ASSESSMENT OF VARIOUS ADJUVANTS IN SPHAEROPHORUS NECROPHORUS TOXOIDS

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Introduction

For many years adjuvants have been employed to enhance immune response to parenteral administration of bacterial or viral antigens (2, 15). On the other hand, various reports have indicated that improper use of immunological adjuvants may also result in adverse systemic and long term effects (3, 10, 15). Although present knowledge on adjuvant use is extensive, most data have been derived from pre-clinical tests on laboratory animals (3, 5) or from extended mass immunization of humans (15). Reliable published information pertaining to the use of adjuvant preparations in large domestic animals remains very limited.

For the past two years, this laboratory has investigated the selection of a suitable toxoid-adjuvant preparation to increase the efficiency of immunizing cattle against infections by *Sphaerophorus necrophorus*, the primary causative organism of bovine liver abscesses. This report aims to focus attention on the value of adjuvants and on the need to assess toxoid-adjuvant preparations based not only on their immunological significance but also on their adverse effects. These factors are particularly evident from results of trial experiments presented here.

Value of Adjuvants

Particulate antigen bound by an adjuvant induces immunization whereas free antigen favours induction of tolerance (14). Weakly immunogenic proteins which induce tolerance will frequently, in the presence of adjuvants, induce antibody formation. In effect, the value of adjuvants lies in their ability to produce a "switch mechanism" from tolerance to immunity. Paraf (12) showed that adjuvants, not only act on the antigen, but also on the cells involved in the immune response.

Freund's studies (4) demonstrated that the activity of an adjuvant was associated with, (i) the establishment of part of the antigen in a persistent form at the site of injection, resulting in a gradual and continual release of antigen which stimulates antibody production, (ii) the provision for a means of transporting the emulsified antigen via the lymphatic system to distant sites such as lymph nodes and the spleen where new sites of antibody formation may be established and (iii) formation and accumulation of mono-nuclear cells appropriate to production of antibody at local and distant sites. The mechanism of action for all adjuvants is basically similar, but the degree of reaction is variable. Adjuvants have less effect on the initial peak of antibody production at eight to 12 days post injection than later on when they give a slow and prolonged rise in antibody production.

Types of Adjuvants

Many materials are capable of acting as adjuvants, however there is no truly universal adjuvant suited to all situations. The ideal adjuvant would combine well with the antigen, remain stable on storage, retain the liquid state at lower temperatures, produce minimal local or systemic reactions, give extended elevated serotitres related to immunity and be innocuous with regard to diagnostic tests for other diseases.

Potash alum (aluminium-potassium sulfate) was used by Roux and Yersin at the Pasteur Institute before 1900 in the production of diphtheria toxoid (2). Alum precipitates bacterial toxins and is useful in the preparation of toxoids. Other aluminium salts, particularly the hydroxide and phosphate are used as adjuvants. Aluminium hydroxide binds the antigen firmly and remains quite stable on storage. Aluminium phosphate is a less efficient absorbent but has been used with purified antigen such as viruses and bacterial toxoids (15).

Considerable work has been done with oily emulsions, either mineral or vegetable (8, 15), of which Freund's adjuvant (4) is the most commonly used. The incomplete adjuvant is a water in oil emulsion of aqueous antigen in paraffin oil; mannide mono-oleate, an emulsifier renders stability to the suspension. Freund's complete adjuvant consists of 5–25 mg of dried heat-killed *Mycobacterium tuberculosis* or *M. butyricum* added to each 10 ml of the above emulsion. The use of the complete adjuvant

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is contraindicated in animals subjected to periodic tuberculin tests. Some workers (10, 11) have considered the local reaction and tissue damage severe following the use of the complete adjuvant while other workers who observed tissue changes histologically (3) attributed the intense reactive changes to the oily adjuvant rather than to the mycobacterium. At the site of injection a localized swelling developed initially, followed by necrosis, inflammation and formation of a granuloma. Scar tissue development was common and abscessation occurred occasionally.

Other substances have been used experimentally in laboratory animals as adjuvants. Amies (1) used sodium alginate which combines with soluble calcium salts in the body to form insoluble calcium alginate. Calcium chloride has also been injected at the same site to ensure the availability of calcium. Local tissue reaction was mild following injection of sodium alginate and necrosis and abscessation were not reported. Gall (5) conducted extensive studies on 142 aliphatic amines and related compounds in combination with diphtheria toxoid in guinea pigs. He found many substances to be active as adjuvants, but none specifically applicable for routine use. Hilleman (8) listed other substances as having been used as adjuvants, such as, calcium phosphate, protamine, acrylamide gel, cholesterol, fatty acids, gram negative bacteria and endotoxins. The adjuvant activity of gram negative bacteria appears to be due to the presence of endotoxin (16). The endotoxic lipopolysaccharide induces cell damage causing release of nucleic acid fragments which may stimulate other cells involved in antibody formation. Other materials of less importance which have been reported to have adjuvant activity include blood charcoal, bentonite and serum albumen, and globulin (17).

