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Importance of the Degree of Antigen Polymerization by Detoxification in Modulating the Immunogenicity of Acellular Pertussis Vaccine

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Abstract For the acellular pertussis vaccine with a high immunogenicity, the concentration, composition and characteristics of acellular pertussis antigens are the crucial points to be considered. Nevertheless, it has not been proved yet whether or not the polymerization degree, one of the characteristics of formalin-detoxified acellular pertussis antigens, has an influence on vaccine potency. Thus, in the present study, the correlations among detoxification conditions of acellular pertussis bulks, their polymerization degrees and their immunogenicities were examined. In addition, the relative importance of pertussis toxoid in vaccine immunogenicity was also investigated. Results show that a lower lysine concentration during detoxification induces highly-polymerized antigens, the immunogenicity has a great dependency on the polymerization degree of antigens, and also pertussis toxoid has a relatively stronger influence on the immunogenicity than other antigens. Accordingly, in the aspect of the potency of detoxified acellular pertussis vaccine, it can be demonstrated that the polymerization of antigens and its degree are the major factors affecting the immunogenicity along with a relatively high content of pertussis toxoid

Keywords: acellular pertussis vaccine, detoxification, immunogenicity, pertussis toxin, polymerization degree

INTRODUCTION

Pertussis is an acute infectious disease of the respiratory system caused by *Bordetella pertussis* infection and has been the cause for higher death rates in children worldwide. In 1994, there have been an estimated 40 million infection cases, from which 360,000 deaths were reported [1]. Whole cell pertussis vaccine against pertussis infection was first licensed in the United States in 1914 [2] and till date it has been widely used to immunize the infants. However, its characteristic local reactivity and fever promoted the development of acellular pertussis vaccine with fewer side effects. Acellular pertussis vaccine was first licensed in Japan in 1981 and many studies for enhancing its immunogenicity have been conducted [3]. Since then, a number of researchers have focused their research on the effect of antigens, such as pertussis toxin, FHA, pertactin, *etc.*, constituting acellular pertussis vaccine and have reported the importance of the pertussis toxin and pertactin [2,4-6], while some researchers, in the case of antigens such as a tetanus toxin and a hemorrhagic principle of Habu snake venom, have reported the importance of the polymerization degree of the toxoid prepared by a detoxification reaction, in triggering immunogenicity [7-9]. In the present study, we have mainly examined whether or not the polymerization degree of the acellular pertussis toxoid, prepared by detoxifying the bulk (antigens) purified from supernatants of *Bordetella pertussis* cultures, has any influence on vaccine immunogenicity in mouse. In addition, the dosage effect of highly-polymerized acellular pertussis bulk as well as the role of PT component was scrutinized with respect to the potency of acellular pertussis vaccine.

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MATERIALS AND METHODS

Preparation of an Acellular Pertussis Bulk, a Detoxified Bulk and a Final Vaccine

Pertussis antigens, including PT (pertussis toxin), FHA (filamentous hemagglutinin), pertactin, and so on, were produced in a culture supernatant of *Bordetella pertussis*, from which an acellular pertussis bulk was prepared by the combination of the processes such as $(NH_4)_2SO_4$ precipitation, diafiltration, and microfiltration. Subsequently, an acellular pertussis bulk was detoxified by formaldehyde and lysine. The buffer used for detoxification was 100 mM phosphate buffer with 1 M NaCl, pH 8.0. Initially formalin was added to become 0.2% and its concen-

tration was increased daily by 0.2% increment until the final concentration reached to 0.6% (72 mM formaldehyde), while the desired concentration of lysine was added at a single time. Detoxification was conducted at 37°C. The detoxified bulks prepared by this procedure were used for the manufacture of final vaccines, in which the aluminum hydroxide gel was fixed at a concentration of 290 μg Al/mL throughout the study.

Polymerization Degree of Acellular Pertussis Bulks

The polymerization degree of acellular pertussis bulks was measured by gel permeation chromatography (GPC) embedded in AKTA explorer (Amersham Pharmacia Biotech, USA) [10,11]. The column and media for separating proteins were XK series (Amersham Pharmacia Biotech, USA) with a dimension of 16 × 70 cm and Superdex G200 prep (Amersham Pharmacia Biotech, USA), respectively. For media equilibrium and protein elution, 100 mM phosphate buffer of pH 7.1 with 0.2 M NaCl was used. Proteins were eluted at a rate of 0.2 mL/min from GPC. In addition, the polymerization degree was re-confirmed by Western blot analysis, in which a mouse anti-FHA IgG, a mouse anti-PT IgG and a mouse anti-pertactin IgG were used as the primary antibodies for detecting FHA, PT and pertactin, respectively. Here, a rabbit anti-horse IgG conjugated to alkaline phophatase (AP) (Sigma, USA) was used as the secondary antibody in all the cases.

