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ADVANCES IN PRODUCTION OF TRADITIONAL VACCINES-A REVIEW

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ABSTRACT

The most successful and effective preventive measure against infection from a particular disease is to get vaccinated. Traditional vaccines use a dead or a weakened pathogenic microbe or a toxin from a pathogen. Introduction of the attenuated or dead pathogen into a healthy individual generates an immune response. A memory of the antigenic specificity is produced in the individual thus immunizing the individual from that particular disease for a long period of time. Some vaccines do require booster doses to retain the memory of antigenic specificity. Various techniques have been developed and are still under development to produce effective vaccines for several diseases. A key development in traditional vaccines is the reduction of booster doses required as well as reduced side effects. Each technique used to produce vaccines has to ensure provision of long-term immunity to the individual, no side effects on the individual due to the vaccine, no relapse or reversion of pathogenicity, and induction of an immune response at a low dosage. This article aims to highlight the progress and failures in the development of different types of traditional vaccines, along with the procedures and techniques used in the traditional vaccine production.

Keywords: Vaccine, adjuvants, toxins, disease, strain improvement, attenuation, polysaccharide carriers, inactivation

Abbreviations

Adjuvant System (AS), Antigen Presenting Cell (APC), β-Propiolactone (BPL), Cross Reacting Material (CRM), delayed type hypersensitivity (DTH), Diphtheria Pertussis Tetanus (DPT), Diphtheria toxoid (DT), Diphtheria-tetanus (DiT), French neurotropic vaccine (YF-FNV), Freund's Complete Adjuvant (FCA), Freund's incomplete adjuvant (FIA), Hemagglutinin (HA), Hemophilus influenza protein D (HiD) Invasive Pneumococcal Disease (IPD), Japanese encephalitis virus (JEV), Live attenuated vaccines (LAVs), meningococcal Outer Membrane Protein Complex (OMPC), Monophosphoryl Lipid A (MPL) Mucous Associated Lymphoid Tissue (MALT), Muramyl Dipeptide (MDP), Neuraminidase (NA)

13- valent polysaccharide conjugate vaccine (PCV13), 23-valent pneumococcal polysaccharide vaccine (PPSV23), Pertussis Toxin (PT), Pertussis toxoid (PT), poly (D, L-lactic/glycolic acid) (PLGA), poly (L-lactic acid) (PLA), Polyribosylribitol Phosphate (PRP), Quillaja Saponaria (QS), RNA-dependent RNA-polymerase (RdRp), Specific Pathogen Free (SPF), Tetanus toxoid (TT), Trivalent inactivated influenza, virus vaccine (TIV), water-oil-water(w/o/w), Yellow Fever (YF)

INTRODUCTION

The history of vaccination started in the 17th century among the Chinese physicians. In the late 1600, Chinese medical practitioners did variolation, that is, dried the pustules of small pox, made incision in a healthy individual and injected the materials taken from the pustule (1). Later in the year 1718, Lady Mary Worley Montagu did the same and prevented small pox in a healthy individual (2). Later, Edward Jenner variolated the materials of cow pox pustules (1, 3), taken from a milk-maid, into a healthy boy and later inoculated the boy with small pox virus. He observed that the boy did not develop small pox (4, 5). This process was then termed as "vaccination". In 19th century Robert Koch, discovered a live attenuated vaccine against cholera and discovered active immunity. He conducted an experiment in which he inoculated the old culture of cholera into the healthy chicken, which did not develop the disease. Later when these inoculated chickens were again inoculated with fresh culture of cholera bacteria, they did not develop the disease, while the ones which were not inoculated developed cholera. In 1881, Louis Pasteur had demonstrated immunization against anthrax by heat attenuation of Bacillus anthracis (6).

As the immunological world advanced into the 21st century various types of vaccines have emerged for various diseases. This development has further advanced into producing modern vaccines like DNA vaccine, RNA vaccine, edible vaccine, hybrid rDNA vaccine. While these modern vaccines may be projected as the future of vaccination, but it is important to understand the rationale adopted in developing and improvising a traditional vaccine. A retrospective study may guide in enhancing the modern vaccine approaches as well as highlight the advancements and limitations of the traditional vaccines. Table 1. represents the four main types of traditional vaccines being used for immunization against infectious diseases. The diseases listed in table 1 have been covered in terms of advances in traditional vaccine development. Traditional vaccines have existed for decades now, and will continue to influence the world of vaccinology as most of these vaccines are considered the gold standard for vaccination.

Table 1: Types of vaccines developed against the respective diseases

Type of Vaccine	Disease
Live Attenuated Vaccine	Vaccinia Influenza Measles Mumps Rubella Smallpox Yellow Fever
Inactivated Vaccine	Polio Rabies Cholera Hepatitis A Influenza
Toxoid Vaccine	Diphtheria Tetanus Malaria
Conjugate Vaccine	Pertussis Pneumococcal infection Meningococcal infection Influenza

1. Live attenuated vaccine

Live attenuated vaccines (LAVs) provide immunity to an individual against a specific disease as it contains attenuated pathogens, hence providing an easy opportunity to the host's immune system to recognize the antigens displayed by these attenuated pathogens and kill the injected attenuated pathogens while retaining memory of the antigenic specificity (7). The mode of action of the vaccine is slow by virtue hence adjuvants are not required to further increase the duration of immunity.

