments). A conjugate L_{31}(human)SA-ara-AMP_{10}, administered at a dose of 10 μg per 1 g b.wt, produced a 50% inhibition of virus DNA synthesis in liver of *Ectromelia* virus infected mice; the same conjugate, injected at a dose 11 times higher (the maximum tested), did not cause any sign of toxicity in mice. However, these conjugates might be immunogenic and consequently produce allergic lesions. By using homologous (i.e. of same species) L-SA this risk can be reduced[9,10] but not excluded a priori since antibodies are produced against some hapten-homologous albumin conjugates[15].

In the present experiments we studied whether ara-AMP and ara-A conjugates prepared with AF or with homologous or heterologous L-SA cause a humoral response and/or a delayed-type hypersensitivity in mice.

Materials and methods. Fetuin (Sigma type III) was desialylated by neuraminidase[16]. Lactose was coupled to ε-NH_2 of lysine residues of human (HSA) (crystalline), rabbit (RSA) (crystalline) and mouse serum albumin (MSA) (fraction V) by reductive amination with cyanoborhydride[17,18]. All SA were from the Sigma Chemical Company. In different preparations of L-SA, increasing amounts of lactose were coupled as a function of time of reaction[13–18]. Ara-AMP (Warner-Lambert) was conjugated to AF and to L-SA by the use of 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide (Fluka)[9,10]. In this coupling, conjugation probably takes place mostly be the formation of an amide bond between the ε-NH_2 group of lysine in the protein and the phosphate

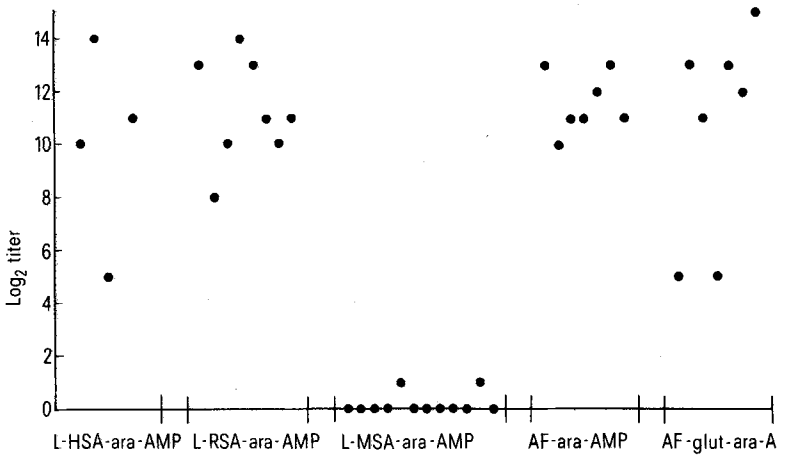

Figure 1. Hemagglutination titers of sera from mice i.v. infused with the monomeric forms of conjugates (AF-ara-AMP_3, AF-glut-ara-A_8, L_{27}-RSA-ara-AMP_9, L_{40}-HSA-ara-AMP_{11}, L_{27}-MSA-ara-AMP_7 (7 animals) and L_{34}-MSA-ara-AMP_8 (5 animals)). Each serum was titrated by using sheep erythrocytes coated with the same conjugate used for immunization. Each point represents the serum titer of an individual mouse.

group of ara-AMP, as shown in an analogous reaction using carbodiimides for coupling thymidilic acid to albumin[19]. The monomeric form of each conjugate preparation was separated from polymers by gel chromatography on Sephadex G-100[9,10]. The presence of polymers was due to the formation of covalent bonds between the protein molecules during carbodiimide coupling[20]. In AF-ara-AMP, the monomer and polymer fractions correspond to 40 and 60% respectively of the whole conjugate preparation. In L-SA-ara-AMP conjugates the monomer percentage increased to about 70%. The molar ratios ara-AMP/protein and lactose/protein were determined as described[9,10]. Unconjugated ara-AMP was recovered as will be described elsewhere. Ara-A was conjugated to AF by the procedure previously described[9]. The drug was first converted to ara-A glutarate which was subsequently linked to AF via its hydroxysuccinimidester (AF-glut-ara-A).