Pertussis Immunogenicity by Mouse Protection Test

The acellular pertussis potency was measured using SPF-grade ICR mice. The test vaccine and reference vaccine (11.9 IU/mL, GreenCross Vaccine Corp., Korea) were diluted 8, 40, and 200 fold, respectively. Each dilution group was composed of 20 mice. After 3 weeks of intraperitoneally immunizing the mice with 0.5 mL of the diluted vaccine, their brains were challenged with 0.025 mL of live *B. pertussis* 18323 (about 200 LD_{50}). After 14 days of the challenge, the acellular pertussis potency was statistically calculated based on the number of surviving mice (The minimum requirements for biological products of Korea, K-FDA).

RESULTS AND DISCUSSION

Effect of the Detoxification by Formaldehyde and Lysine on the Characteristics of Acellular Pertussis Bulk

There have been numerous reports on the detoxification of virulent proteins by formaldehyde itself or both formaldehyde and amino acids [9,12-15]. The present study shows the effect of the detoxification by both formaldehyde and lysine on the characteristics of acellular pertussis bulk, characterized by SDS-PAGE, Western blot and gel permeation chromatogram, and on the composition of major acellular pertussis antigens including FHA, PT and pertactin. In the present case, 72 mM of formaldehyde and 40 mM of lysine were used to detoxify the acellular pertussis bulks and the other detoxification conditions are described in Materials and Methods. As a result, some characteristic changes on detoxified acellular pertussis bulk have been observed and are reported as follows. Initially, Western blots revealed that FHA proteins, the major factor in the attachment of *B. pertussis* onto the epithelial cell, were polymerized at a relatively high proportion during detoxification, while, in the case of PT and pertactin, only a small amount was modified into higher molecules (Fig. 1). Subsequently, changes in the relative composition of antigens, especially the increase of pertactin ratio, was observed by SRID (single radial immunodiffusion) assay, following detoxification (Table 1). Finally, the overall shift of peaks by detoxification was definitely confirmed by the gel permeation chromatograms, in which the major peak of acellular pertussis bulk, presumed to be mostly polymerized FHA proteins, moved significantly forward after detoxification (Fig. 2). Basically, detoxifying toxic proteins with both formaldehyde and lysine is thought to induce their aggregation and precipitation through a polymerization process forming an excessive network of intermolecular cross-linking between homologous or heterologous species. The intermolecular cross linking resulting in polymerization appears to be induced by the protein-derived Schiff base produced by the reaction of acellular pertussis antigens and formaldehyde. Besides, it is thought that acellular pertussis antigens can

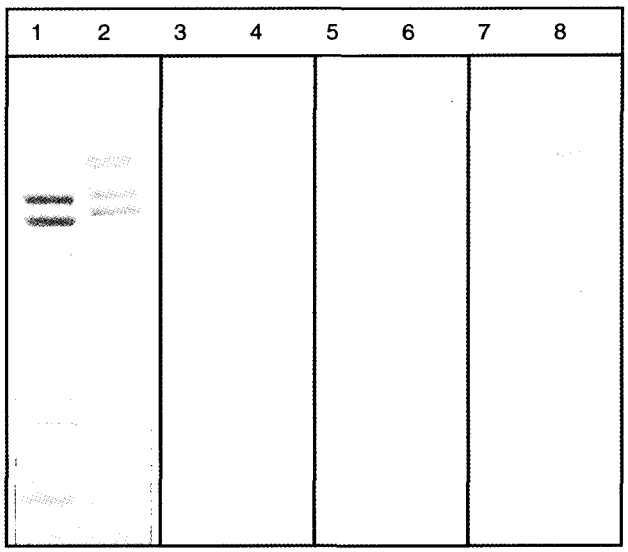

Fig. 1. Comparison of the migration pattern of antigens before and after detoxification. Lane 1: SDS-PAGE of acellular pertussis bulk; Lane 2: SDS-PAGE of detoxified acellular pertussis bulk; Lane 3: Western blot analysis of pertussis toxin in acellular pertussis bulk; Lane 4: Western blot analysis of pertussis toxoid in detoxified acellular pertussis bulk; Lane 5: Western blot analysis of pertactin in acellular pertussis bulk; Lane 6: Western blot analysis of pertactin in detoxified acellular pertussis bulk; Lane 7: Western blot analysis of FHA in acellular pertussis bulk; Lane 8: Western blot analysis of FHA in detoxified acellular pertussis bulk.