Attenuated pathogens are pathogens whose virulence has been decreased by artificial means. In the past, LAVs were produced by continuous passages and selections. Even though LAVs have been successfully produced for a number of RNA viruses and is one of the most successful type of vaccine, the empiric methods of production would sometimes lead to reversion of the virus into a wild lethal strain (8). Rational methods have been devised for the production of attenuated pathogens as it reduces the probability of reversion of the virus into a wild strain (9). The strategies for development are listed below;

1.1 Degeneration of Quasispecies

In case of viral pathogens, the population is not a single genotype but a mixed genotype due to rapid mutations in the viral RNA, hence it is essentially a "quasispecies". This quasispecies is necessary for the survival and virulence of the pathogen as it provides the pathogen a better chance of survival when infecting an individual by granting the virus the ability to adapt to a new environment when infecting a host(10). Degradation in this quasispecies can be brought about by propagating the virus in an atrophic host resulting in loss of genetic diversity for successful infection of the trophic host(11). The oral poliovirus vaccine was produced by limiting the quasispecies of the poliovirus by

conducting several passages of Mahoney type-1 strain and Saukett type-3 (12) strain in rats and mice with subsequent passages in cell cultures.

The virulence of a pathogen was observed to decrease if the overall genotypic diversity of the population was restricted by subjecting the viral population to continuous genetic variation and competition (13). For example, the Measles vaccine is produced by inoculating and passaging the pathogen in chicken embryonic fibroblast resulting in the attenuation of the virus (10). The vaccine for yellow fever was produced in a similar manner, here two strains of Yellow Fever (YF) were independently produced by different groups. The French neurotropic vaccine (YF-FNV) was produced by taking a wild strain and passaging the strain 128 times in intracerebral mouse brain (14) while the Asibi strain was produced by another wild strain that had been passaged in mouse culture followed by chick embryo tissue (15,16). The Asibi strain has also been attenuated by passaging in HeLa cells (17). An experiment on prolonged cultivation of YF virus in vitro demonstrated that the virulence showed a decrease for cultivation in chick embryo tissue while the virulence was retained in a prolonged cultivation in mouse tissue (16).

The controversial Urabe strain for mumps which has been stopped being used as a vaccinating strain as it caused meningitis and encephalitis (18) was produced by attenuating the pathogen in chick fibroblast (8, 19). While the Jeryl Lynn strain of Mumps has been produced owing to this concept by inoculating them into Specific Pathogen Free (SPF) Chicken Embryonic Fibroblasts (20) and cell cultures of chick embryo (19,21). Strains like Rubini strain (22), have been obtained by isolating the pathogen from the patient followed by passages SPF chicken fibroblasts and MRC-5 cells (15,23). The Leningrad-3 strain is a combination of 5 strains of mumps and L-Zagreb strain is a further sub-cultivation of the Leningrad-3 strain in chicken fibroblasts (24, 25).

Influenza vaccine is produced using a similar strategy where dominant strains of Influenza A (H1N1 and H3N2) and Influenza B are collected for the next infective season(26) and propagated in embryonated chicken eggs which are typically 2 weeks old(27–29). Since such large-scale production of single use bioreactor is not possible to meet the demands for bulk production of vaccine, the host has been changed to a mammalian cell line which is typically the Vero cell line or in case of Flucelvax which is produced by Novartis uses the MCDK cell line (30).

The vaccine for smallpox which was obtained from milkmaids suffering from

cowpox can be considered the first ever vaccine using this concept(3,5,31). The Variola virus had not been inoculated in another host, however, it can be deemed that cowpox was an already existing attenuated model of smallpox present in cows that would mildly infect humans. Thus, when Edward Jenner inoculated James Phipps with cowpox pathogen followed by Variola virus, James survived without developing smallpox (3–5).

In case of bacterial pathogens, a synthetic quorum sensing environment that induces expression of attenuated factors can be constructed and induced on the population of the pathogen (32). Thus, by inducing a bottleneck to prevent the expansion of genetic diversity of the population, the virulence of the pathogens decreases. Live attenuated cholera vaccine uses this strategy where a deletion of virulence factors (33) and RS1 of the El Tor strain (34, 35) was carried out which rendered the strain incapable of recombining with exogenic genetic material of target host (36, 37).

Attenuation of a pathogen can be confirmed by checking the markers for virulence in the pathogen's genome sequence. This indicates that there are certain genes that are responsible for the reduced virulence of a pathogen. Hence by targeting genes and "switching them on or off" a virulent pathogen can be engineered to be non-virulent and in case of vaccines it can be engineered to be attenuated. These engineered pathogens are attenuated in nature either due to low replication rate or due to decrease in expression of toxins or both (38). Polymerase based attenuation relies on mutation or replacement of a specific residue on the pathogenic RNA-dependent RNA-polymerase (RdRp) such that the genetic variation of the quasispecies is lowered. Site-directed mutation mediated through PCR overlapping is used to mutate a specific residue (39). The mutation is aimed at increasing the fidelity of RdRp. By increasing the fidelity, the number of mutations occurring is lowered hence a loss in genetic variation is evident (40). This method reduces the probability of the attenuated virus from becoming a wild-type even after several passages thus effectively reducing chances of reversion.

