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Basic aspects of immunomodulation through active immunization

R.H. Meloen

Institute for Animal Science and Health (ID-DLO), P.O. Box 65, NL-8200 AB Lelystad, The Netherlands

Abstract

This paper reviews how immunomodulation through active vaccination has evolved in the past 25 years. Although initially it progressed isolated from the main stream of immunological research and vaccine development, lately it merged with this main stream and is taking full advantage of the newest developments in vaccinology. The first immunomodulation vaccine is already on the market, while various others are close to it. Not in the least because one of the major stumbling blocks of immunomodulation through active vaccination, the inherent low immunogenicity of 'self' antigens, has in a number of other cases been solved. Most progress has been made in veterinary applications and has helped to formulate practical rules, necessary to break immunotolerance. It is not unlikely that these rules will be used to design better immunomodulation vaccines to be used in humans; notably to control fertility or combat tumours.

Keywords: Immunomodulation; Vaccine; Self antigen; Immunotolerance

1. Introduction

In this paper I will focus on active immunization against 'self' molecules, notably hormones, but not on active immunization against 'foreign' molecules derived from, for instance, pathogens. Active vaccination against pathogens is one of the most applied and most cost-effective preventive practices in human and veterinary medicine. One of the outstanding properties of active vaccination is that, to this day, all successful vaccines have been developed without any detailed reference to the knowledge about the immune system. All vaccines that have found their way to the market were solely developed by focusing on their efficacy in the 'target' animal and by detailed analyses of the antigens themselves, in combination with modern biotechnological techniques. This approach has been extremely successful and has led to a whole generation of new vaccines in the past decade. Thus, advancement of vaccine development has become to depend on fundamental knowledge of antigen structure, antigen function and the interaction of antigen with receptor or antibody.

Although an enormous amount of literature is available on both active immunization against foreign and against 'self' antigens, work on 'self' antigens has been largely ignored by those working on active vaccination against 'foreign' antigens. Nonetheless, both sides may learn from each other. 'Anti-self' research may benefit from the experience with 'foreign' antigens, while 'anti-foreign' research may benefit from experience with respect to the active induction of anti-self immunity, which could produce new leads for the understanding of spontaneous auto immune diseases or the development of anti-tumour vaccines.

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2. Active immunization against 'self'

The first efforts to study the effects of active immunization against hormones were done with luteinizing hormone (LH) (Wakabayashi and Tamaoki, 1966; Quadri et al., 1966; Pineda et al., 1968). Subsequently, a vast array of protein-, peptide- and steroid hormones have been tried, including follicle-stimulating hormone (FSH) (Torjesen and Sand, 1975; Wickings and Nieschlag, 1980; Al-Obaidi et al., 1986), human chorionic gonadotrophn (hCG) (Talwar et al., 1976; 1992; 1993), thyroid-stimulating hormone (TSH) (Melmed et al., 1980), inhibin (Henderson et al., 1984; Scanlon et al., 1993), growth hormone (GH) (Beattie et al., 1992), luteinizing hormone-releasing hormone (LHRH) (Fraser and Gunn, 1973; Ladd et al., 1990; Adams and Adams, 1992; Thau, 1992; Hoskinson et al., 1990), vasopressin (Kamoi et al., 1977), somatostatin (Varner et al., 1980), testosterone (Thomson et al., 1985; Hillier et al., 1973), progesterone (Kaushansky et al., 1977) and many others (Ohlson et al., 1981; Yamada et al., 1978; Bettencourt et al., 1993; Ronayne et al., 1990; Skinner et al., 1984; Mettler and Czuppon, 1985; Travis, 1993).

The target of these studies were manifold: they included efforts to control fertility by providing new contraceptives for humans and animals (Lincoln, 1992; Talwar et al., 1992; Tallberg et al., 1985; Reeves et al., 1989), to boost productivity of farm animals, to increase meat quality and ease of animal management (Adams and Adams, 1992; Finnerty et al., 1994; Hoskinson et al., 1990; Reeves et al., 1989; Prendiville et al., 1992; Crowe et al., 1994a, 1994b; Bonneau et al., 1994), to combat disease (Tallberg et al., 1985; Thau, 1992) or to better understand endocrine regulation (Bettencourt et al., 1993; Martin et al., 1986; Ohlson et al., 1981; Safir et al., 1987).

