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Experimental squalene adjuvant II. Harmlessness and local reactogenity

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Abstract

Model experiments on laboratory animals (guinea pigs) were carried out to test the possible allergic reaction (possibility of sensitisation) to the repeated administration of an experimental lipoid adjuvant prepared on the basis of squalene (experimental squalene adjuvant—ESA). No significant differences were observed between the animals sensitised-provoked with ESA and control animals. In order to evaluate the local tissue reactivity (local reactogenity), also with regard to the process dynamics to the administration of ESA, comparative patho-anatomical and patho-histological examinations of tissues were carried out in the location of adjuvant administration. The examinations indicated very low local reactogenity of the experimental lipoid adjuvant prepared in our laboratory. The test of pyrogenicity also confirmed the safety of ESA, the labelled lysate sensitivity λ was under 0.25 IU/cm^3.

Keywords: Squalene adjuvant; Sensitisation; Local reactogenity; Pyrogenicity

1. Introduction

Adjuvants have been used extensively for almost 80 years to potentiate the immune response in both experimental immunology and preparation of effective vaccines. Simple but elegant experiments carried out by Ramon [1] in 1925 showed that the antitoxin response to tetanus diphtheria toxoid were potentiated when the vaccines were applied in combination with, for example, inorganic salts, oil, tapioca, pyogenic bacteria and similar. These experiments proved that some vaccine components other than the antigen itself are important for adequate immune response and initiated research concerning potentiation of effectiveness of vaccines that has continued up to this day.

The research in the field of adjuvants became an important factor in the development of new and more effective vaccines. While the number of substances with adjuvant effect increases tremendously the mechanism of their action remains frequently unknown. The approach to the use of suitable adjuvants that will potentiate the effectiveness of vaccines should be rational and targeted and because of that the knowledge of the mode of action and overview of adjuvant types may limit the preparation of effective vaccines. Information about mechanisms of effect help in construction of combined adjuvants in which the effect of individual components may sum up and increase further the overall supporting effect.

A number of combined adjuvants have been prepared up to this day. The aim of combining various types of adjuvants was to obtain a product with different properties that would induce required immunological responses. The best known combination is Freund's complete adjuvant (FCA). Improved or modified FCA, corresponding to the current requirements, prepared by Ciba–Geigy (combination of muramyldipeptid (MDP) and metabolizable oil—squalene). Another well known combination is Titer Max, developed also as an alternative to FCA [2]. It is a mixture of block co-polymers and oil squalene. A number of combined adjuvants is based on emulsion of the oil-in-water type. The best known of them are SYNTEX based on oil squalene, MDP and block co-polymer Pluronic-L121 [3] and RIBI adjuvant (squalene, MPL, TDM, CWS) [4]. Adjuvants used in human or veterinary medicine is necessary individually tested for their effectiveness and harmlessness [5]. The development

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of new vaccine adjuvants (combined adjuvants) has been hampered by their unacceptable reactogenicity [6].

We prepared in our laboratory an emulsion of oil-in-water type based on esters of fatty acids, namely izopropylester of palmitic acid with the use of block co-polymer Poloxamer 105 (pluronic polyol) as an emulsifier with marked adjuvant activity [7,8]. Pluronic polyols are a family of non-ionic surfactants currently used as drug carriers for antibiotic, anti-inflammatory and anti-neoplastic agents [9]. A disadvantage of this adjuvant is that it can be used only as a diluent or solvent suitable for resuscitation of lyophilised vaccines. Poloxamer 105 itself ensures adequate emulsification only at higher temperatures of homogenisation (50 °C) which disturb integrity of the immunising antigen.

On the basis of need to develop an adjuvant suitable for preparation of liquid vaccines, we used squalene as an oil component in combination with an appropriate mixture of emulsifiers which ensures adequate emulsification of oil and the water component already at laboratory temperature. Tests were carried out to determine its effectiveness and to validate its areactogenity.

2. Materials and methods

2.1. Study of possible sensitisation to the administration of the experimental squalene adjuvant (ESA)

An adjuvant of the oil-in-water type was prepared. The oil component was squalene (Merck, Germany); Poloxamer 105 (ICI, England) and Abil-Care (Merck, Germany) were used as emulsifiers. Detailed description of the preparation was presented in the first part of this paper [10].

2.1.1. Experimental animals

Fifteen laboratory guinea pigs of average weight 250 g were used.

2.1.2. Experimental scheme

The model experiments tested the possible sensitisation of guinea pigs after repeated administration of the experimental lipoid adjuvant on the basis of a skin-fold thickness (ST) comparing the animals sensitised intradermally on Day 14 or 28 after administration and the non-sensitised animals that received the same dose of the adjuvant (intradermally) at the time of provocation of the sensitised animals. The scheme of the experiment is presented in Table 1.