1.2 Removal of Codon Biasness

A codon is degenerate in nature, that is, there are multiple codons coding for the same amino acid. In an organism the genome has degenerate codons but it has been observed that among synonymous codons certain codons are expressed more, thus a codon bias exists for a certain species (41, 42). For example, in the case of polioviruses a change in the genome sequence of type-2 strain by increasing the frequency of CpG and UpA dinucleotides

results in the decrease of codon bias followed by attenuation of the virus due to lowered expression of toxins and lowered replication rate which was found to be conserved after several passages in HeLa cells (41, 43, 44).

Deoptimized codons has several advantages complying to the strategy of vaccine development. Firstly, the deoptimized codons express proteins that are identical to the wild-type and the attenuated type but the translational efficacy is greatly reduced. Hence the antigenicity is not affected and the immune response elicited is similar to the response towards a natural infection. Secondly, this technique can be applied to a large number of viruses as it is a systemic approach rather than an empiric one. Finally, the point mutations are brought about in thousands of synonymous codons, thus, minimizing the possibility of reversion to a wild lethal strain.

1.3 Auxotrophic Mutation

Auxotrophic mutants of a strain are incapable of producing a naturally synthesizable compound by the normal strain, thus requiring the naturally synthesizable compound to be additionally present in the media for normal functioning of the auxotrophic strain. Auxotrophic strains are usually mutated by deleting or silencing a gene that is responsible for production of a growth-linked product. As a direct result of this deletion, the mutant strain is unable to proliferate at a rate similar to that of the normal strain.

In live attenuated vaccines the use of auxotrophic mutants is possible due to the fact that the mutant will be unable to receive sufficient quantity of metabolite required for growth inside the host system. To make the vaccine more effective and less revulsive the strain is further manipulated to decrease the invasive and toxin production capability. A successful model of such a vaccine can be observed for *Shigella enterica* and *Shigella flexneri* (45). While a hypoxanthine, thiamine and adenine auxotroph of *S. typhi* (46) constructed through two deletion mutations from the Ty2 and CDC 10-80 strain has been reported as a possible candidate for live-oral vaccine (47).

2. Inactivated Vaccine

Unlike Live Attenuated Vaccines (LAVs), inactivated vaccines provide immunity to the host by inducing an immune response in the host towards the injected virus that has been killed or inactivated via chemical or thermal means such that it cannot further replicate or survive in the host organism. Several novel agents and methods of inactivation have been described (48) such as Ascorbic acid used for preparation of rabies vaccine (49), psoralen induced inactivation for dengue vaccine (50), UV treatment and Gamma irradiation for inactivated Influenza A vaccine (51, 52),

ethylenimine derivates, heat inactivation for polio virus (53), formaldehyde, β-Propiolactone, and many more (54, 55). Rabies vaccine was produced by binary ethylenimine after propagating the virus in BHK cells, however, vaccine inactivation by this method is less stable (56). Nonetheless, usually formaldehyde and β-Propiolactone are widely used for production of inactivated vaccine because of higher efficiency and stability retention. Inactivated vaccines are second to LAVs in stimulating an immune response that mimics an immune response to a natural infection, however, the immune response generated by inactivated vaccine is weaker compared to the immune response generated by LAVs, thus, requiring "booster" injections and immunological adjuvants to provide a stronger immune response against the pathogen.

Inactivated vaccines are stable and can be easily maintained when compared to LAVs. Also, inactivated vaccines can be used in multivalent combinations to provide immunity against different strains and viruses in a single dose. Inactivated vaccines can be further classified as whole virus vaccines, these vaccines contain completely killed/inactivated virus; Split virus vaccines, the viruses are disrupted using a detergent to make split virus vaccine; subunit virus vaccines, produced by only purifying out the antigen from the virus such that the purified antigen can mimic the stimulation of a natural immune response.

2.1 Heat Inactivation

Inactivation by heat treatment is the simplest technique that can be used for inactivating viruses. Further inactivation is followed by chemical inactivation which may result in an increase in vaccine toxicity (57). For unknown viruses, the virus sample is usually heated to a sub 100ºC for a long duration or heated over 100ºC for a short duration. The thermal inactivation point determines the lowest temperature that would suffice to inactivate an unknown virus when treated for 10 minutes. The temperature is increased by intervals of 10ºC from the first exposed temperature, and the interval is reduced to 5ºC when inactivation first occurs (58). A common drawback of heat inactivation is the denaturing of the RNA and DNA strands as well as proteins, resulting in ineffective vaccines. Despite this drawback, both Hepatitis A and Hepatitis B vaccines are produced by heating the virus to 56ºC for 30 minutes (59). A study demonstrates that heat inactivation at a temperature of 65ºC for a period of 15 minutes(60) was sufficient to completely inactivate poxvirus, picornavirus, toga virus, coronavirus, orthomyxovirus, rhabdovirus, herpes virus, lentivirus, and retrovirus while parvovirus and papovirus were

inactivated with heat treatment for 90 seconds at 103ºC(61).

