Safety Evaluation of Immunological Adjuvants for Biologics

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Adjuvants have been applied to potentiate the immune responses to many antigens in practical vaccination as well as in experimental immunology for more than seven decades. The development of new subunit vaccine candidates has been greatly accelerated by advances in the fields of protein purification, peptide synthesis and recombinant DNA technology. These antigens are usually not or are weakly immunogenic and cannot, therefore, be used effectively in the absence of vaccine adjuvants that can induce sufficient levels of protective immunity under acceptable safety condition to be considered for clinical use. The major advantage of using adjuvants with routine human vaccines was the development of earlier, higher and longer-lasting immunity especially after primary immunization compared to soluble vaccines.

There is a great need to find adjuvants which effectively potentiates the immune response and exhibits safety in terms of reactivity and side effects. Only vaccine adjuvants for use in humans currently licensed by the FDA are aluminum compounds but they are not active with all immunogens and stimulate only humoral responses containing IgE antibody production. It has even been suggested that the periodic use of vaccines adsorbed onto aluminum compounds could be related to an increased incidence of allergic diseases. Moreover, it is also known that aluminum-adjuvanted vaccines produce a high incidence of local side reactions such as redness, pain, swelling, persistent granulomas, and sterile abscess. In addition, many reports pointed out the neurotoxicity of aluminum compounds that may play a role in pathogenesis of Alzheimer's dementia and the dialysis-associated encephalopathy syndrome. An alternative to aluminum adjuvants may be calcium phosphate adjuvant that has been used for the diphtheria-pertussis-tetanus group of vaccines. It is a constituent of the human body and offers a greater guarantee for the absence of adverse reactions and is safer than aluminum hydroxide. Both aluminum and calcium adjuvants are inexpensive and simple to formulate.

In the present study, we evaluated *in vitro* and in *vivo* toxicities of aluminum hydroxide and calcium phosphate adjuvants having extremely different physical properties. We compared also adjuvanticities of the adjuvants aiding tetanus toxoid (TT) in IgG antibody production in guinea pigs.

The following four materials were examined : 1) aluminum hydroxide gel

(Al-gel, 1 mg Al/ml) ; 2) aluminum hydroxide suspension (Al-sus, 1 mg Al/ml) ; 3) calcium phosphate gel (Ca-gel, 1 mg Ca/ml) ; and 4) calcium phosphate suspension (Ca-sus, 1 mg Ca/ml).

Lactate dehydrogenase (LDH) release assay using guinea-pig peritoneal macrophages (Mø) and peritoneal polymorphonuclear leukocytes (PMN) were used to measure the cytotoxicity of four materials. The method, which detected the LDH activity of injured and surviving cells simultaneously, was widely recognized to be sensitive. Incubation of Mø or PMN with Al-sus released the largest amount of LDH. LDH release of Mø with Al-gel was 50% of that of the positive control (Triton X-100) and that of PMN with Al-gel was similar to that of the negative control. Neither of the calcium compounds released LDH on incubation with Mø or PMN. Hemolytic effects were assayed with human, mouse rabbit and sheep 2% red blood cells (RBCs) suspensions. The hemolysis ratio was expressed as the hemoglobin released as a percentage of that of the positive control. Both Al-gel and Al-sus were mildly hemolytic, while Ca-gel elicited a significantly higher hemolytic effect than any other material. Ca-sus was also hemolytic. The hemolytic activity of each gel depended on the gel dose. We suggested that the physical properties of those compounds may be one of the factors affecting the reactogenicity, especially hindering PMN and Mø in phagocytosis.

Furthermore, to investigate a cell damage of Mø treated with vaccine adjuvants, morphological changes of Mø were observed by scanning electron microscopy. Solubilizing membrane proteins of some Mø incubated for 30 min were removed with PHEM buffer containing 0.5% CHAPS (a non-denaturing zwitterionic detergent) to observe for phagocytosis and cytoskeleton networks. Neither Ca-gel nor Ca-sus caused cell damage when the compounds were treated with 10% FCS-containing culture medium before incubation. To the contrary, both Al-gel and Al-sus were very toxic to Mø even when the compounds were treated with FCS-containing medium. Especially, Al-sus revealed a high cytotoxicity. The toxic effect of these aluminum compounds, however, disappeared when the Mø were treated with cytochalasin B, suggesting that the toxicity of aluminum compounds depends on phagocytosis.

Vascular permeability at the site of intracutaneous injection was assayed by the dye method in guinea pigs. The method used to estimate the vascular permeability was used to assess the degree of edema arising in the skin. More Evans' blue was extracted from the skin site injected with Ca-sus than with any other material. The amount with Al-gel was much larger than that with Al-sus, whereas Ca-gel elicited little or no vascular permeability-increasing effect.

Al-gel elicited granulomatous inflammatory reactions consisting mainly of Mø with foamy cytoplasm, small lymphocytes and giant cells at the injection sites for

up to 8 weeks or longer. Ca-gel also induced active inflammatory reactions consisting of neutrophils and foamy Mø associated with many multinuclear giant cells for at least 4 weeks. Severity of local tissue irritation due to Ca-gel was essentially similar to that due to Al-gel except for the duration of the inflammatory reactions. Ca-sus induced local reactions completely ceased by the 4th week, while Al-sus-induced reactions were seen up to the 8th week.

The adjuvant activity of Al-gel for TT was significantly stronger than that of any other adjuvant material, whereas those of Ca-gel and Ca-sus were not seen at a dose of 3 mg calcium phosphate per milliliter. Al-sus-TT at a dose of 3 mg aluminum hydroxide per milliliter induced very low levels of antibody. These results suggest that calcium phosphate adjuvant may not be an alternative to aluminum hydroxide adjuvant. Accordingly, as a substitute for aluminum compounds, we considered liposome-like vesicles composed of ornithine-containing lipids (OrnL). OrnL extracted from *Flavobacterium meningosepticum* have been reported to have various biological activities such as B-cell mitogenicity and Mø activation to generate interleukin-1 and prostaglandin E_2. Completely antigen-entrapped OrnL vesicles including phosphatidylglycerol and cholesterol induced a significantly greater enhancement of IgG antibody production than did Al-gel using OVA or TT as an antigen in BALB/c mice. Secretory or mucosal immune responses are known to play important roles in the establishment of protective immunity to microbial infections through mucosa. Most pathogens attack their animal hosts at mucosal surfaces where parenteral IgG serves as an inefficient barrier. Strategies that actively promote induction of mucosal IgA antibody and mucosal cellular immunity are needed to deal with the plethora of respiratory, gastrointestinal and urogenital pathogens that confront the animal kingdom.

Studies on the potent adjuvants for mucosal immunization are in progress in our laboratory.

Additional lecture

Approval of Permission of Production for Biological Products in Japan

To develop a new biological product, approval of permission of production by the National Council of Pharmacy must be obtained according to the Drugs,

Cosmetics and Medical Instruments Act. The materials requested are the methods of production used for development of the product (fractionation, extraction, synthesis, biotechnology, etc.), data on properties obtained by basic tests for physicochemical properties of the product, results of the tests for quality standards given in the Minimum requirements for Biological Products, moreover, data on preclinical and clinical tests to obtain information on safety and efficacy.