REVIEW ARTICLE

CONTRIBUTIONS OF BIOTECHNOLOGY TO THE FORMULATION OF VACCINES AGAINST PARASITES OF VETERINARY IMPORTANCE

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SUMMARY: Despite the existence of efficacious parasiticides, parasitic diseases of food animals still cause enormous damage to agriculture and human nutrition. Development of parasite resistance to parasiticides in the last years has aggravated the problem and forced to look for alternatives to the current control. Anti-parasite vaccines appear as a feasible option. Development of an effective anti-parasite vaccine, however, requires identification of the protective antigen(s), its purification, and its production in useful quantities. A rational method for the identification of putative protective antigens have been developed only recently. Purification was undependable until the techniques of affinity chromatography and production of monoclonal antibodies provided the necessary microscale range and monospecific reagents. Judicious use of these techniques permit now to obtain pure molecular species and investigate the amino acid composition of individual epitopes.
Production of useful quantities of the antigen relies on gene cloning. Starting with parasite tissues that produce the desire antigen in abundance, it is possible now to synthesize the corresponding gene, incorporate it into bacteria, and harvest the antigen from the bacterial culture. Because the weak immunogenicity of parasite protective antigens, vaccination must produce stronger immunity than natural infections. New methods of administration of immunogens with immunomodulating agents or as part of replicating viruses or bacteria assure that the antigen will generate a strong and sustained response. Biotechnology is a complex and expensive science but provides research opportunities that were unthinkable a few year ago.

KEY WORDS: antigens biotechnology, parasites, techniques, vaccines.

INTRODUCTION

In the United States alone, veterinary parasitism causes losses equivalent to 9.7% of the total income from the cattle industry, 27.9% of the sheep industry, 4.6% of the swine industry and 4.8% of the poultry industry (BURRIDGE, 1982). Because control of food animal parasitism is quite efficient in this country, it is reasonable to assume that the losses are more dramatic in nations without the economic and technological resources available in the United States. This not only illustrates the enormous damage to economy and human nutrition provoked by animal parasitism but also the limited success of the current procedures of control. The fact that many parasites are becoming resistant to antiparasitic drugs (NOLAN & SCHNITZERLING, 1986; PRICHARD, 1990) adds to the cost and inefficiency of the control. A further reason for alarm is that pharmaceutical companies may be reluctant to spend large sums on the development of new drugs that may exhibit a useful life too short to recover their investment. As a result, we may soon find fewer and less efficacious antiparasitic drugs in our therapeutic arsenal.

It is only natural that, faced with these prospects, the specialists have turned to look for alternatives to the conventional control of parasitism, A modern alternative with ancient roots is the selection of animal breeds genetically resistance to parasites. The first report on such a breed was probably the discovery of trypanosome resistant cattle in West Africa in 1906 (see DOLAN, 1987).

Nowadays, cattle resistant to Boophilus microplus have been bred (UTECH & WHARTON, 1982) and sheep resistant to Haemonchus contortus have been identified (ALBERS et alii, 1987). The mechanisms of resistance remain largely unknown, however, and improvement on immunity often occurs at the expense of production characteristics. Only recently work has began toward the identification and cloning of the genes responsible for resistance. The task is enormous and getting practical results may take decades.

VACCINES AGAINST PARASITES

Another approach rooted in old findings is the development of vaccines against parasites.

Observations of resistance in rats to a second infection with Trypanosoma lewisi and immunization of cattle against T.

brucei were reported around the turn of the century (see TALIAFERRO, 1929). An immense amount of work on immunity to parasitic infections has been done subsequently (see JACKSON, HERMAN & SINGER, 1970; BARRIGA, 1981; COHEN & WARREN, 1982; SOULSBY, 1987).
Working with concepts and techniques that would be regarded as primitive today, dedicated workers succeeded in the 1950's and 1960's in developing schemes against *Eimeria*, *Dictyocaulus*, *Ancylostoma*, or *Babesia* (see STUART, 1960; JONES & NELSON, 1970; MILLER, 1978; CALLOW, 1977). In every case, these were mild infections with attenuated organisms that, nevertheless, protected reasonable well against further infections. Possibly the first effective parasitic vaccine containing selected antigens (and probably the last to be attempted with conventional technology) was that developed against *Taenia ovis* in the 1970's (RICKARD & BELL, 1971). Then biotechnology came to the rescue.