2.3 Inactivation by Formaldehyde

Formaldehyde is the simplest aldehyde with the formula CHOH and usually acts as a reducing agent unless a stronger reducing agent is added to the reaction mixture. It brings about various modifications like methyl groups, methylene bridges and Schiff bases in proteins thus resulting in the inactivation of the proteins. The specific mechanism of inactivation is still under study (62).

Generally, formaldehyde stock solution is diluted to get a final concentration of 0.4% formalin, this concentration requires an inactivation period of up to 3 weeks for higher titers of the virus and an incubation temperature of 2oC – 7oC. However, to inactivate more potent batches of virus, the concentration of formalin was lowered to approximately 0.025% – 0.012% and a higher temperature of 37oC – 40oC was used as observed in the preparation of Salk's vaccine (63). Therefore, higher the concentration of formalin and higher the temperature, the rate of inactivation will be faster though a loss of immunogenicity might be observed due to degradation and destruction of the toxin. Thus, the inactivation time should be optimized such that the immunogenicity is not lost while also assuring complete inactivation of the virus. Once inactivation of the virus is done, the residual formalin is removed using sodium bisulphate (54).

Japanese encephalitis virus (JEV) was cultured in Vero cells and formalin-inactivated followed by purification to obtain the antigens. The genetic analysis of all JEV isolates confirmed that they comprised of a single serotype (64). This is information is valuable for vaccine design (65). A study on a strain obtained from Vellore, India demonstrated that inactivation by formalin at 22ºC was faster and cheaper than inactivation by formalin at 4ºC (66).

Formalin-inactivated Hepatitis A virus showed appropriate immune response in a study (57), thus, suggesting vaccine development by formalin inactivation (67). The Inactivated Poliovirus Vaccine is produced by inactivation of three strains of poliovirus, Mahoney type-1, MEF-1 and Saukett type-3, using formaldehyde, however, a failure in inactivation of the Mahoney strain due to resistance to formaldehyde had resulted in the replacement of Mahoney strain with Brunenders strain type-1(12).

In case of the live attenuated strains of influenza, which are subtypes of Influenza A (H1N1 and H3N2) and two antigenically distinct lineages of Influenza B, obtained from serial passages in embryonic chicken eggs or Vero cells, depending on the host used, when further treated with formalin

(68), it was observed that the surface glycoproteins hemagglutinin (HA) as well as neuraminidase (NA) characteristics were retained while effectively killing the cells resulting in formation of an inactivated vaccine (28, 69). Hence, the vaccine is multivalent and a whole virus vaccine in nature, containing toxins from more than 1 strain (27). This whole virus vaccine when treated with Triton-X100 resulted in the production of split virus vaccine (70).

Cholera vaccine is produced by treating classical as well as El Tor Ogawa and Inaba subtypes of Vibrio cholerae O1 serotype with formalin (35). In addition to the formalin killed pathogen, CT B subunit may also be included, however, the inclusion of the CT B subunit requires a buffer of sodium bicarbonate to protect the CT B subunit from acidic degradation by gastric juices (71).

2.4 Inactivation by β-Propiolactone (BPL)

β-Propiolactone is a four-ringed lactone and is highly reactive towards nucleophiles. BPL is stable in concentrated liquid forms but readily degrades in aqueous solution due to hydrolysis into non-toxic and non-carcinogenic products. This results in complete elimination of BPL from the reaction mixture thus eliminating the requirements of techniques for further removal of BPL from the product. Thus, BPL poses an advantage over formaldehyde inactivation methods as residual formalin has to be removed from the product. However, if excess BPL is present, it has to be neutralized using thiosulphate. The inactivation time of viruses using BPL is shorter compared to formaldehyde inactivation method. Also, the temperature used during inactivation is low thus reducing the risk of denaturation of the epitopes due to thermal degradation.

BPL is used for inactivation of Rabies virus. The PM strain and Flury HEP strain were adapted in WI-38 for several passages and a viral pool was prepared in BHK cell line. These strains were then chemically inactivated by BPL at 0.025% concentration at 4ºC for various lengths of time (72). The PV/VERO-Paris strain of rabies was inactivated by BPL after culturing in Vero cells (73).

However, BPL directly interacts with the nucleic acids to inactivate the virus. DNA and RNA are irreversibly alkylated and acylated by BPL, specifically reacting with N-7 of guanosine and with N-1 of Adenosine to some extent. Due to this modification Gp is misread as Ap by the polymerase resulting in numerous irreversible point mutations in the genome of the virus, hence rendering it inactive. Therefore, the proteins expressed are altered due to this nucleic mutation. Also, it has been observed in a study that BPL interacts with 9 amino acids hence directly

altering proteins. A loss in immunogenicity occurs due to this alteration. Unlike formaldehyde inactivation, the concentration and the temperature of BPL varies based on the virus being inactivated.

