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Comparative Study on the Adsorption Rate and Antibody Level of Bulk Purified Diptheria Toxoid on Chemical Adjuvants : Aluminium Phosphate, In Situ Gel and Calcium Phosphates

***P.H. Preethy, M.P. Ayyappadas, R. Renugadevi, S. Savetha, C. Geetha**
Department of Biotechnology, School of Life Sciences, RVS College of Arts and Science, Sulur 641 402, Coimbatore

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***Corresponding author:**

E-mail: preethy@rvsgroup.com,
Phone: +91-9894481962

ABSTRACT

Vaccines often contain preservatives, adjuvants, in addition to pathogen specific immunogen. It contain inactivated bacterial toxin. Vaccines also contain preservative that prevent bacterial or fungal contamination (e.g. thiomersal); adjuvants that enhance antigen specific immune responses (e.g. aluminium and calcium salts). Although alum is the most commonly used vaccine adjuvant, it has some limitations for use with the next generation recombinant antigens. In the present study the use of alternative adjuvant formulation with calcium phosphate and in situ was explored. In this study the stability of old gel along with new gel was checked and showed 100% adsorption rate of old $AlPO_4$ gel which was same as that of new gel. In situ and calcium phosphate were also used as an alternative adjuvants. Calcium phosphate gel showed the increased efficacy of the DT group of vaccine and did not show any adverse effects. The study using in situ gel yield lesser aluminum content but it shows greater adsorption rate. Finally the adsorption rate and antibody level of the gels were compared. Calcium phosphate shows higher density by comparing the adsorption rate with aluminium phosphate gel. Calcium phosphate gel shows less percentage of adsorption rate but the antibody levels were greater. Comparison of aluminium phosphate with in situ gel shows the same result.

INTRODUCTION

Diphtheria is an upper respiratory tract illness caused by non-invasive infection of nasopharyngeal tissues by toxigenic *Corynebacterium diphtheriae*, a facultative anaerobic Gram-positive bacterium [1]. The lethal toxin produced at the site of infection enters the blood stream from where it reaches to other organs, causing damage and causing death. The infection starts with the multiplication of bacilli in the nasopharynx. The production of diphtheria causes local necrotic lesions and a host inflammatory response. This results in a typical grayish diphtheria pseudo membrane's on the tonsils or elsewhere in the nasopharynx. The major consequences of Laryngeal diphtheria are respiratory suffocation and obstructions. The toxin shows no organ specificity, although frequent complications are myocarditis and polyneuritis caused by demyelination of the peripherial nervous system.

Diphtheria toxin is a cytotoxic protein having a molecular weight of 58,350 Da. It consists of a single polypeptide. Proteolysis yields two fragments (A and B) which are held together by a disulfide bond. Diphtheria toxin inhibits cellular protein synthesis in eukaryotes by inactivating elongation factor-2 through ADP-ribosylation [2, 3].

The toxin binds to a specific receptor on the cell surface [4, 5]. There it is internalized by receptor mediated endocytosis and finally, the enzymatically active fragment is translocated to the cytosol where the A fragment inactivates elongation factor 2. EF-2 is needed for translocation of tRNA from A site to the P-site of the ribosome during translocation and stop protein synthesis.

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe. The agent stimulates the body's immune system to recognize the agent as foreign body, destroy it, and “remember” it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters.

The word “adjuvant” is derived from a Latin word Adjuvare which means to help. So an adjuvant is a helper to the antigen. An adjuvant is also a compound administered with the antigen or one that provides a mode of presentation of antigen that will enhance the immune response towards the antigen.

The present study is to compare the adsorption rate and the antibody level of Bulk Purified Diphtheria Toxoid (BPDT) on chemical adjuvants such as Aluminium Phosphate ($AlPO_4$) Adjuvant (2 years Old and Newly prepared), In situ Adjuvant ($AlPO_4$) and Calcium Phosphate ($Ca_3(PO_4)_2$). By preparing vaccine, checking for pH, identity, adsorption rate and for the potency and antibody level.

MATERIALS AND METHODS

Preparation of aluminum phosphate gel

9.126 gm of Trisodium Orthophosphate was taken and dissolved in 10 ml of 65C hot distilled water then 11.376 gm of Potassium Aluminium Sulphate was taken and dissolved in 10 ml of 65°C hot distilled water. The above solution was made up to 100 ml, by adding distilled water and mixed thoroughly, adjusted the pH to 5.12 and allowed it to stand for 46 hrs. After that removed the supernatant and then collected the precipitate and centrifuged the precipitated at 2200 rpm for 10-15 min. Discarded the supernatant. Then added normal saline to the collected precipitate and made up to 100 ml and kept in shaker for 48 hrs. The supernatant was discarded by centrifugation at 2200 rpm for 15 min and then homogenized. Then made the volume up to 100 ml and kept for incubation [6].