58 female Swiss mice (30–35 g) and 4 male Wistar rats (300–320 g) were used for experiments on antibody production. In mice, 2 procedures of immunization were followed. First, the monomeric forms of the conjugates (AF-ara-AMP_3, AF-glut-ara-A_8, L_{27}-RSA-ara-AMP_9, L_{40}-HSA-ara-AMP_{11}, L_{27}-MSA-ara-AMP_7 and L_{34}-MSA-ara-AMP_8) were i.v. infused in order to mimic the way of administration which should be followed in the clinical use of the conjugates[21]. Every day for 4 consecutive days mice received, in 2 h, 1.4–1.5 ml of NaCl 0.9% containing 420–450 µg of conjugate infused into a tail vein by a peristaltic pump, Gilson Miniplus II. This course was repeated after a 4-week rest. 1 week after the 2nd course, mice were bled from the retroorbital plexus under ether anesthesia. During infusion each mouse was housed in a small, perforated, closed, plexiglass tube which allowed very limited movement. Only the tail remained outside the tube and it was immobilized by elastoplast strips. Up to 8 mice were simultaneously infused.

In the 2nd procedure mice received, s.c., 0.2 ml of water in oil emulsion (Freund's complete adjuvant) containing 100 µg of the polymeric form of conjugate (L_{31}-HSA-ara-AMP_{10}, L_{27}-RSA-ara-AMP_8, L_{27}-MSA-ara-AMP_6) injected in 2 different sites (0.1 ml in each site). After 1 month animals received a s.c. booster dose of 200 µg of the conjugate in 0.2 ml of 0.9% NaCl. A week later the animals were bled. In rats a total volume of 0.5 ml of complete Freund's adjuvant emulsion containing 500 µg of L_{27}-MSA-ara-AMP_6 (polymeric form) was injected s.c. at 2 different sites. After 1 month animals received a s.c. booster dose of 1 mg of conjugate in 1 ml of 0.9% NaCl; a week later they were bled.

Antibodies against the conjugate were measured by the passive haemagglutination test according to Ling et al.[22],

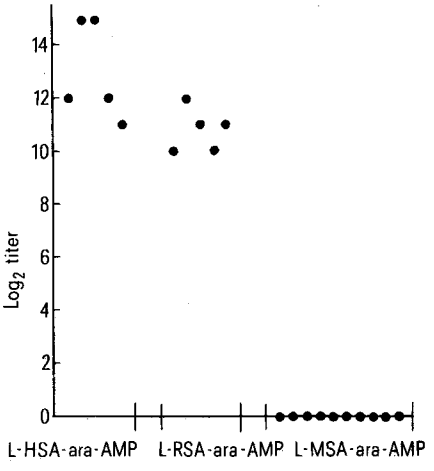

Figure 2. Hemagglutination titers of sera from mice which received the polymeric forms of conjugates (L_{31}-HSA-ara-AMP_{10}, L_{27}-RSA-ara-AMP_8, L_{27}-MSA-ara-AMP_6) s.c. in Freund's complete adjuvant. Each serum was titrated by using sheep erythrocytes coated with the same conjugate used for immunization. Each point represents the serum titer of an individual mouse.

with the following slight modifications. Defibrinated sheep blood was mixed with Alsewer's solution prepared as in Campbell et al.[23], and the antigen to be coated was added to the erythrocyte pellet in the amount of 30 µg in 150 µl NaCl 0.15 M. After coating, blood cells were suspended in 20 mM Na-phosphate buffer, pH 7.4 in 0.14 M NaCl. In order to detect non-agglutinating ('incomplete') antibodies, the antigen binding capacities of sera from mice which received L-MSA-ara-AMP and L-HSA-ara-AMP (administered by the 2 different procedures) were also measured by radioimmunoassay (according to the ammonium sulphate method of Minden and Farr[24]). Radioactive antigens were prepared by coupling ara-AMP to L-MSA and to L-HSA which had been previously labeled with [^{3}H] iodoacetic acid (203 mCi/mmole; NEN) according to Wilson[25]. The conjugates L_{24}-[^{3}H]MSA-ara-AMP_9 and L_{22}-[^{3}H]HSA-ara-AMP_{10} (monomeric forms) had spec. act. of 3.5×10^4 DPM/µg and 3.7×10^4 DPM/µg respectively. They were soluble in 50% ammonium sulphate. Sera were tested in amounts up to 100 µl. Where smaller amounts of tested sera were used, serum volume was brought to 100 µl with nonimmune mouse serum, as carrier. The limit of sensitivity of the assay was about 0.5 µg IgG per ml of serum. Antibodies binding [2,8-^{3}H]ara-A (22 Ci/mmole; Amersham), D-[1-^{3}H]galactose (9.3 Ci/mmole; Amersham) or [D-glucose-1-^{14}C]lactose (59 mCi/mmole; Amersham)

were measured by radioimmunoassay according to Minden and Farr[24].