Not unexpectedly, active vaccination against hormones of the fertility axis (i.e., targeting LHRH, LH, FSH, hCG) have been most widely studied for their potential to modulate fertility, not least to provide new contraceptive procedures (Figs. 1 and 2). Later on, these studies were extended to 'self' proteins involved in conception and reproduction, for instance, targetting zona pellucida proteins, sperm antigens, etc. (Lincoln, 1992; Prasad and Rajalakshmi, 1976; Talwar, 1978; Talwar et al., 1993).

Within the avalanche of reports on active immunization against hormones produced in the last 28 years, two hormones of the fertility axis stand out: one is a small peptide hormone, LHRH, the other one is a large protein hormone, hCG. Therefore, aspects of active vaccination against these two hormones will be discussed in this paper.

Vaccination against LHRH has been studied intensively because if LHRH is fully neutralized the fertility axis maybe shut off completely (Fig. 1). In animal production this would allow much desired alternatives to surgical castration of male piglets and bulls (Meloen et al., 1994; Finnerty et al., 1994; Hoskinson et al., 1990; Bonneau et al., 1994). Furthermore, LHRH vaccination would help to bring down costs of extensively farmed heifers that are due to unwanted pregnancies, would resolve management problems of bulls, would form a useful tool to maintain proper ratios of animals in wildlife reserves, and would ease management problems in zoos. Vaccination is probably the only practical way to control fertility of stray animals, notably dogs and foxes. Stray animals form a threat to human health because they form a reservoir of pathogenic microor-

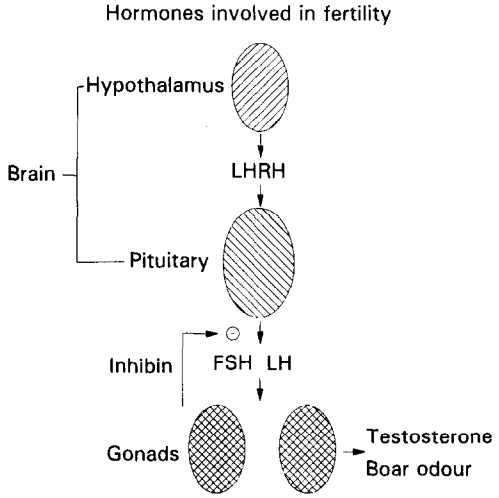

Fig. 1. The hormones of the fertility axis are often targetted for immunomodulation. For example, the induction of a successful immune response against LHRH will completely shut off all reproductive activity. This is a desired goal to prevent the occurrence of boar taint in non-castrated male piglets. Alternatively, the selective neutralization of FSH would stop spermatogenesis but would leave libido intact. This would produce a new contraceptive method for males. Finally, selective inhibition of inhibin has been applied to increase fertility.

ganisms. This problem is not only present in developing countries, but is also on the increase in the cities of developed countries (Carter, 1990).

Vaccination against LHRH is also suggested for use in men suffering from prostate cancer and as a contraceptive, in combination with testosterone supplementation, for men (Thau, 1992; Ladd, 1993). Furthermore, an anti-LHRH vaccine has recently been approved for commercial veterinary use. As such, it forms the first commercial synthetic anti-peptide and anti-hormone vaccine (Hoskinson et al., 1990).

Vaccination against hCG is capable of providing a cheap, long-lasting, easy to apply contraceptive for females, especially in the developing world, because it can be combined with established vaccination procedures. Such contraception is urgently needed if one takes into account that each year the world population increases by 100 million people, 95% of whom are born in developing countries, and each year more than 50 million abortions are performed, of which more than 45% occur under unsafe conditions (Lincoln, 1992).

From studies of active vaccination against hormones, it is posssible to draw general rules with respect to the antigen itself, the form in which it is presented, the vaccine formulation (dose, adjuvant), the vaccination procedure, and the evaluation of the efficacy, mode of action, possible side effects and reversibility.