Table 1. Comparison of the antigen composition before and after detoxification by both 72 mM of formaldehyde and 40 mM of lysine

Components	Antigen composition in acellular pertussis bulk (%)	
	Before detoxification	After detoxification
PT	6.6	6.3
FHA	89.0	87.4
PRN	4.4	6.3

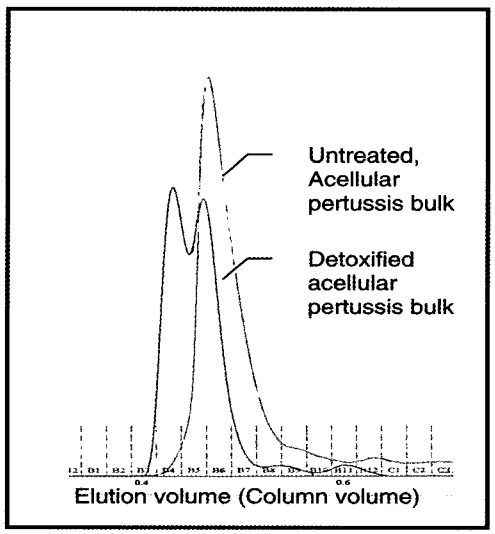

Fig. 2. Comparison of a gel permeation chromatogram of acellular pertussis bulk with that of its detoxified form. Red line: Acellular pertussis bulk, Untreated; Blue line: Detoxified acellular pertussis bulk.

also react with the lysine-derived Schiff base formed by the preceding reaction of lysine and formaldehyde, by which they end up as non-polymerized proteins. Thus, it is presumed that the two kinds of reactions mentioned above can occur simultaneously during the detoxification by formaldehyde and lysine, and the preference for each reaction depends significantly on the ratio of reagents as well as the characteristics of proteins, participating in the detoxification reaction. Therefore, detoxifying the acellular pertussis bulk is thought to not only vary its antigen composition, by which pertactin content can increase relatively, but also results in the preferential shift of FHA in Western blot by its preferential polymerization with acellular pertussis antigens and also the overall shift of peaks on gel permeation chromatograms. Accordingly, it can be concluded that the detoxification of acellular pertussis bulk by formaldehyde and lysine eventually leads to biophysical and biochemical changes of the constituting components, including the polymerization, aggregation and precipitation of antigens, and the following composition change. In addition, the physico-chemical modifications of the antigens can potentially affect their immunogenicity, which is

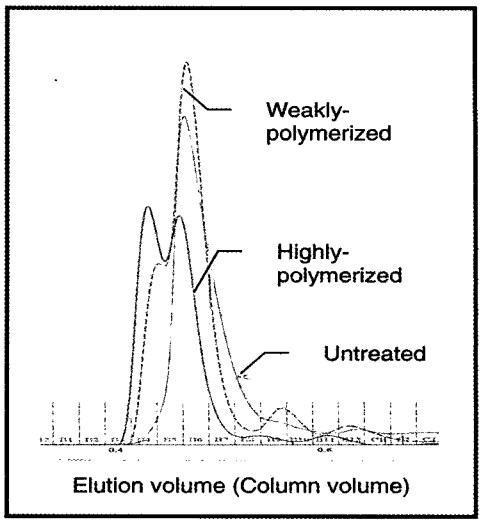

Fig. 3. Changes in gel permeation chromatograms of detoxified acellular pertussis bulks according to detoxification conditions. Red line: Acellular pertussis bulk, Untreated. Blue dot line: Acellular pertussis bulk detoxified by 72 mM formaldehyde and 90 mM lysine, Weakly-polymerized; Blue line : Acellular pertussis bulk detoxified by 72 mM formaldehyde and 40 mM lysine, Highly-polymerized.

important for vaccine performance [7-9,15,16]. Hence, in the present study, the detailed effect of antigen components, contents and its polymerization degree on vaccine immunogenicity was investigated as follows.