The possible sensitisation of animals was also evaluated on the basis of animal behaviour—changes in behaviour and feed intake. Animals were examined clinically every day for the period of 1 month following the provocation. The animals were subjected to additional examination for local allergic reactions carried out by measurement of the skin-fold thickness at the site of the intradermal administration after 1, 24, 48 and 72 h and 7 and 14 days. To eliminate the individual differences between the single animals the changes in the skin-fold thickness were determined as multiples (reaction factor *f*) of its value before the i.d. administration of ESA.

$$f = \frac{\text{ST at the respective time interval after the administration}}{\text{ST before the administration}}$$

Statistical evaluation of results of skin reactivity was carried out by means of the Student's *t*-test.

2.2. Patho-morphological determination of tissue reactivity (local reactogenity) to the administration of ESA

Another model experiment on guinea pigs was carried out to test the local reactivity (local reactogenity) to administration of the experimental adjuvant in a dynamic process. The evaluation was based on patho-histological examination of the site of intramuscular administration of the adjuvant (thigh inguinal muscles) on Day 1, 14 and 28 after the administration.

2.2.1. Experimental animals

Fifteen conventional guinea pigs of body weight 250 g were used, five animals in each group. Experimental squalene adjuvant was administered intramuscularly to the left thigh using a medial approach. The volume of inoculum was 0.5 cm^3 for all animals.

2.2.2. Examination of guinea pigs

24 h and subsequently 14 and 28 days after the administration of adjuvants, three animals from each group were killed with aether and the site of administration was subjected to

Table 1
The scheme of the experiment intended for validation of reactogenity of the experimental adjuvant

Group[a]	Sensitisation	Provocation		Note
		Day 14th	Day 28th	
1 a	i.m.—0.5 cm^3	i.d.—0.1 cm^3	–	Measurement of the skin-fold thickness
1 b	i.m.—0.5 cm^3	–	i.d.—0.1 cm^3	
C	–	i.d.—0.1 cm^3	–	Clinical observation
		–	i.d.—0.1 cm^3	

i.m.: intramuscular administration; i.d.: intradermal administration; C: control group.
[a] Number of animals in group: 5.

patho-anatomical examination. A sample of muscular tissue of dimensions 0.5 cm × 0.5 cm × 0.5 cm was taken from the site of administration and examined histologically.

The patho-anatomical examination consisted in macroscopical examination of local changes in tissues and changes in corresponding lymph nodes. The samples of tissues (excisions) from the sites of administration of adjuvant and of corresponding inguinal lymph nodes, obtained for patho-histological examination, were fixed in cold formalin, embedded in paraffin and the sections were stained with haematoxylin-eosin or van Gieson method, respectively. Samples of tissues and lymph nodes taken from the right thigh of the respective animals served as a control.

2.3. Test of pyrogenicity of ESA

The adjuvant substances without antigen were tested on pyrogenicity. Test of pyrogenicity was carried out in accordance with the rules of European Pharmacopoeia [11] in vitro, by gel–clot method: limit test and semi-quantitative test. Following this prescription, the labelled lysate sensitivity (λ) for injection substances should be lower than 0.25 IU/cm^3.

Research was conducted according to the principles presented in the "Guide for Care and Use of Laboratory Animals", published by the State Veterinary Office of the Slovak Republic, Bratislava.

3. Results

3.1. Experiment A

Results of skin reactivity are presented in Table 2. Differences in the skin-fold thickness between the animals sensitised and control animals were insignificant in all time intervals after provocation.

3.2. Experiment B

3.2.1. Patho-anatomical examination

Macroscopical examination of the site of adjuvant administration and inguinal lymph nodes showed no changes in comparison with extremities or inguinal nodes in the control.

3.2.2. Patho-histological examination

24 h post-administration: more marked oedemisation of intermuscular connective tissue was observed. Vessels were moderately dilated. Metabolised portions of the adjuvant were detected. Due to the oedema slight dehiscence of muscle fascicles was observed. Nuclei of muscular cells were undisturbed and striation of muscular cells was preserved. Observed were foci of intensive heterocellular infiltration with predomination of neutrophilic granulocytes.

14 days post-administration: less pronounced oedemisation of intermuscular connective tissue was observed. Vessels were moderately dilated. Moderate dehiscence of muscle fascicles due to the presence of oedema persisted. In contrast with the previous finding (after 24 h) foci of slight infiltration with predominance of neutrophilic granulocytes were observed. Granulomatous character of reactions prevailed in the examined tissue The process was limited to the intermuscular connective tissue and only rarely penetrated into the muscles in the form of narrow bands.

28 days post-administration: the histological picture showed no histopathological changes in comparison with the controls.

3.3. Experiment C

The result of gel–clot: limit test and semi-quantitative test showed, that the value λ of experimental squalene adjuvant (i.e. squalene + poloxamer 105 + abilc-are + water) was under 0.25 IU/cm^3.