Preparation of in situ gel

Taken 23.33 ml of Sodium Phosphate and added 2.5 ml of Aluminum Chloride and made up to 100 ml with distilled water. Kept in shaker for 2 hrs. pH was adjusted to 6.

Preparation of calcium phosphate gel

Taken 14.196 gm of Disodium Hydrogen Phosphate and mixed it with 11 gm of Calcium Chloride by using distilled water and made up to 200 ml. Mixed well and allowed it to stand for 48 hrs, after adjusting the pH to 5.12. After 48 hrs removed the supernatant and collected the precipitated. Centrifuged the precipitate at 2200 rpm for 10-15 min, discarded the supernatant and collected the precipitated again. Added normal saline to the collected precipitated and made up to 100 ml and keep it in shaker for 48 hrs. Discarded the supernatant by centrifugation at 2200 rpm for 15 min and then homogenized. Then made the volume up to 100 ml and kept for incubation.

Formulation of aluminum phosphate gel

Old gel (26/07)

- Diphtheria toxoid = 2 ml
- Aluminum Phosphate gel = 7.52 ml
- 1% Thiomersal = 0.81 ml
- Normal Saline = 89.67 ml

Old gel (35/07)

- Diphtheria Toxoid = 2 ml
- Aluminum Phosphate gel = 8.46 ml
- 1% Thiomersal = 0.79 ml
- Normal Saline = 88.75 ml

New gel (01/09)

- Diphtheria toxoid = 2 ml
- Aluminum Phosphate gel = 9.85 ml
- 1% Thiomersal = 0.76 ml
- Normal Saline = 87.39 ml

New gel (02/09)

- Diphtheria Toxoid = 2 ml
- Aluminum phosphate =9.17 ml
- 1% Thiomersal = 0.76 ml
- Normal Saline = 88.06 ml

New gel (03/09)

- Diphtheria Toxoid = 2 ml
- Aluminum Phosphate gel = 6.85 ml
- 1% Thiomersal =0.82 ml
- Normal Saline = 90.33 ml

Formulation of in situ gel

- Diphtheria Toxoid = 2.04 ml
- 1% Thiomersal = 0.96 ml
- 20% sodium chloride = 0.39 ml

Formulation of calcium phosphate gel

- Diphtheria Toxoid = 2.0 m
- Thiomersal = 0.96 ml
- Gel Content = 30.0 ml
- Normal Saline = 67.04 ml

Estimation of aluminium

Transferred 2 ml of vaccine sample into a 250 ml conical flask. 1 ml of concentrated Sulphuric acid and 6 drops of concentrated nitric acid were added. Heated the solution over flame until dense white fumes evolved. If charring was noticed added a few more drops of concentrated Nitric acid and heated further to get a colorless solution. Added 10 ml of distilled water and boiled again to obtain a clear solution. Added another 25 ml of distilled water. After cooling added 2 drops of methyl orange solution and neutralized the contents by addition of Sodium Hydroxide solution drop wise until the color changes from pink to golden yellow. Transferred 25 ml of accurately measured EDTA solution. Added 10 ml of acetate buffer solution. Heated the flask over a flame to boil the solution.

Added 1 ml of PAN indicator and titrated the hot solution against the $CuSO_4$ solution taken in a burette to get an end point of purplish brown color. Simultaneously carried out a blank determination using water instead of the test sample, which undergoes all the above steps.

Estimation of chloride

Standard preparation

Taken 3 ml of Silver Nitrate and 2 ml of Nitric acid in a conical flask. Added 6 ml of Ferric alum into it. Heated it for 1- 2 mins. After fumes appeared, cooled it for a min. Then titrated it against 0.02 N Thiocyanate on burette until the appearance of reddish brown color as end point.

Preparation of gel sample

Taken 1 ml of gel sample in a conical flask Added 3 ml of Silver Nitrate and 2 ml of Nitric acid into it. Then added 6 ml of Ferric alum. Heated it for 1- 2 mins. After fumes appeared, cooled it for a min. Then titrated it against 0.02 N Thiocyanate on burette

till the appearing of reddish brown color as end point.