2.5 Inactivation by Psoralen

Treatment of viruses by psoralen is a relatively mild method to inactivate the virus. 4′-aminomethyl 4,5′-8-trimethylpsoralen in an inert environment along with UV radiation has shown to be an effective inactivator for bluetongue virus (74,75) and immunodeficiency viruses (76). Psoralen inactivates virus by intercalating between the base pairs of double stranded nucleic acids hence inhibiting replication of DNA. The viral protein structure is preserved after inactivation and no residual toxicity is observed however, inactivation by psoralen is not cost effective (77).

3. Toxoid Vaccine

Toxoid is a bacterial endotoxin whose properties have been suppressed by using chemicals such as formalin but maintaining the immunogenicity. On vaccination, the immune response is generated by the host body, the immunological memory is built against the molecular markers of the toxoid but there is no manifestation of the disease in the host. However, no strong immune response is elucidated, therefore booster doses are necessary.

The objective is to prepare a toxoid in such a way that the toxin is irreversibly inactivated by chemical methods. This inactivation can be done by using oxidizing agents such as hydrogen peroxide, aldehydes, sodium peroxides, N-chloro-4-methyl-benzene-sulfonamide sodium salt (chloramine–T), performic acid, dioxane peroxide, periodic acid, sodium permanganate, sodium hypochlorite (78). The oxidizing agents used could be organic or metallic. Hydrogen peroxide is mostly preferred because of easy handling, easy availability and is cost effective (78).

While aldehydes such as glutaraldehyde or formaldehyde form Schiff bases. Schiff bases are chemically unstable and may lead to reversible reaction where the toxoid converts into an active toxin (62).

The recent method developed for treatment of the partially isolated protein toxin is with an oxidizing agent in the presence of small amount of metal ion. This metal ion will chemically inactivate the toxin but maintain its immunogenicity. The oxidizing agent will oxidize the peptide at specific positions in the peptide chain where amino acids like cysteine, cystine, methionine, tryptophan or tyrosine are present (78).

For example, tetanus disease is caused by endotoxin secreted by the clostridium tetani bacteria into the body. Toxin from clostridium tetani is isolated from the isolates of clostridium tetani using a purifier (79). The toxin in pure state is harmful, hence to inactivate its effect

formaldehyde or aluminum salts are used to neutralize the toxin. This toxin would not produce a strong immune response, therefore, an adjuvant, usually aluminum, is added (80). The vaccine for pertussis may act as an adjuvant for tetanus vaccine, hence the vaccine is administered as a combo dose of Diphtheria Pertussis Tetanus (DPT) vaccine.

A study was conducted to check efficiency of acellular vaccine of pertussis toxin inactivated by hydrogen peroxide (81). Infants were vaccinated with either Diphtheria toxoid (DT) or Tetanus toxoid (TT) or Pertussis toxoid (PT) or as a combined trivalent form. These combinations showed no adverse effects although a slight local inflammation was observed in case of PT when compared to DT (82). Furthermore, the recipients of DPT toxoids diagnosed with pertussis had cough for shorter duration than the DT recipients, while fewer had whooping cough and vomiting (83). This study concluded that an acellular pertussis vaccine is pharmacologically inert, safe and protects against pertussis (84).

3.1 Adjuvants

The term adjuvant means to help; thus, an adjuvant is a material that helps the vaccine to enhance its immune response. In the decades long evolution of vaccines many adjuvants have been in clinical trials but were not accepted because of their high toxicity and side effects such as depot development at the site of administration by mineral compounds or oil-based adjuvants or biodegradable polymers and liposomes (85). Freund's Complete Adjuvant (FCA), Muramyl Dipeptide (MDP), Pertussis Toxin (PT), Monophosphoryl Lipid A (MPL) are the widely used adjuvants as they act like immuno-stimulators (86).

An ideal adjuvant has minimal side effects where local and systemic reactions are minimized, elicits maximal response with less antigen, doesn't cause carcinogenicity, hypersensitivity or teratogenicity. It should be stable and biodegradable as well (86). Also, it should possess physical properties such as high surface area, high pI, and good capacity for adsorption of positively charged proteins (87).

Aluminum hydroxide, aluminum phosphate and alum-precipitated compounds are the most common adjuvants for human use. Protein antigens such as diphtheria and tetanus toxoids can also be purified by using aluminum phosphate and aluminum hydroxide in the presence of anionic compounds like bicarbonates and sulphates (88). Protein antigens can also be purified by using ammonium sulphate, and further purified using ultrafiltration and chromatography. Aluminum hydroxide shows better adsorption for both TT and DT as compared to aluminum phosphate. Studies have also shown that serum

proteins absorb 10-20 times more on aluminum hydroxide. Thus, aluminum hydroxide is a superior adjuvant than aluminum phosphate as it showed similar results for FCA (86). Another method of adsorption is to incubate the positively charged gel and the antigen at optimal pH of 6, stirring it continuously overnight (89). Vaccines using calcium phosphate have also been prepared in a similar manner but a severe Arthus type was observed in case of TT (90).

In a comparative study to check efficiency of adjuvants for TT purified by ammonium sulphate, ultrafiltration and chromatography and adsorbed onto aluminum phosphate or calcium phosphate or stearyl tyrosine, it was observed that aluminum phosphate generated a significantly high titre value of IgG in the first dose, the second highest titre value was followed by calcium phosphate while stearyl tyrosine did not yield impressive titre values. However, in the second dosage the difference in the titre values were negligible for all the three adjuvants used. It should be noted that TT purified by chromatography showed the highest titre values for all the three adjuvants (91).