2.1. The antigen

In contrast to 'foreign' antigens, 'self' antigens are poorly immunogenic when used in a vaccine (Reeves et al., 1989). Thus, all sorts of practical approaches have been taken to overcome this lack of immunogenicity. The major ones are:

(1) coupling of the antigen to a carrier molecule, e.g., bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH) or tetanus toxoid (TT);
(2) changing the native structure of the hormone molecule either by changing its structure (e.g., by denaturation) or by changing its configuration (e.g., by the application of heterodimers or by truncation or dimerization of the molecule);
(3) applying intact molecules from a different species.

2.1.1. Coupling of the antigen

Because small antigens like LHRH (ten amino acids) or vasopressin (nine amino acids) tend to be poorly immunogenic, especially as they are 'self' antigens, such molecules need to be coupled to others to increase their molecular weight and to make them sufficiently 'foreign'. Normally, large protein molecules are used, notably KLH or TT; the latter molecule can even be used for humans. Other protein molecules have been used as well (Hoskinson et al., 1990; Talwar et al., 1990). Although the carrier molecule chosen may affect the efficacy of the antigen in different ways, no particular rules are known except one, i.e., the carrier molecule should be 'foreign' to the target species.

The coupling itself is normally effected by using bifunctional chemical linkers like *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Lerner et al., 1981). In the case of MBS, the linker molecule is first attached to the free amino groups present in protein. Then the MBS-activated protein will link to a free SH group (e.g., from cystein) present in the peptide or protein molecule. Because the cystein, necessary for the coupling, can be introduced at any position within the amino acid sequence when the peptide is synthesized, the chemical link can be hooked up to either end of the peptide or any position in between. For LHRH, the effect of different linkage positions have been studied and marked effects have been observed in the induced immune responses (Goubau et al., 1989a, b; Silversides et al., 1988; Ladd, 1993). However, only one report suggests that the linkage may have a biological effect (Ladd, 1993). Instead of chemical linkage the coupling can be effected genetically using recombinant methods as shown recently for inhibin and ovalbumine (Geary and Reeves, 1994).

2.1.2. Changing the native structure

Denaturation or conformational change may sufficiently alter antigenic sites of 'self' molecules to induce the immune system to respond with the production of antibodies which cross react with the native antigen (Talwar et al., 1976). For small peptide molecules it has been reported that dimerization further increases its immunogenicity. We have obtained very good results applying a tandem repeat of the amino acid sequence of LHRH (Fig. 2; Meloen et al., 1994; Oonk et al., 1993).

For large molecules like hCG or FSH, which contain an α- and a β-subunit, the isolated subunits themselves can be used, e.g., the β-hCG subunit (Talwar et al., 1976; Shahani et al.,1991). Also, 'hetero dimers' or 'hybrid molecules' have been applied, i.e., an artificial

a) Peptides used:
monomer: pEHWSYGLRPG(C)
dimer: pEHWSYGLRPGQHWSYGLRPG(C)