Effect of the Polymerization Degree on Vaccine Immunogenicity

The following experiments were conducted to investigate the effect of polymerization degree and antigen components on the immunogenicity, which was assayed by the mouse protection test. Different concentrations of lysine were used to induce several degrees of polymerization. As a result, the acellular pertussis bulks with various polymerization degrees were prepared, which were confirmed by the gel permeation chromatograms (Fig. 3). As shown in Fig. 3, the considerable amount of acellular pertussis antigens was shifted evidently towards the peak with higher molecular weight, in the presence of low lysine concentration. The results show that lysine concentration has a significant effect on the degree of intermolecular cross-linking of antigens. In addition, the change in antigen composition according to the lysine content was observed by SRID, in which the lower lysine conditions finally induced the higher pertactin contents, while PT and FHA contents were not visibly affected (Table 2). The immunogenicity of the acellular pertussis bulks with these different characteristics was examined (Table 2). The resulting analysis showed a strong dependency of the immunogenicity on detoxification conditions. More specifically, immunogenicity was highest in the acellular pertussis bulk detoxified by 40 mM of lysine, in which the two

Table 2. Effect of the lysine contents with a fixed formaldehyde concentration (72 mM) on the characteristics of antigens and the immunogenicity of acellular pertussis bulk

Division		Lysine content (mM)		
		40	60	90
Polymerization degree		High	Middle	Low
Composition (%)	PT	6.3	6.9	6.9
	FHA	87.4	89.0	89.5
	PRN	6.3	4.1	3.6
Potency (Units/mL)		11.9 ± 3.2	5.2 ± 1.5	2.5 ± 1.2

Table 3. Acellular pertussis vaccines with different antigen compositions

Vaccines	Antigen content (%)		
	FHA	PT	Pertactin
Vaccine with mainly FHA	93.0	6.0	1.0
Vaccine with mainly both FHA and PRN	73.5	4.7	21.8
Vaccine with mainly both FHA and PT	51.2	48.3	0.6
Vaccine with similar contents of FHA, PT and PRN	31.6	47.0	21.3

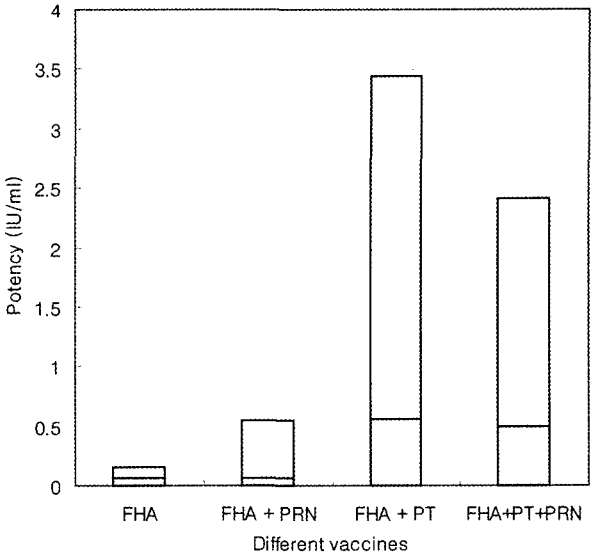

Fig. 4. Effect of vaccine components on the immunogenicity of acellular pertussis vaccine. FHA: Vaccine with mainly FHA; FHA + PRN: Vaccine with mainly both FHA and PRN; FHA + PT: Vaccine with mainly both FHA and PT; FHA + PT + PRN: Vaccine with similar contents of FHA, PT and PRN; Gray-colored column: lowest assay value; White-colored column: highest assay value.

characteristics with high polymerization degree and high pertactin content were observed. There have been some reports, which specify that the toxoids with high polymerization degree are favorable for inducing the antibody of a hemorrhagic principle isolated from the venom of a Habu snake [9], and that PT and pertactin contribute largely to the immunogenicity of acellular pertussis vaccine [2,4-6]. Therefore, a series of studies were conducted to evaluate the factors, which are more important for immunogenicity. To investigate the role of antigen components in detoxified acellular pertussis vaccine, some vaccines with different concentrations of components but with similar total concentrations were prepared as shown in Table 3. As a result, with 10 fold increase in relative PT content (FHA → FHA + PT, FHA + PRN → FHA + PT + PRN), the vaccine immunogenicity showed a surprising rise, but in case of the significant rise of relative pertactin content (FHA → FHA + PRN, FHA + PT → FHA + PT + PRN), it didn't contribute much to the immunogenicity (Fig. 4). Therefore, it is presumed that PT has a major effect on the immunogenicity of acellular pertussis vaccine prepared in this study, while pertactin doesn't have much influence contrary to our expectations. Considering the results so far, the reason for an increase in the vaccine immunogenicity at low lysine contents seemed to be mainly due to the high polymerization degree of antigens. Moreover, this notion can be supported not only by some reports, which talk about that large and insoluble antigens are more immunogenic than small and soluble ones because the former are more readily phagocytosed and processed by macrophage [17], but also by general methods which have long been used for vaccines, such as the induction of antigen aggregation by formalin treatment and the attachment of antigens onto insoluble alum gels [12,13,16,18-22]. Accordingly, it can be concluded that the polymerization degree of antigens is one of the major factors, which bring about a significant rise in the immunogenicity of detoxified acellular pertussis vaccine.