Another comparative study demonstrated the efficiency of aluminum hydroxide and calcium phosphate for Diphtheria-Tetanus toxoid. The aluminum hydroxide adjuvant expressed higher titre values for both diphtheria and tetanus toxoid compared to calcium phosphate as adjuvant (92). Antibody titers were assessed in pregnant women for vaccine prepared by adsorption of purified tetanus toxoid on calcium phosphate and vaccine prepared by adsorption of purified formalinized tetanus toxoid on aluminum phosphate. It was observed that aluminum phosphate as an adjuvant showed higher titre values compared to calcium phosphate as adjuvant (93). Hence, it can be inferred that alum adjuvants have a higher efficiency when compared to calcium phosphate adjuvant.

A study on school children was conducted to check for adverse reaction for vaccination against diphtheria-tetanus (DiT), either vaccination by aluminum adsorbed DiT or non-adsorbed DiT. This study concluded with results demonstrating that local reactions were frequent in the non-adsorbed DiT vaccine, however, the titre values were slightly higher in the second dosage for the non-adsorbed vaccine (94).

Liposomes made of dioxyethylene cetyl ether, cholesterol and oleic acid, non-phospholipid in nature, are used as adjuvants in TT and DT. Studies have shown that they gave higher response in comparison to FCA aluminum phosphate. TT encapsulated vaccines sustained more antibody levels as compared to TT mixed with liposomes. Encapsulated liposomes

showed higher anamnestic response than aluminum phosphate adsorbed to TT (95).

RTS,S is a fusion of repeat regions of circumsporozite protein (R), T-cell epitope (T) to Hepatitis B surface antigen (S) (96). Malaria vaccine is a formulation of RTS, S with MPL and QS-21 (derived from Quillaja Saponaria) as immunostimulants in either Adjuvant System (AS) 01 or AS02 (97). AS01 constituting a liposomal suspension showed better immunogenicity and efficacy as compared to AS02 which constituted an oil-in-water emulsion (98, 99).

Freund's complete adjuvant is a water-in-oil emulsion with killed mycobacteria in the aqueous phase. This is one of the most potent adjuvants as it confers a delayed type hypersensitivity (DTH) response by directing T lymphocytes to acquire a Th1 pattern (100, 101). FCA causes local reactions which are long lasting, ulcers at the site of injection and toxicity in humans (102). Freund's incomplete adjuvant (FIA) is a water-in-oil emulsion but lacks mycobacteria. FIA is prepared by using paraffin oil with mannide mono-oleate as surfactant. It generates immune response by release of antigen from oil to stimulate innate immunity (103). The toxicity of FIA is less, hence it is fit for human vaccine formulations. Both the adjuvants act by prolonging the life of antigen and stimulating its delivery to immune system (104).

3.2 Micro-Particles

Microspheres can exhibit immunostimulant property, thus act as antigen delivery vehicles (105). Micro-particles are used as adjuvants for prolonged and controlled release of the toxoid, and also reduce the number of booster doses required to the extent of making toxoid vaccine a single dose vaccine (106). Micro-particles are prominently used in the preparation of DT. The diphtheria toxoid is encapsulated in the microparticle using polylactide-co-glycolide polymer using the solvent evaporation technique (107). Various combinations were made with different size of the microparticles and their characteristic release. These were tested on the Sprague rats and the antibody response was monitored and compared to alum immunized control groups for a period of 32 weeks. It was observed that microparticle with single trapped antigen DT, had better response than combination of DT & TT because of presence of antigenic competition among both the antigens resulting in poor antigen presentation (108). It was also observed that microparticles with single polymer were less effective for long term antibody formation where as a combination of vaccine with antigen and alum adsorbed to it gave the best response in formation of

antibodies titre and duration of response (108).

The advantage offered by microparticles in vaccine development is their biodegradable nature, ability to generate potent immune response, and ability to control release of antigens by manipulating their composition, molecular weight and crystallinity of the polymer (109, 110). The variation in size of the microparticle controls the particle uptake by the Antigen Presenting Cell (APC). The smaller microparticle (<10 μ) are taken by the macrophages while the bigger ones are not taken by the macrophages as these particles exhibited stronger adjuvant activity compared to microparticles greater than 10 μ (109, 111).

The microparticles are prepared by solvent evaporation method in which the larger microparticles with DT toxoid are diluted, emulsified using silverson homogenizer and polymer solution in methylene chloride (112). This emulsification is added to distilled water containing polyvinyl alcohol resulting in the formation of a water-oil-water (w/o/w) emulsion. The microparticles were obtained after evaporating the w/o/w emulsion. Smaller microparticles can be prepared by varying polymer concentration and stirring speeds (112).