Estimation of calcium

Taken 2 ml of Calcium phosphate gel in a test tube. Added 2 ml of distilled water and mixed it well. Added 1 ml of 4% Ammonium Oxalate in it. Mixed well and allowed to stand for overnight. Shaken it at frequent intervals (For complete precipitation). After precipitation of Calcium, centrifuged and removed the supernatant without disturbing the precipitate. After removing the supernatant, keep the supernatant centrifuged tubes inverted on the filter paper and allowed to drain off (For 5 mins). Wiped the mouth of the tube, Run 3 ml of the 2% of Ammonia down the inside of the tube and mixed with glass rod. Centrifuged it again, drained off the supernatant, wiped the mouth and added 2 ml of normal H_2SO_4. Mixed it by using glass rod, boiled it, removed and kept the mixture at 70-75 C. Then titrated it against 0.01 N Permanganate on burette, till the appearance of Faint Pink color as end point and persisted for few minutes. Simultaneously carried out a blank determination using water instead of the test sample, which undergoes all the above steps.

Limit of flocculation (LF) test

Physiological method

A representative sample for the whole batch was used for this test. The toxin was distributed into 5 or 6 flocculation tubes in 1 ml amounts graded doses of standard antitoxin (Adjusted to 100 LF / ml) is added so as to cover the expected range. The contents were mixed well and kept in a water bath maintaining 50º C ± 1º C. Care was taken to keep at least 1 / 3rd of the contents in the tubes, well above the water level in the water bath. The tube which contains the optimum concentration of toxin and antitoxin flocculates 1st and the corresponding unitage of the antitoxin was taken as the LF value of the toxin. The time taken for the flocculation was noted as KF. This serves as a useful indicator of the quality of the toxin if the KF is short; the quality of the antigen was good and vice–versa.

Total Lfcontent

Added 0.5 g of Tri – Sodium Citrate to 10 ml of vaccine. (Final bulk or final lot). Incubated the mixture in the water bath at 52º C for one hr. Centrifuged at 2500 rpm for 15 min and collected the supernatant. Pipetted out 1 ml each into five flocculation tubes. Added 0.4, 0.45, 0.5, 0.55, and 0.6 ml of DATF (20 LF / ml) and accordingly made up the volume to 2 ml with normal saline. Used a piece of butter paper to cover the mouth and inverted the tube thrice to mix the contents. Placed the tubes in a water bath, which was brightly illuminated from behind and maintained at 52º C. Observed the flocculation using a magnifying glass. Note the tube where the flocculation occurs at the earliest and assign the unitage. Noted the time of first flocculation (KF).

Adsorbed Lf content

Taken 10 ml of vaccine in a centrifuge tube. Centrifuged at 2500 rpm for 15 min and discarded the supernatant. Added 5 % tri–sodium citrate solution to the precipitate to made up the volume to 10 ml. Mixed well and incubated the mixture at 52ºC in a water bath for 1 hr. Centrifuged at 2500 rpm for 15 mins and collected the supernatant. Pipetted out 1 ml each into 5 flocculation tubes. Added 0.3, 0.35, 0.4, 0.45 and 0.5 ml of DATF (20 LF/ml) and accordingly made up the volume to 2 ml with normal saline. Used a piece of butter paper to cover the mouth and inverted the tube thrice to mix the contents. Placed the tubes in a water bath, which was brightly illuminated from behind and maintained at 52ºC. Observed the flocculation using a magnifying glass. Noted the tube where the flocculation occurs at the earliest and assigned the unitage. Noted the time of first flocculation (KF).

Calculation

Rate of Adsorption = Adsorbed LF Content / Total LF Content X 100 %

The potency test

The potency test was then performed by injecting 0.5 ml of each sample into the guinea pig. Booster dose was given after fifth week of inoculation. Bleeding was done on the sixth week. Serum was separated and titrated.