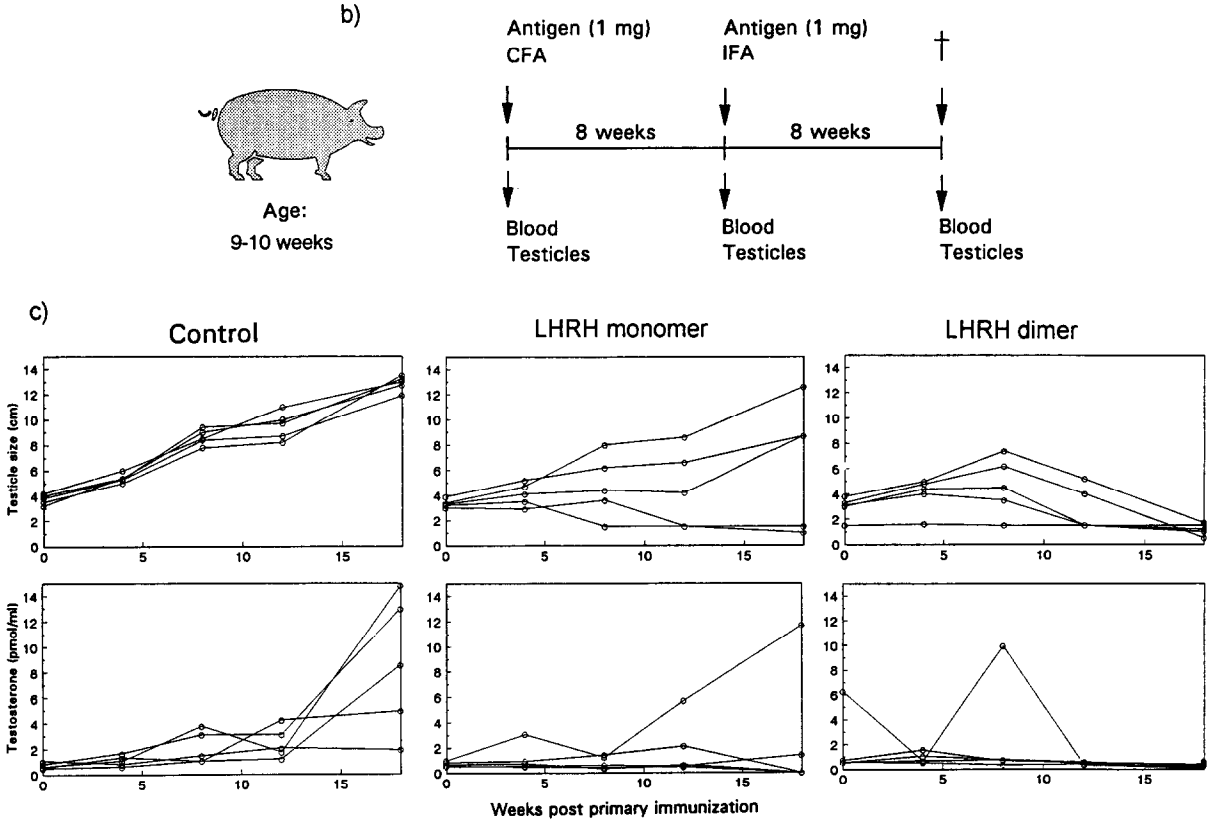

Fig. 2. Typical immunomodulation experiment to block the action of LHRH in pigs, to prevent the occurrence of boar taint. (a) Two antigens are used: the monomer is the native LHRH peptide with an extra Cys added to the C-terminus to facilitate coupling to KLH. The tandem is a tandem repeat of the native amino acid sequence also with an extra C-terminal Cys added to facilitate coupling to KLH (pE is pyro Glutamic acid). The dose indicated is the amount of peptide applied. (b) Vaccination procedure: small piglets are vaccinated twice, the first time at 9–10 weeks of age, the second time 8 weeks later. The pigs are slaughtered when they are approx. 26 weeks old, at the normal slaughter age. (c) The success of the vaccination is indicated by the size of the testicles at slaughter. Testicles weighing less than approx. 100 g (8–9 cm in size) have grossly impaired functions and do not produce androstenone, one of the steroids responsible for boar taint. The control group were sham-vaccinated animals. All animals in the tandem group had very small testicles, in contrast to the monomer group, where also non-affected, or partially affected, testicles were found (Oonk et al., 1993; Meloen et al., 1994).

combination of an α-subunit from one species combined with the β-subunit of another species (Lerario et al., 1978; Talwar et al., 1993). Furthermore, the use of the C-terminal part of the β-subunit appears to be a fairly good inducer of anti-hCG antibodies (Thanavala et al., 1980; Stevens, 1986).

The ultimate approach would be to use only small peptides instead of the intact hormone molecules to induce an immune response which inactivates the biological activity of the intact hormone. This approach is particularly appealing because it allows specific targeting of the immune response and because the production

cost of peptide vaccines will be low. For instance, in the case of FSH it might allow the induction of specific anti β-subunit antibodies which will not cross-react with LH. This would result in cheap peptide vaccines which will specifically neutralize FSH, while leaving LH active. Such vaccines would be the ideal contraceptive vaccine for human males as it is assumed that, if FSH is neutralized, spermatogenesis is blocked while libido is maintained due to the activity of LH. The real problem of this approach is to define the amino acid sequence of the peptide(s) which can be used in anti-FSH vaccines. We ourselves are presently trying to define such epitopes, using advanced and systematic peptide based methods, including PEPSCAN (Grootenhuis et al., 1989; van Amerongen et al., 1994; Westhoff et al., unpublished results). This approach has been used to define useful immunogenic peptides for pathogens including foot-and-mouth disease virus (FMDV), HIV, corona viruses and herpes virus (Meloen and Barteling, 1986; Goudsmit et al., 1988; Middeldorp and Meloen, 1988; Jacobs et al., 1990; Posthumus et al., 1990; Langedijk et al., 1993). It also allowed us to develop the first synthetic peptide vaccine which gives full protection against a viral disease (canine parvovirus) in the target animal itself (Langeveld et al., 1993; 1994a; 1994b).