4. Discussion

The first part of this study focused on testing of the effectiveness of supporting activity of the experimental adjuvant in association with the use of inactivated rabies vaccine and porcine parvovirus one [10]. The second part concentrated

Table 2
Changes in the skin-fold thickness at the site of intradermal application of ESA; expressed as multiples of the thickness before the application (*f*)

Time of evaluation after provocation	Reaction factor, *f*		
	Guinea pig groups[a]		
	1 a	1 b	C
1 h	1.89 ± 0.10	1.89 ± 0.08	1.89 ± 0.07
24 h	1.80 ± 0.09	1.82 ± 0.09	1.73 ± 0.10
48 h	1.78 ± 0.13	1.69 ± 0.06	1.66 ± 0.07
72 h	1.61 ± 0.07	1.45 ± 0.15	1.64 ± 0.14
Day 7th	1.60 ± 0.07	1.45 ± 0.14	1.56 ± 0.12
Day 14th	1.38 ± 0.11	1.36 ± 0.10	1.46 ± 0.11

[a] Number of animals in group: 5.
C = control group.

on testing of the harmlessness—possible development of allergic reaction to the repeated administration of the adjuvant and observation of local tissue reactivity (local reactivity). The adjuvant was tested on guinea pigs, the extremely sensitive species of laboratory animals. Substances with adjuvant action can be of different origin, chemical composition and mechanism of action, however, due to their possible toxicity and reactogenity only some of them are suitable for practical use. For example, aluminium hydroxide is the substance most frequently used to potentiate effectiveness of veterinary and human vaccines [12]. It was determined that this adjuvant can induce fibrosarcomas in some recipients after subcutaneous administration [13,14]. Development of necrotising granulomas and sterile abscesses [15,16], also severe local reactions such as erythema, subcutaneous nodules and contact hypersensitivity were described after administration of aluminium compounds [17]. Non-aluminium adjuvants could not readily replace aluminium adjuvants. New generation vaccines will probably need new generation adjuvants [12].

Oil adjuvants prepared on the basis of mineral oils can also give rise to post-vaccination local reactions, e.g. granulomas, haemorrhagic lesions, serous infiltrations and long-term persistence of non-metabolisable components of oil emulsion at the site of administration [2,18]. Total reactions to the application of oil adjuvants—development of metastatic granulomas and so-called adjuvant arthritis were recorded [19–21]. Development of FIA-induced paresis in guinea pigs on the basis of development of granulomatous lesions in the spinal canal was described [21]. In the late 1980s of the past century Krejèí et al. [22] described total anaphylactoid postvaccination reaction in cattle in the Czech Republic that developed after application of oil vaccines. According to Toman et al. [23] these total reactions were caused by the use of Tween-80 as an emulsifier and the negative influence of mineral oil was not proved.

In the 1990s, development of multifocal granulomatous myositis was described after the use of combined adjuvant RIBI (MPL, squalene, TDM, CWS, Tween-80), and similar with adjuvant SYNTEX (squalene, MDP, Pluronic) [24]; after the use of adjuvant Titer Max (squalene, Pluronic) necrotising pyogranulomatous myositis was observed [2,24]. The reactogenity of the lipoid adjuvant prepared in our laboratory on the basis of izopropylpalmitate (IPP) as an oil component was tested also pathologically–histologically [7]. An unambiguously granulomatous diffuse character of reactions was observed, limited to the intermuscular connective tissue without long-term persistence of residua of the oil component of the adjuvant in the tissue.

Because of excellent adjuvant properties of the naturally occurring hydrocarbon precursor of cholesterol—squalene—and its non-toxicity and total metabolisability [25] we decided to prepare in our laboratory a squalene-based, combined adjuvant of the oil-in-water type. Despite the above mentioned observations of some authors who described some adverse reactions to the use of combined squalene-containing adjuvants, the reactions cannot be ascribed to the action of squalene alone. The results of this study, focused on evaluation of the experimental squalene-based adjuvant with regard to its harmlessness, failed to prove the possible development of total reaction to the adjuvant administration. Also the results of pathological–histological examination of the site of administration of this adjuvant indicated its low local reactogenity. It should be noted that some tissue reactivity is inevitable for good adjuvant action (e.g. accumulation of immunocompetent cells).

With the tested experimental adjuvant, the granulomatous character of reactions was limited to the intermuscular connective tissue and no histopathological changes or presence of adjuvant residua were observed as early as 1 month after administration of the adjuvant which indicated rapid consolidation of changes.

With regard to the results obtained by testing the supportive effect of experimental squalene adjuvant on rabies vaccine and porcine parvovirus one effectiveness [10] as well as results presented in this study, we can unambiguously recommend the use of this metabolisable adjuvant for the purpose of potentiating the effect of inactivated veterinary vaccines without the risk of development of postvaccination complications in animals.

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