Experimental Trials

This trial was undertaken to determine the antibody response in cattle to injections of *S. necrophorus* cells and cell fractions alone and combined with different adjuvants.

Thirty-six grade yearling cattle were utilized in the studies. The strain of *S. necrophorus* used was isolated from a bovine liver abscess. The isolation and identification procedures employed are described in a previous study (6). Three antigens were prepared; washed whole cells, sonicated cells which had been disrupted by ultrasound and a cytoplasmic fraction, separated from the cell wall by centrifugation. These preparations were formalinized with 0.5% formaldehyde and held for at least two weeks at room temperature before being combined with the various adjuvants. The concentration of antigen was standardized according to the protein content in the antigen-adjuvant preparation. All preparations contained approximately 2.5 mg protein per ml. Higher concentrations of protein antigen up to 10 mg per ml resulted in minimal titres, possibly indicating a paralysis of the immunological system (8). Freund's incomplete adjuvant, sodium alginate and aluminium hydroxide (10%) were combined in equal parts with the antigen and emulsified by forcing through a syringe with an 18 gauge needle. Alum was prepared according to the method as outlined by Kawamura (19). Serum antibodies against their homologous antigens were detected by the double agar diffusion precipitin test.

Most injections of the antigen-adjuvant preparation were given by a commonly used route, subcutaneously in the neck just anterior to the shoulder. A small number of injections, as listed in Table I and in preliminary trials, were given intramuscularly in the neck also. Other animals were injected intraperitoneally on the left side above the rumen to determine if this could be a useful alternative route of administration.

Results

A summary of our findings is given in Table I. On subcutaneous injection, whole bacterial cells combined with each of the adjuvants produced the most severe reaction of any of the antigens. In general there was an acute local reaction accompanied by an increased temperature for two or three days and frequently an abscess was formed at the site of injection. Sonicated cells caused a less severe reaction than whole cells and cytoplasmic fraction gave no prolonged reaction. Differences in tissue reaction were not only dependant on the cellular antigen used, but also on the type of adjuvant. Freund's incomplete adjuvant caused more reaction than alum or aluminium hydroxide with each of the bacterial preparations and offered no particular advantages over the alum. In general higher titres were obtained without objectionable reactions using alum. Aluminium hydroxide did not give prolonged titres and sodium alginate was not readily available for further trials.

The overall results of the trial indicated subcutaneous injection was the route of choice to immunize animals and sonicated cells or cytoplasmic fractions were the preferred antigens.

TABLE I

SUMMARY OF THE EFFECTS OF ANTIGEN INJECTIONS IN CATTLE

Number of Animals	Injection: Antigens (in 0.5% formaldehyde)	Injection: Number	Injection: Interval (weeks)	Injection: Route*	Antibodies**	Remarks
4	Cytoplasm + alum ppt'd	2	6	SC	+	local reaction short duration
2	Cytoplasm + alum ppt'd	2	4	SC	+	local reaction short duration
2	Whole cells + alum ppt'd	2	6	SC	+	abscess at site
2	Sonicate + alum ppt'd	2	6	SC	+	induration at site
2	Sonicate + alum ppt'd	2	4	SC	+	induration at site
2	Cytoplasm + Aluminium hydroxide	2	4	SC	+	low titres, local reaction short duration
2	Cytoplasm + Freund's	2	4	SC	+	local reaction short duration
3	Cytoplasm + Freund's	2	8	SC	+	local reaction short duration
2	Cytoplasm only	6	2 days	SC	+	prolonged titres, local reaction short duration
1	Cytoplasm + Freund's	2	8	SC IM	+	muscle abscess
2	Whole cells + Freund's	2	4	SC	+	acute local reaction
2	Whole cells + Freund's	1	—	SC	+	acute local reaction
2	Sonicate + Freund's	1	—	SC	+	local induration
4	Cytoplasm + alum ppt'd	2	6	IP	−	bloat and/or colic
2	Cytoplasm + Freund's	2	6	IP	−	bloat and/or colic
2	Cytoplasm + sodium alginate	2	6	IP	−	bloat and/or colic

*SC = subcutaneously − IM = intramuscularly − IP = intraperitoneally

**+ denotes the presence of precipitin lines in the double agar diffusion precipitin test

Discussion

The intramuscular route was contraindicated in beef cattle due to the extensive diffuse swelling in the tissue surrounding the injection site and the undue discomfort for the animal following injection. There was also a high risk for abscess formation with the resultant loss in carcass value. Similarly, intraperitoneal injection of cytoplasmic fraction in combination with sodium alginate, alum or Freund's incomplete adjuvant was objectionable because it induced colic in the injected animal and severe bloat was frequently observed soon after injection. The intraperitoneal route did not result in detectable antibody levels in any animals, probably due to the lack of an adequate antigenic depot (12) as the material would be rapidly absorbed and widely dispersed in the lymphatic system. Alum appeared to be a satisfactory adjuvant, particularly since the cell material was a toxoid which could be absorbed by aluminium salts.