2.1.3. Applying intact molecules to heterologous species

This approach is used when the amino acid sequence of the hormone differs between species (Faulkner, 1975; Neubauer and Schone, 1978; Melmed et al., 1980).

For example, human FSH in rabbits readily induces anti-FSH antibodies which are specific for human FSH. Amino acid sequences of human FSH and rabbit FSH differ by approx. 15% (Westhoff, personal communication; Rose et al., 1991), which is apparently sufficient to induce antibodies.

3. The presentation form of the antigen

The physical form in which the antigen is presented to the immune system is important for the induced immune response, especially when small antigens are used. From studies with peptides derived from 'foreign' antigens it is known that up to 1000-fold more efficient immune responses are induced if the peptide is presented on nanometer particles (i.e., particles sized between 20 to 100 nm) in a repetitive form. For instance, a peptide derived from FMDV, when coupled to KLH, has to be applied at a dose of approx. 100 μg to obtain a satisfactory response (Bittle et al., 1982). This FMDV peptide can be expressed on the N-terminus of the hepatitis B core protein. This protein spontaneously forms 27 nm particles; each particle exposes the N-terminus carrying the FMDV peptide multiple times in a symmetrical fashion. Only 0.1 μg per dose needs to be applied to induce the same immune response obtained with approx. 100 μg of the KLH-coupled peptide (Clarke et al., 1987). Other examples of highly immunogenic combinations are combinations of peptides and hepatitis B surface antigen (Delpeyroux et al., 1986) and yeast Thy protein (Adams et al., 1987). Most, if not all, small nonenveloped symmetrical viruses (particles between 25 and 100 nm) can be used in similar minute amounts to obtain good responses; this strongly suggests that nanometer particles which carry small antigens in a symmetrical repetitive fashion may form superb immunogens.

Similarly, one would expect that peptides, properly exposed on ISCOMs (immunostimulating particles between 20 and 100 nm which can be engineered to expose antigens in a repetitive manner) would be more effective than carrier protein coupled peptides. However, although rumours of promising results have been around for some time, solid published data are still not available.

Much can also be gained by producing molecules which carry tandem repeats of an antigen. For example, FMDV peptides expressed as a tandem on galactosidase, although less immunogenic than on a nanometer particle, were much more immunogenic than the similarly expressed single peptide (Broekhuijsen et al., 1986). We have shown that when LHRH is synthesized as a tandem repeat, this tandem repeat is far more effective than the native monomer (Fig. 2; Meloen et al., 1994; Oonk et al., 1993). Taking this further, the ultimate molecule one could think of is a single branched synthetic molecule carrying many LHRH molecules (Flegel et al., 1990).

4. Vaccine formulation

Antigens (and especially 'self' antigens) without any adjuvants will only rarely induce a useful immune

response. If they do, such responses are often suboptimal, requiring larger doses and multiple injections. Substantially better results can be obtained by using adjuvants (i.e., immunostimulants). Therefore, antigens are normally mixed or emulsified with an adjuvant. A wide range of substances and formulations are known which improve the induced immune response, but their mode of action is not well known and is subject to much speculation. However, it is agreed that adjuvants provide two major functions. One is the reservoir function: most adjuvants store the antigen and release it slowly into the circulation, thus continuously stimulating the immune system; the other one is loosely defined as producing a local inflammation which activates, in a non specific way, the immune system (for instance induction of cytokines, recruitment of cells, etc.) (Stevens, 1993; Dalsgaard et al., 1990).