Effect of the Dosage Levels of Highly-polymerized Acellular Pertussis Bulk on Immunogenicity

Six acellular pertussis vaccines with highly-polymerized acellular pertussis bulks, detoxified by both 72 mM of formaldehyde and 40 mM of lysine were prepared and were subsequently investigated for the dose-response characteristics of antigens. In the present case, the amount of acellular pertussis bulk in vaccines ranged from 2 to 19.2 μg PN/mL, while aluminum hydroxide gel was fixed at 290 μg Al/mL in all the cases. As a result, a strong correlation between the dose of acellular pertussis bulk and its immunogenicity is shown at low dose range (Fig. 5). Instead, the immunogenicity between 8.4 and 19.2 μg PN/mL remained stabilized without any significant variation. Similarly, the dependency of the dose of a polymerized antigen on its immunogenicity was reported on the polymerized HR1B toxoid detoxified by both formaldehyde and lysine, in which the toxoid dose within the experimental range had a significant effect on the level of circulating anti-HR1B antibody [9]. Accordingly, it can be concluded that the highly-polymerized acellular pertussis bulk prepared in the study had a typical dose-

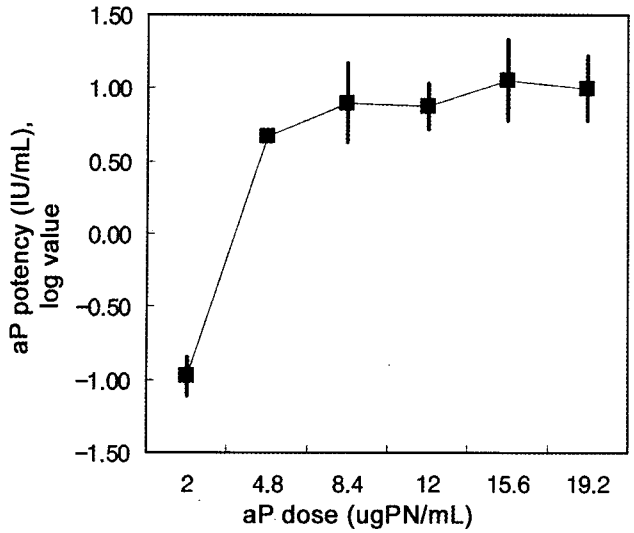

Fig. 5. Correlation between the dose of detoxified acellular pertussis bulk and its immunogenicity. aP dose : Dose of acellular pertussis bulk in vaccines; aP potency: Potency of detoxified acellular pertussis vaccines.

response pattern similar to the polymerized HR1B toxoid and its optimal dose for vaccine was around 8 μg PN/mL.

CONCLUSION

The detoxification of acellular pertussis bulks by formaldehyde and lysine induced some biophysical and biochemical modifications of antigens constituting acellular pertussis vaccines, including their polymerization and also notable rise in pertactin content after the filtration of final detoxified bulks. These modifications also led to changes in immunogenicity of the acellular pertussis vaccines and the detailed investigation revealed that the polymerization of antigens and its degree are the major factors for any significant increase in immunogenicity. In addition, the vaccines from highly-polymerized antigens exhibited a typical dose-response pattern and the dose of detoxified bulk for the optimal immunogenicity was around 8 μg PN/mL.

REFERENCES

[1] Global programme on vaccines (1996) *State of the World's Vaccines and Immunization*. World Health Organization, Geneva.

[2] Edwards, K. M., M. D. Decker, and E. A. Mortimer Jr. (1999) Pertussis vaccine. pp. 293-344. In: S. A. Plotkin and W. A. Orenstein (eds.). *Vaccines*. W. B. Saunders company, Philadelphia, USA.

[3] Yasuo, M. and T. Atsuko (1996) Pertussis vaccine. pp. 39-51. In: O. Akira (ed.). *Vaccine Handbook*. Maruzen Publisher, Tokyo, Japan.