Biodegradable microspheres is a type of micro-particle made of poly (L-lactic acid) (PLA) or poly (D, L-lactic/glycolic acid) (PLGA) (113). By solvent extraction or solvent evaporation method in multiple emulsion systems biodegradable microsphere for tetanus toxoid was prepared (112, 114, 115). The protein was analyzed at different physical conditions to check for its antigenicity and integrity. A partial loss in antigenicity was observed because of lyophilization and the nature of the organic solvent (116). Varied sizes (ranging from 3kDa-130kD) of PLA and PLGA showed good protein loading efficiency (117). Protein release is influenced by polymer weight and composition. A study demonstrated that large microspheres degraded slowly hence having a low titer value while smaller microparticles exhibited a burst and continuous increasing release rate (117). PLA and PLGA showed constant release pattern and it was observed that the release rate of PLA was lower than that of PLGA (114). The microencapsulated vaccines are more immunogenic as compared to the fluid vaccines which were determined by the IgG levels (113), although the duration and response of antibody did not differ much (118).

Microspheres may be used to carry antigens at the targeted delivery sites for example transfer of TT vaccine in Mucous Associated Lymphoid Tissue (MALT) (119). The microspheres are made of polystyrene, poly (methyl methacrylate),

poly (hydroxybutyrate), poly (DL-lactide), poly(-lactide) with varying ratios of lactide to glycolide. When the drug was orally administered it showed good absorption in the payer's patches in the small intestines. The microspheres coated with ethyl cellulose, acetate hydrogen phthalate or cellulose triacetate showed very less uptake. The tissue penetration is specific and is limited to the diameter of 10 µ. However, microspheres with diameter lesser than 5 µ were transported through the efferent lymphatics within the macrophages while the ones with a greater diameter remained fixed at the payer's patch (120). The effective delivery of microspheres in MALT is associated with their ability to produce secretory IgA. Microspheres are prepared using endotoxin or coumarin-6 dye by using microencapsulation procedures. It was observed that hydrophobic particles are readily phagocytized by the reticular-endothelial system (120).

A study was conducted for passive immunization in pregnant women in their last trimester. They were injected with trivalent inactivated influenza virus vaccine (TIV) or TT. After observing the maternal blood at the time of delivery and the infant blood at 5 weeks and 2 months the antibody responses were studied and T-lymphocyte response was determined by proliferation of lymphocyte cells. It was noted that more than one antibody was found in maternal blood for influenza in all TIV vaccine while in TT it was 9 of 13. High number of IgG antibodies was present in the cord and the infant serum. Significant blastogenic response was seen in influenza A and B in maternal cells as compared to in cord or infant lymphocytes for TIV immunized woman (121). It was observed that infants had higher levels of vaccine specific IgG antibody, however, no transfer of specific T-lymphocyte response or production of neonatal IgM antibody was evident (122–124). The vaccine specific antibodies transferred to infants behaved similar to those that were passed naturally. Their half-life remained similar whether they were result of recent or past immunization or infection. There was no detection of vaccine specific antibody IgM (125).

4. Conjugate Vaccine

A conjugate vaccine is a covalent vaccine that is made by joining a weak antigen to a strong antigen, resulting in an increase in the immunogenicity of the weak antigen. The weak antigen is usually a polysaccharide that is attached to a strong protein antigen. Recently, peptide/protein or protein/protein conjugates have also been developed.

The response of B cell to capsular polysaccharide is T cell independent, implying that the B cells can produce antibodies without the help of T cells.

Normally, polysaccharides cannot be loaded by themselves on MHCs of the Antigen Presenting Cells (APCs) because MHCs bind only peptides, therefore by conjugating a polysaccharide a T cell response can be induced(126). The target polysaccharide antigen is linked to the carrier peptide which is made available to bind the MHCs molecule for activation of T cell. T cells generate a vigorous immune response and a long-lasting immune memory, hence conjugate vaccine is very effective in prevention of invasive bacterial diseases (127).

4.1 Polysaccharide conjugatevaccine

Polysaccharide conjugate vaccine is covalently attached to the carrier protein to provide epitope for T cell independent antigen. The polysaccharide of known molecular size is chemically purified for generation of chemically reactive groups that can form a bond with the carrier protein. The methods used for polysaccharide activation are periodate oxidation of vicinal hydroxyls, and cyanalation of hydroxyls (128). Size of the polysaccharide may be noted after purification as low molecular size impurities result in inefficient conjugation.

Following aspects are considered for conjugation; polysaccharide to protein ratio and the percent nonconjugate saccharide. Yield and conjugate stability play a determining role as well. Usually, less than 20% of the activated polysaccharide becomes conjugated, this yield can be increased by improving the conjugation methods by generation of highly reactive groups (128).

4.2 Carrier protein

Carrier protein bind to the MHCs of APC. The carrier proteins used in conjugate vaccine preparation are genetically modified Cross Reacting Material (CRM) of diphtheria toxin, Tetanus toxoid (TT), meningococcal Outer Membrane Protein Complex (OMPC), Diphtheria toxoid (DT) and Hemophilus influenza protein D (HiD)(126). These carrier proteins are effective in increasing the vaccine immunogenicity but they vary in quantity and avidity of antibody they carry.