One of the least effective adjuvants is aluminum hydroxide gel, onto which antigen is adsorbed. This adjuvant does not cause adverse reactions and is at present the only one accepted for use in humans. On the other hand, the best known adjuvant is Freund's Complete Adjuvant (FCA). It is a mineral oil-based adjuvant (containing cell wall extract of mycobacterium tuberculosis) in which the aqueous antigen solution is emulsified to form a water-in-oil emulsion. It is thought to provide a good reservoir and also to activate the immune system well; however, it often causes unwanted side reactions at the injection site and, in the case of cattle, causes the animal to appear positive when tested for tuberculosis. Therefore, it cannot be used in humans and is only rarely applied in animals on a routine basis. Despite this, it is still used very often in laboratory animals because it forms more or less the 'golden standard' for adjuvants: it provides the upper range of effectiveness of adjuvants, it is well defined, and easy to obtain and use. Thus all adjuvants normally fall in the range between aluminum hydroxide (lower level) and FCA (upper level).

An excellent and concise review has recently been published by Stevens (1993), in which the use of adjuvants is discussed with respect to anti-fertility vaccines. It is claimed that by using TT coupled antigens administered in slow release particles, vaccine efficacies can be obtained which are close to that attainable with FCA and are yet fully acceptable for human use.

5. Vaccination procedure

Ideally the number of vaccinations should be kept at a minimum, preferably one. This has been achieved in the case of a number of anti-disease vaccines for veterinary use and is the ultimate target of the WHO with respect to anti-fertility vaccines (Stevens, 1993).

However, even at best and, in contrast to 'foreign' antigens, vaccines against 'self' need to be applied at least twice and often many more times to obtain full efficacy. This poses another practical problem: while two vaccinations are under certain circumstances still acceptable, more than two vaccinations will seriously preclude general applicability. In the past it was reported once that, after a single vaccination session, full efficacy under laboratory conditions was achieved; however, it required many injection sites at the same time, which is not at all practical (Fraser et al., 1974). However, recently effective one shot vaccines (for GnRH in heifers and bulls and for PGF in heifers) have been developed (W.J. Enright, personal communication).

Furthermore, recent data suggest that one shot vaccines may be possible as well using a mixture of slow release particles to deliver a primary and booster vaccination at the same time. Fast 'dissolving' particles provide the primary antigenic dose while slowly 'dissolving' particles can be timed to release their contents when a booster immunization is required (Stevens, 1993).

6. Evaluation of efficacy

Protection against disease induced by vaccines is still poorly understood. Because the ultimate protection experiments in target animals are often very expensive (i.e., in case of cattle or horses) or virtually impossible (in humans), much effort has been devoted to define parameters which correlate with protection: for example, antibodies which neutralize a virus. Unfortunately, at best such parameters only correlate with protection under very restricted experimental conditions. The same applies to animal models: correlations are sometimes found but they do not negate the requirement for protection assays in the target animal itself.

The determination of the efficacy of immunomodulation vaccines is not an exception to the above. For

instance, immunocastration vaccines based on LHRH may have different efficacies in rats and swine; therefore, immunocastration trials for swine need to be done in swine, although studies are much more laborious than in rats. Another more important and general drawback of immunomodulation through active vaccination is the lack of adequate responses in 'all' animals treated. In the case of immunocastration of male piglets, it was only reported once that 'all' male swine had fully regressed testicles using an LHRH vaccine (Meloen et al., 1994; Fig. 2). Using another LHRH vaccine, only 80% of pregnancies of heifers were prevented (Hoskinson et al., 1990). This pregnancy rate is generally too low because, especially in the area of fertility, 95 to 100% is often mandatory. Another aspect which must be considered is that immunomodulation through active vaccination usually appears to be reversible; i.e., the response disappears after a while (Ladd et al., 1989). Although this is desirable, as in the case of contraceptive vaccines in humans, it also poses another serious problem, because reversibility is likely to vary with each individual and, therefore, is difficult to control.

Finally, vaccination should not be accompanied by unwanted side effects. Especially in the case of vaccines for contraception, much concern has been expressed about the possible occurrence of unwanted side effects. In contrast to a large variety of such concerns (Chard and Howell, 1991; Rose et al., 1991; Berger, 1987; Dirnhofer et al., 1993), actual data in humans are nonexistent, including ongoing trials (Lincoln, 1992), while the scarce data in animals do not support these concerns (Ladd et al., 1989; Upadhyay et al., 1989; Giri et al., 1990).