PART- I Immunization of guinea pigs

No. of Guinea Pigs immunized	: 2 (for each gels).
Weight of Guinea Pigs	: 250-300 grams.
Vaccine Dilution	: 1 ml Vaccine + 99 ml Sterile Normal Saline.
Dose	: 1/50 SHD in 1 ml.
Route of Injection	: Subcutaneous.
Date of Immunization	: 19-01-2010
Booster dose on	: 20-02-2010
Date of bleeding	: 08-03-2010

PART – II Measurement of antibodies

(carried Out On Flanks Depliated G.pigs)

Date of Titration	: 11-03-2010
No: of Sera Titrated	: 2 Sera For Each Gel.
Toxin Used	: F 21/00
Lt/100 Dose of the Toxin	: 0.00016 ml
Reference Standard Used	: DNR Batch No: 1/06 (10 I.U/ ml)

Reference Dilution

(To get 0.1 IU/ ml).	: 0.1 ml DNR+9.9 ml of normal saline
Incubation period	: 1 hr at room temperature.
No. of spot Inoculated	: 1 spot/dilution.
Dose and route	: 0.2 ml / spot, intradermal

Sera titration

Individual Sera Tested for	a) 1 Unit	(1/10 Dilution)
	b) 2 Unit	(1/20 Dilution)
	c) 3 Unit	(1/30 Dilution)
	d) 4 Unit	(1/40 Dilution)

Individual Mixtures: 1.0 ml dilution Serum + 0.5 ml diluted Toxin + 0.5 ml Borate buffer.

RESULTS

Gel preparations

Different types of gels such as aluminium phosphate, in situ and calcium phosphate are prepared

Estimation of aluminium

Mg of Aluminium/ ml = (blank titre-test titre)×0.2698×4.52/volume of sample titre

Table No.1: Old gel

Batch No.	Blank Titre (ml)	Test Titre (ml)	Vol. of Sample Tested (ml)	Al Content (mg/ml)
26 / 07	24.5	17.3	0.2	43.90
35 / 07	24.5	18.1	0.2	39.02

Table No.3: In situ gel

Batch No.	Blank Titre (ml)	Test Titre (ml)	Vol. of Sample Tested (ml)	Al Content (mg/ml)
IG 01 / 09	24.2	14.6	2	1.29
IG 02 / 09	24.2	15	2	1.24
IG 03 / 09	24.2	15.1	2	1.22

Table No. 5: In situ gel

Batch No.	Standard Titre (ml)	Unknown Titre (ml)	NaCl Content (mg)
IG 01 / 09	7.5	0.8	7.839
IG 02 / 09	7.5	1	7.605
IG 03 / 09	7.5	1.5	7.02

Mg of Sodium Chloride per 100 ml = (ml of Titrated Standard – ml of Titrated Unknown) ×117

Formulation study

Table No.7: Aluminium content

Sample	Batch No.	Before Formulation (mg/ml)	After Formulation (mg/ml)
Old Gel	26/07	43.90	2.69
Old Gel	35/07	39.02	2.83
New Gel	01/09	33.50	2.69
New Gel	02/09	35.97	2.96
New Gel	03/09	48.17	2.83
In Situ Gel	IG-1	1.29	1.26
In Situ Gel	IG-2	1.20	1.13
In Situ Gel	IG-3	1.24	1.06

Table No.9: Calcium content

Sample	Batch No.	Before Formulation (g/ml)	After Formulation (g/ml)
Calcium Phosphate	1	3.15	2.15
Calcium Phosphate	1.5	3.35	2.95
Calcium Phosphate	2	3.25	2.35

Table No.2: New gel

Batch No.	Blank Titre (ml)	Test Titre (ml)	Vol. of Sample Tested (ml)	Al Content (mg/ml)
01 / 09	24.5	19	0.2	33.50
02 / 09	24.5	18.6	0.2	33.50
03 / 09	24.5	16.6	0.2	48.17

Estimation of chloride

Mg of Chloride = (ml of Titrated Standard – ml of Titrated Unknown) ×20.

Table No. 4: In situ gel

Batch No.	Standard Titre (ml)	Unknown Titre (ml)	Cl_2 Content (mg)
IG 01 / 09	7.5	0.8	134
IG 02 / 09	7.5	1	130
IG 03 / 09	7.5	1.5	120

Table No.6: Estimation of calcium

Batch No.	Molar (M)	Titrated Blank (ml)	Titrated UnknowN (ml)	Calcium Content (mg/ml)
CP 01 / 10	1	0.05	3.2	3.15
CP 02 / 10	1.5	0.05	3.4	3.35
CP 03 / 10	2	0.05	3.3	3.25

Mg of calcium/ ml = (titration of unknown- titration of blank) × 0.2 × 100/2

Table No.8: Sodium chloride content

Sample	Batch No.	Before Formulation (g/ml)	After Formulation (g/ml)
IN SITU GEL-1 (IG I)	IG-I	0.78	0.84
IN SITU GEL-2 (IG II)	IG-II	0.76	0.81
IN SITU GEL-3 (IG III)	IG-III	0.70	0.80