CRM197 is a nontoxic variant of diphtheria toxin. A point mutation by glycine substituting glutamic acid at position 52 of the polypeptide sequence results in elimination of enzyme activity and toxicity. CRM 197 has more lysyl side chains available for conjugation and does not require inactivation by formaldehyde. It requires formaldehyde detoxification and is obtained at about 100% purity. The size is about 63kD (129). For example, meningococcal serogroup C vaccine was developed by conjugating it with CRM197 (130).

Diphtheria toxoid (DT) is derived from Corynebacterium diphtheriae, detoxified by

formaldehyde and purified from ammonium sulphate fractionation and distillation. The size is about 63Kd (131). A tetra valent Meningococcal vaccine of serogroups A, C, Y and W135 was conjugated to diphtheria toxin is one such example (130).

Tetanus toxoid (TT) are isolated from clostridium tetani, by detoxification with formalin and purified with ammonium sulphate and filter sterilized before use. It's about 140kD in size (132). Meningococcal serogroup C vaccine can also be conjugated to tetanus toxoid and was the first to be introduced in UK (130, 133, and 134). Meningococcal serogroup A vaccine has been produced by using tetanus toxoid as carrier protein (135) and was observed to be efficient in providing immunization to toddlers and school children (136).

OMPC is isolated from N meningitidis serogroup B strain 11 outer membrane protein complex. It is purified by detergent extraction, ultracentrifugation, defiltration and sterile filtration. The size is about 37kD (137).

HiD is an antigenically conserved surface lipoprotein isolated by solubilization with sonication and sarcosyl-extraction by SDS-PAGE. It is used in a non-acylated active form. The size is 42kD (138).

HiB Polyribosylribitol Phosphate (PRP) conjugate vaccines showed local reactions like redness, pain and swelling in infants. It was observed that the inflammatory reactions were less frequent in children who received the vaccine with carrier proteins D and CRM, as compared to the frequency of the inflammatory reactions for carrier proteins OMP and T after 3 doses of vaccine (127). The first injection showed high rate of irritability, crying and fever in T but not in subsequent vaccines. HiB-OMP showed lymphadenopathy, hypersensitivity, abscess and febrile seizures (126).

The vaccine stability is by changes in the molecular size of polysaccharide and percent free polysaccharide. It has been reported that saccharides of shorter chain lengths are better to develop T-cell dependent antibody responses (139). Oligosaccharide-T (average length 14.5kDa) is a superior immunogen compared to average of 27kDa long chain. But presence of conformational epitopes is an important determinant of the optimal length of the oligosaccharide used in the conjugated vaccine (140).

Pneumococcal conjugate vaccine is a bacterial polysaccharide conjugated with a carrier protein and several valency variants of this vaccine exist. In a placebo-controlled trial in adults of age more than 65 years, the efficacy of 13- valent polysaccharide conjugate vaccine (PCV13) was determined (141) for vaccine type-community acquired pneumonia and

vaccine type-invasive pneumococcal disease (142). It was observed that PCV13 had significant efficacy in prevention of pneumococcal disease in adults over 65 years of age (143). Another study demonstrated that non-conjugated pneumococcal vaccine is not effective in preventing pneumonia (144) the converse was demonstrated in the case of PCV13 against 23-valent pneumococcal polysaccharide vaccine (PPSV23) (145).

In HIV infected adults, streptococcus pneumoniae is the serious infection. The 23 valent polysaccharide vaccine is not effective (146). In a placebo-controlled trial of seven valent conjugate vaccine in patients who were infected with Invasive Pneumococcal Disease (IPD), it was observed that the vaccine protected HIV infected adults from recurrent IPD of vaccine serotype or serotype 6A (147). In young children, Streptococcus pneumoniae causes acute respiratory tract infection which is the major cause of mortality (148). In a study conducted to evaluate the 9 valent pneumococcal conjugate vaccine efficacy it was noted that in children without HIV, the vaccine reduced occurrence of IPD because of serotypes (149). While, in children infected with HIV the efficacy was nearly more than 50%. This vaccine has reduced the occurrence of vaccine serotype and antibiotic resistant invasive pneumococcal disease in children with and without HIV injection (149, 150). Also, multivalent vaccine is effective against IPD and radiological pneumonia (151). In another study the efficacy and immunogenicity of CRM197 pneumococcal conjugate vaccine was studied, heptavalent pneumococcal conjugate was proved to be effective for invasive disease in young children. It also showed to have no increase in disease in non-vaccine serotypes (152). It was also shown to provide immunity to HIV infected adults from recurrent pneumococcal infection (146).

CONCLUSION

Traditional vaccines have been a gold standard in immunizing against a number of infectious diseases over the years. These vaccines have also been improved and improvised to increase their efficacy while reducing the dosage and toxicity. The 21st century has produced various developments for vaccination in the form of recombinant DNA vaccine which provides long-term immunity like LAVs but do not consist an attenuated organism like inactivated vaccines; however, rDNA vaccines are still under preclinical and clinical trials for a majority of diseases. Another development in modern vaccinology is edible vaccines, however, the pre-clinical trial results are not as promising as rDNA vaccines. Nanotechnology has been developed to produce antigen carrying vehicles, the

biocompatibility of the nanoparticles and rate of antigen release from the nanoparticles are the limiting factors of this development.

Conflict of interest

The authors declare no conflict of interest.

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