Nevertheless, animal systems are excellently suited to study side effects. We have been involved in studies on the LHRH neuron in the median eminence in swine vaccinated against LHRH. Sometimes, in LHRH-vaccinated animals nerve endings of LHRH neurons appeared to be truncated. However, in other LHRH-vaccinated animals this effect was not observed. Furthermore, other releasing hormones did not appear to be affected (Molenaar et al., 1993; Oonk, personal communication). Whether or not this effect was transient and has any positive or negative physiological significance remains to be seen. However, Crowe et al. (1994b) found that after Prostaglandin F2α vaccination in heifers, undesired side effects could occur.

On the other hand, a potential 'worst case' trial with respect to side effects was reported; active vaccination against cholesterol resulted in actual lowering of the cholesterol level without any apparent adverse side effects (Travis, 1993). This is surprising because cholesterol is an integral part of all cell membranes.

7. Mode of action

The immune system has evolved to recognise very efficiently 'foreign' antigens while, on the other hand, the same system has learned to ignore 'self'. Thus, trying to vaccinate against 'self' is like trying to make the immune system do something it is not really designed for. Indeed, at best, immune reactions against 'self' are in general far weaker than reactions against 'foreign'.

By trial and error a number of methods have evolved which trick the immune system into mounting a mostly moderate response against 'self'. Such methods rely on alteration of the antigens, application of strong adjuvants, repeated vaccination, etc., as described previously.

Superficially, the mode of action of anti-'self' vaccines appears to be mediated by antibodies. However, some inconsistencies do exist between biological activity in vivo and in vitro measurement of antibody response. For instance, in the case of immunocastration experiments in pigs with LHRH vaccines, LHRH binding activity of antibodies does not correlate well with biological activity in vivo, i.e., sometimes fully regressed testicles occur in the presence of low antibody binding activity, while also the reverse is seen (Meloen et al., unpublished observations). Numerous explanations can be thought of. For example, one could argue that not LHRH binding but rather inactivation of LHRH activity would be the preferred antibody parameter to measure. Similarly, anti-hCG vaccination appears to be highly effective in neutralizing the activity of hCG in vivo, although it was shown that anti-hCG antibodies do not neutralize in vitro the LH activity of hCG (Dirnhofer et al., 1993). Further clues about the mode of action of anti-self vaccines may be learned from data obtained from more basic studies of the induction or breakdown of immune tolerance. This has been well studied because it forms the basis for auto immune disease. In particular auto-immunity

against cells, often via cell membrane exposed proteins, is the focus of intense interest (Zinkernagel et al., 1990; Sinha et al., 1990). Induction of auto immunity against soluble proteins, which appears to be of little relevance for most auto immune diseases, but is of relevance to immunomodulation, has only recently attracted attention (Goodnow et al., 1988; 1989; 1990). These latter studies suggest that anti-'self' antibodies will in general have only average affinities for the native 'self' antigen. Antibodies with high affinities against 'self' may not occur because B-cells that could produce such antibodies are silenced. No critical role has been suggested for the origin of the T-cell epitopes. Apparently they can be 'borrowed' from 'foreign' carrier proteins to which the 'self' antigen is coupled.

Although these data are in accordance with the results obtained by trial and error, they have at present not produced any new leads towards the design of better immunomodulation vaccines. Thus, in general the precise mode of action still needs to be resolved. Resolution of this mode of action may help to design more effective vaccines for immunomodulation by active immunization and may help to improve immuno-therapies based on tumor vaccines (Nossal, 1993).

8. Conclusion

Immunomodulation through active vaccination has come a long way. In the past 25 years it has progressed slowly, isolated from the mainstream of immunological research and vaccine development. Lately, it has merged with this mainstream and is taking advantage of the newest technological developments in vaccinology, involving precise epitope mapping (Beattie et al., 1992; Westhoff et al., unpublished observations), recombinant DNA approaches (Talwar et al., 1993), developments in delivery systems and adjuvant systems (Stevens, 1993), and useful concepts from classical vaccinology (Meloen et al., 1994).

A major stumbling block for successful vaccination against peptides for immunomodulation purposes is the variation in response beween animals, but this appears to be solvable. Also, serious side effects have been predicted but have generally not yet materialized. Indeed, promising, very useful medical and veterinary vaccine applications are in the process of being realized.

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