Percentage of adsorption rate

Table No. 11: Aluminium gel

Sample	After 15 Days (%)	After 30 Days (%)
Old Gel I	80.00	100
Old Gel Ii	76.92	100
New Gel I	66.66	100
New Gel Ii	84.61	100
New Gel Iii	75.00	100

Table No.10: pH estimation

Sample	Batch No.	Before Formulation	Adjusted	After Formulation
Old Gel	26/07	5.50	6.0	6.96
Old Gel	35/07	5.20	6.0	6.56
New Gel	01/09	4.80	6.0	6.35
New Gel	02/09	5.01	6.0	6.36
New Gel	03/09	4.98	6.0	6.27
In Situ Gel	IG-I	2.84	6.0	6.40
In Situ Gel	IG-II	2.75	6.0	6.23
In Situ Gel	IG-III	2.72	6.0	6.16
Calcium Phosphate	1M	3.12	6.0	6.06
Calcium Phosphate	1.5M	3.25	6.0	6.10
Calcium Phosphate	2.0M	3.68	6.0	6.0

Table No. 12: In situ gel

Sample	After 15 Days (%)	After 30 Days (%)
In Situ Gel I	61.53	100
In Situ Gel II	69.20	100
In Situ Gel III	75.00	100

Table No. 13: Calcium phosphate gel

Sample	After 15 Days%	After 30 Days%
Ca_3PO_4 GEL I	91.66	100
Ca_3PO_4 GEL II	72.72	100
Ca_3PO_4 GEL III	81.80	100

Table No. 14: I Immunization of guinea pigs

G. Pig No.	Colour Code	Sex	1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week	7th Week
1.	NC	Male	✓	✓	✓	✓	✓	✓	✓
2.	NC	Male	✓	✓	✓	✓	✓	✓	✓
3.	RH	Male	✓	✓	✓	✓	✓	✓	✓
4.	RH	Male	✓	✓	✓	✓	✓	✓	✓
5.	RB	Male	✓	✓	✓	✓	✓	✓	✓
6.	RB	Male	✓	✓	✓	✓	✓	✓	✓
7.	RT	Male	✓	✓	✓	✓	✓	✓	✓
8.	RT	Male	✓	✓	✓	✓	✓	✓	✓
9.	BT	Male	✓	✓	✓	✓	✓	✓	✓
10.	BT	Male	✓	✓	✓	✓	✓	✓	✓

NC - No Colour - Control
RH - Red Head - Old Gel
RB - Red Back.- New Gel
RT - Red Tail – In Situ Gel
BT - Blue Tail - Calcium Phosphate Gel

Table No.15: Toxin dose confirmed based on the control titration

Toxin Dose (ml)	DNR Dose (IU)	Mixture Table				Observation
		Diluted Toxin (ml)	Diluted DNR (ml)	Borate Buffer (ml)	Total Volume	After 48 hrs (Reaction Size in mm)
0.00016	0.008	0.5	0.8	0.7	2.0	20×25
0.00016	0.009	0.5	0.9	0.6	2.0	20×22
0.00016	0.010	0.5	1.0	0.5	2.0	15×15
0.00016	0.011	0.5	1.1	0.4	2.0	Necrosis
0.00016	0.012	0.5	1.2	0.3	2.0	Necrosis

Table No.16: Sera titrationH estimation

Serum No.	Observation (after 48 hrs)	Results in (I.U/ ml)	Serum No.	Observation (after 48 hrs)	Results in (I.U/ ml)
OG 1	No Reaction	>4	IG 1	No Reaction	>4
a)			a)		
b)			b)		
c)			c)		
d)			d)		
OG 2			IG 2		
a)			a)		
b)			b)		
c)			c)		
d)			d)		
NG 1			CG 1		
a)			a)		
b)			b)		
c)			c)		
d)			d)		
NG 2			CG 2		
a)			a)		
b)			b)		
c)			c)		
d)			d)		

OG – Old Gel
NG – New Gel
IG – In Situ Gel
CG – Calcium Phosphate Gel
Result: each serum contains > 4 IU/ml.

DISCUSSION

Adsorption rate comparison from the above study

The constant unit of the toxoid was mixed with standard antitoxin (200 Lf/ml). The Lf of the toxoid was found to be 60-80% and Kf was 5-20 mins.