In some instances precipitating antibodies were not produced following the initial injection, however, most animals developed serotitres within a week following the booster. Longer intervals between injections of up to six and eight weeks produced higher serotitres when compared to intervals of two weeks or less. For acceptance in feedlot operations, a schedule of immunization should be devised using an effective product requiring not more than two injections.

Summary

Formalinized whole cells, sonicated cells and cytoplasmic fractions of *S. necrophorus* in combination with various adjuvants were injected into yearling calves by different routes. Alum precipitated sonicate and cytoplasmic antigens administered subcutaneously appeared more acceptable immunizing agents because of their ability to immunize without producing extensive tissue reactions and damage. The formalinized whole cells caused severe inflammatory reactions and were unsuitable for routine use. By contrast, the alum precipitated sonicate and cytoplasmic antigens appeared more acceptable.

Some information on adjuvants which may be useful in a large animal immunization programme is presented and reviewed.

Résumé

On a injecté à des veaux d'un an, et par diverses routes, des cellules entières formolées, des cellules soumises aux vibrations ultra-soniques et des fractions cytoplasmiques de *S. necrophorus* combinées à divers adjuvants. Les cellules soumises aux vibrations ultra-soniques et précipitées à l'alun, ainsi que les antigènes cytoplasmiques, en injection sous-cutanée, s'avérèrent de meilleurs agents immunisants parce qu'ils réussissaient à immuniser sans provoquer de dommages tissulaires diffus. Les cellules entières formolées provoquèrent des réactions inflammatoires graves et s'avérèrent impropres à un usage routinier. Par contre, les cellules soumises aux vibrations ultra-soniques et les antigènes cytoplasmiques ont semblé plus acceptables.

Les auteurs donnent et commentent des renseignements sur les adjuvants qui pourraient s'avérer utiles dans l'élaboration d'un programme d'immunisation, chez les grands animaux.

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BOOK REVIEW

Diseases of Birds. Treatment, Management and Care of Cage and Wild Birds. (Volgelkrankheiten. Behandlung, Haltung and Pflege von Zierund Wildvögeln.) Uta Ebert. Published by Schaper Verlag, Hannover. 1972. 324 pages. Price DM 89, – (German).

The increasing popularity of pet birds in private homes demands more knowledge of their management and diseases. The author has had many years of experience at the "VOGEL-KLINIK" of the Veterinary College, Hannover, Germany. In addition to writing from her own experience she has included research results and observations reported in the international literature. The book is composed of five chapters.

The introductory chapter provides useful information with regard to scientific terminology for the various avian orders, families, subfamilies or genera. The text covers most of Germany's avifauna, as well as, commonly imported birds.

The second chapter deals with the healthy bird and contains well organized and comprehensive sections on general care, feeding and management. This is followed by details of specialized handling of psittacines, hardbills, softbills, birds of prey, owls and other wild and zoo birds. Several pages refer to the raising of wild nestlings of the two types: *nidicolous* (all passerine birds, psittacines, birds of prey) and *nidifugous* birds, subdivided into self-feeding and beak-fed birds (game chicks, waterfowl, gulls). The sketches of birds used in this chapter are very attractive, although they are of doubtful scientific value.

Chapter three, the major portion of the book, is devoted to diseases. Practical methods are suggested for obtaining a proper anamnesis, for clinical examination including capture and restraint, and for laboratory procedures. Diseases are dealt with in four main sections, i.e. psittacines – canaries and other foreign and indigenous hardbills – softbills – birds of prey, owls and other wild and zoo birds. In each of these sections the diseases are described according to organ involvement. Special emphasis is placed on the psittacines and excellent tables for quick reference are included which summarize clinical and therapeutic information. More photographs illustrating clinical abnormalities and pathological lesions would improve this chapter.

Surgical aspects and anesthesia of cage and wild birds are thoroughly discussed in chapter four.

The final chapter provides suggestions for establishing a Pet Bird Practice, as well as an alphabetical compilation of drugs recommended in avian practice. References at the end of the book are made to the international literature. A comprehensive index terminates the text.

The author tends to use the first person singular pronoun throughout the text. However, this does not detract from the value of the information presented. Printing, layout, paper and binding are good. This book is an appreciable contribution to the avian sector of veterinary medicine and can, as a practical guide, be well recommended to veterinarians and others concerned with cage or wild birds.

Regrettably, a translation of the German text is not available. *G. Speckmann.*