2 procedures of immunization were also followed in the experiments on delayed hypersensitivity. In the first, according to the schedule of Crowle and Hu[26], female Swiss mice (30–35 g) received 2 s.c. injections, given 1 week apart, of 0.1 ml of water in oil emulsion (Freund's complete adjuvant) containing 0.25 mg of the polymeric forms of conjugate (L_{27}-RSA-ara-AMP_8, L_{27}-MSA-ara-AMP_6). 10 animals were used for each conjugate. 6 week after the last injection, 200 µg of conjugate in 20 µl physiologic phosphate buffer (pH 7.4) was injected into the left hind foot pad[27]; the same amount of solvent without antigen was injected in the pad of the opposite foot. The footpad reaction was read subjectively[28] within the following 48 h. In the 2nd procedure, 8 mice received the monomeric form of L_{34}-MSA-ara-AMP_8 administered in 2 infusion cycles, using the same doses and modalities as in experiments on antibody production. 10 days after the last cycle, hypersensitivity was tested by the footpad reaction.

Results and discussion. As shown in figures 1 and 2, high titers of antibody binding conjugates were found, by the passive hemagglutination test, in sera from mice treated with AF-ara-AMP, AF-glut-ara-A, L-HSA-ara-AMP or L-RSA-ara-AMP. In carbodiimide coupling some free carboxylic groups of protein are probably substituted by the N-acylisourea residue formed from the carbodiimide[29] which could increase the immunogenicity of ara-AMP conjugates. However AF-glut-ara-A, prepared by a coupling procedure which does not produce side reactions[9], was found to induce antibody production as strongly as AF-ara-AMP. Not withstanding the different modes of administration of L-SA conjugates (i.v. infusion of monomer or s.c. injection of polymers in Freund's adjuvant) no substantial differences in antibody titers were observed. In none of the mice which received L-MSA-ara-AMP (either administered in polymeric form in Freund's adjuvant or i.v. infused in monomeric form) were antibodies against the conjugate detected by the passive haemagglutination test (figures 1 and 2). Antibodies against L-MSA-ara-AMP were revealed in the sera of all rats injected with this conjugate (Log_2 titers: 14, 14, 13, 14). This indicates that coating of L-MSA-ara-AMP to erythrocytes had been successful. All the sera from mice, immunized with L-HSA-ara-AMP conjugates, bound the labeled antigen in radioimmunoassay. The antigen binding capacities of these sera ranged from 5 to 590 µg of conjugate per ml of serum (mean value 168 µg) and correlated very well with the hemagglutination titers. None of the sera from mice treated with L-MSA-ara-AMP conjugates bound the antigen in radioimmunoassay.

No conjugate, whether infused or s.c. injected, produced detectable antibodies to ara-A, D-galactose or lactose in (figures 1 and 2). Antibodies against L-MSA-ara-AMP were revealed in the sera of all rats injected with this showed any reaction in the injected foot. Of the 10 mice tested for cell-mediated hypersensitivity to L-RSA-ara-AMP only 3 showed a reaction in the tested foot; the reaction was strong but peaked at 3–6 h and then rapidly subsided, thus having the characteristics of an Arthus reaction more than of a delayed-type hypersensitivity response[27]. This was confirmed by histological examination which showed predominance of polymorphs.

In L-SA-ara-AMP conjugates a large number of molecules of lactose, ara-AMP and probably of the urea derivative of carbodiimide are linked to albumin. In spite of these substitutions, which probably modify the conformational structure of the native protein, mice recognize the conjugates prepared with homologous L-SA as 'self' molecules and do not give an immune response against them. This immunological tolerance can be explained by the finding that, unlike B cells, T lymphocytes involved in delayed hypersensitivity[30] and in helper activity in antibody production[31–33] do not discriminate in mice between the native and denatured forms of some proteins.

In conclusion the present experiments show that conjugates of ara-AMP or ara-A prepared with AF or with heterologous L-SA are strong antibody inducers whereas ara-AMP conjugates prepared with homologous L-SA are not immunogenic, at least in mice.

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