Old gel

From the above table it clearly shows that the stability of the old Aluminium Phosphate gel was maintained. The above data's shows all parameters of old Aluminium Phosphate gel such as Aluminium content, adsorption rate, pH, ionic strength was maintained in a controlled manner. The adsorption rate clearly shows that even after storage of Aluminium Phosphate gel for 2 years stability was maintained as such, and also for final vaccine pH was maintained "between" 6-7.

pH and gel surface of gel makes the toxoid to get adsorbed over the surface of gels. It was clearly shown by adsorption rate results after 15 days. From the above results the adsorption rate of adjuvants has higher surface area and so gives good results of adsorption rate.

Stability of old Aluminium Phosphate gel was confirmed by comparing it with new Aluminium Phosphate gel. It shows percentage of adsorption rate of old Aluminium Phosphate gel which was same as that of new gel. By this we confirmed that the stability was still maintained.

New gel

Mostly alum containing adjuvants are used for the DTP group

of vaccines. From the above table, it shows 60-80% of adsorption rate after the 15 days of incubation period. By further allowing the new gel to get matured it shows 100% of adsorption rate. Due to the nature of the Aluminium Phosphate compounds having higher surface area it gives better adsorption rate. By maintaining the pH 5-6 after preparation of gel, it increases the binding capacity, thus results in the higher adsorption rate. Aluminium salts can be used for the maintaining the experimental conditions such as temperature, concentration, and pH. By comparing the percentage of adsorption rate with alum containing adjuvants it has given better results, and also by comparing the new Aluminium Phosphate with old gel, the percentage of adsorption rate results greater.

In situ gel

From the above results, alternative preparation of Aluminium Phosphate gel by in situ method showed better adsorption rate and it has many advantages. In situ method yields lesser aluminium content but it gives greater adsorption rate as like that of Aluminium Phosphate gel. In situ method the formation of $AlPO_4$ takes place in the presence of the diphtheria toxoid in the mixing vessel it self. But the pH was maintained with in the range of 6-7. In situ method yields good level of Sodium Chloride production, so there is no need of adding large quantity of Sodium Chloride.

Calcium gel

From the above data for Lf test shows 80% of adsorption rate is for Calcium Phosphate gel. This shows good results after 15 days of incubation period**.** Calcium Phosphate gel shows good adsorption rate of DT group of vaccines. Calcium Phosphate gel yields higher calcium content and adsorption rate. The binding capacity of diphtheria toxoid over the Calcium Phosphate gel was determined by doing Lf test. By allowing the gel for further maturation period after 30 days it gives 100% adsorption rate. Due to the presence of Calcium content in our body it does not show any adverse effects.

Calcium Phosphate gel shows high density. Due to its higher density, result shown better adsorption rate, and also in calcium phosphate gel it can maintain 3 mg/ml of calcium content easily. Due to the presence of calcium content in the body it can be solubilization by the fluid and it can be evenly distributed to the targeted site and got excreted by urine, presence of excess of calcium doesn't show any adverse reactions, further studies may be continued. By this way Calcium Phosphate gel increases the efficacy of the DT group of vaccines

CONCLUSION

Even though the immunogenicity of antigens absorbed onto aluminium adjuvants are high, there are a number of disadvantages of using alum like increased sensitivity to alum [7] and local granuloma formation at injection sites [8,9]. The excessive amounts of Aluminium compounds may suppress immunity by covering the antigen with mineral compounds or the Aluminium compounds and may be cytotoxic to macrophages. The disadvantages of alum-based adjuvants also include the severity of local tissue irritation, the longer duration of the inflammatory reaction at the injection site, strong Th2 responses, minimal induction of cell-mediated immunity, and a propensity to elicit undesirable immunoglobulin E (IgE) responses [10-13]. Alum compounds have also been shown to increase the levels of potential undesirable homocytotropic antibodies in animal species [14, 15]. Furthermore, alum-based vaccines are frequently ineffective for the induction of antiviral immunity [16]. For these reasons, new adjuvants are being developed to enhance the immunity against weak antigens. New-generation adjuvants are designed to induce minimal side effects, enhance the duration of the immune response, and concurrently stimulate humoral, cellular, and mucosal immune responses. But in case of Calcium Phosphate, the excessive amounts do not cause any harmful effects as the body needs calcium in higher amount. Calcium helps in the contraction of heart as it is pumping blood through the body. If there is increase in quantity of Calcium, the body has some very well understood mechanisms to bring the balance back to normal. Taking large amount of calcium does not upset the heart beat. Calcium ions are essential in converting electrical impulses into chemical signals in the brain and also in blood clothing.

Thus considering the adverse effects of Alum as adjuvant, Calcium is recommended as safe adjuvant in vaccines

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