[4] Cherry, J. D., J. Gornbein, U. Heininger, and K. Stehr (1998) A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. *Vaccine* 16: 1901-1906.

[5] Hewlett, E. L. and S. A. Halperin (1998) Serological correlates of immunity to *Bordetella pertussis*. *Vaccine* 16: 1899-1900.

[6] Storsaeter, J., H. O. Hallander, L. Gustafsson, and P. Olin (1998) Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. *Vaccine* 16: 1907-1916.

[7] Huet, M. E., E. Relyveld, and S. Camps (1992) Simplified activity evaluation of several tetanus vaccines. *Biologicals* 20: 35-43.

[8] Relyveld, E. H., B. Bizzini, and R. K. Gupta (1998) Rational approaches to reduce adverse reactions in man to vaccines containing tetanus and diphtheria toxoids. *Vaccine* 16: 1016-1023.

[9] Seiji, S. O., O. S. Tamotsu, K. Satoru, and M. Tyoku (1984) Effect of pH and lysine during detoxification of a hemorrhagic principle of HABU snake venom (*Trimeresurus, Flavoviridis*) with formalin on the immunogenicity of the toxoid. *Japan J. Med. Sci. Biol.* 37: 225-231.

[10] Guan, Y. X., H. X. Pan, Y. G. Gao, S. J. Yao, and M. G. Cho (2005) Refolding and purification of recombinant human interferon-γ expressed as inclusion bodies in *Escherichia coli* using size exclusion chromatography. *Biotechnol. Bioprocess Eng.* 10: 122-127.

[11] Lim, I. H., K. J. Lee, E. K. Lee, M. R. Choi, G. W. Lee, Y. Yoon, D. H. Park, and K. H. Jung (2004) High-yield purification and chracterization of recombinant human leukotactin-1 in *Pichia pastoris*. *Biotechnol. Bioprocess Eng.* 9: 1-6.

[12] Bae, C. S., G. Y. Lim, J. S. Kim, and B. K. Hur (2003) Quadrivalent combined vaccine, including diphtheria toxoid, tetanus toxoid, detoxified whole cell pertussis, and hepatitis B surface antigen. *J. Microbiol. Biotechnol.* 13: 338-343.

[13] Bae, C. S., K. N. Park, S. J. Ahn, J. S. Kim, and B. K. Hur (2002) Novel formulation of quadrivalent combined vaccine including diphtheria toxoid, tetanus toxoid, acellular pertussis antigens and hepatitis B surface antigen. *J. Microbiol. Biotechnol.* 12: 787-792.

[14] Linggood, F. V., F. S. Muriel, A. J. Fulthorpe, A. J. Woiwod, and C. G. Pope (1962) The toxoiding of purified diphtheria toxin. *Brit. J. exp. Path.*14: 177-188.

[15] Scheibel, I. and C. P. Elo (1965) Irreversible detoxification of purified diphtheria toxin. *Acta Pathol. Micrbiol. Scand.* 65: 117-128.

[16] Gupta, R. K., E. H. Relyveld, E. B. Lindbald, B. Bizzini, S. Benefraim, and C. K. Gupta (1993) Adjuvants: A balance between toxicity and adjuvanticity. *Vaccine* 11: 293-306.

[17] Goldsby, R. A., T. J. Kindt, and B. A. Osborne (2000) *Kuby Immunology*. 4th ed., W. H. Freeman and Company, NY, USA.

[18] Cooper, P. D. (1994) The selective induction of different immune responses by vaccine adjuvants. pp. 125-158. In: G. L. Ada (ed.). *Strategies in Vaccine Design*. Landes Company, Austin, USA.

[19] Gupta, R. K. and B. E. Rost (2000) Aluminum compounds as vaccine adjuvants. pp. 73-74. In: D. T. O'Hagan (ed.). *Vaccine Adjuvants: Preparation Methods and Research Protocols*. Humana Press, New Jersey, USA.

[20] Gupta, R. K. and G. R. Siber (1995) Adjuvants for human vaccines-current status, problems and future prospects. *Vaccine* 13: 1263-1276.

[21] Jenson, O. M. and C. Koch (1988) On the effect of $Al(OH)_3$ as an immunological adjuvant. *Acta Pathol. Microbiol. Immun. Scand.* 96: 257-264.

[22] Shirodkar, S., R. L. Hutchinson, D. L. Perry, J. L. White, and S. L. Hem (1990) Aluminum compounds used as adjuvants in vaccines. *Pharm. Res.* 7: 1282-1288.

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