BIOTECHNOLOGY IN IMMUNOPARASITOLOGY

It is difficult to say what biotechnology is. A precise definition is so inclusive as to be almost meaningless (see COOMBS, 1986). In immunoparasitology, biotechnology is generally understood as the use and manipulation of bioregulatory macromolecules (antigens, antibodies, and nucleic acids) to generate products of greater biological value. For example, the identification of antigens by western blot, their purification by high pressure liquid chromatography (HPLC), the generation of their specific monoclonal antibodies, the isolation of their corresponding mRNA, the synthesis of complementary double-stranded DNA, the inclusion of these strands into a plasmid , the incorporation of DNA-coded protein secreted by the cells are all events that belong to what we call biotechnology. Associate techniques such as multiplication of DNA fragments (polymerase chain reaction or PCR), macromolecule sequencing, gene mapping, vector design, and many others are also legitimate parts of biotechnology. Any recent book on techniques of molecular biology or immunology will provide a robust sample of the procedures that biotechnology embraces.

BASIC PROBLEMS IN THE FORMULATION OF VACCINES TO PARASITES

Development of a vaccine against parasites confronts three fundamental practical problems: identification of the protective antigen(s), its purification and its production in useful quantities. Answers to questions such as the mechanism or nature of the resistance are important and desirable but not essential to the success of a vaccine. As a rule, natural parasitic infections are weak inducers of immune resistance. This is because, through evolution, parasites have reduced the immunogenicity of their protective antigens (DAMIAN, 1989) or developed mechanisms to depress the host's immune response (BARRIGA, 1984; WEIKEL & WHELEN, 1986). One of the most obvious mechanisms to reduce the immune response to protective antigens is the release by the parasite of a large number of potent antigens irrelevant to resistance but able to inhibit the response to the weaker relevant antigens. An effective vaccine, then, must increase the weak immunogenicity of the protective antigens and present them free of competing materials. An essential requisite to achieve this goal is the clear identification and subsequent purification of the antigen(s) responsible for the generation of resistance.

IDENTIFICATION OF PUTATIVE PROTECTIVE ANTIGENS

Only recently a rational method for the identification of these antigens has been developed (BARRIGA *et alii*, 1991; BARRIGA & MAJEWSKI, 1993) but it still requires confirmation and assay in other infections. The method is based on the comparison of the antigens recognized by the serum of an infected but non-resistant host with the antigens recognized by the serum of an infected and resistant host. The parasitic materials to which only the resistant host reacts are very likely to contain the protective antigens.

1. THE SERA

Production of infected but non-resistant hosts takes advantage of the weak immunogenicity of the protective antigens. One or a few light infections with the parasite under study usually result in antibody responses to non-protective antigens but not in manifestations of resistance. If one insists with further or heavier infections, there is a moment when manifestations of resistance appear.
Depending on the infection, resistance may be expressed in different ways such as a sudden drop in the yield of the infection, less fertile parasite, smaller parasites, less pathology, etc. Sera obtained during the infection immediately before and immediately after the appearance of manifestations of resistance are the most adequate for comparison. If the antibody titers to parasite antigens are too low at this time, it may be necessary to increase the titer by concentrating the antibodies. This is easily done by purifying the immunoglobulins in a volume of serum and redissolving them in a smaller volume of solvent.

2. THE PARASITE EXTRACT

An important consideration to demonstrate antigens associated with resistance is to make sure that the parasite extract actually contains such antigens. It is possible that the traditional extracts obtained by homogenization of adult parasites do not contain the protective antigens at a detectable concentration. Excretory-secretory extracts obtained by incubation of living parasites in appropriate media may be more suitable in these case. A common problem with excretory-secretory extracts is that the antigens are too diluted. In these case, one can concentrate the whole extract or separate its proteins in overlapping groups (by molecular weight exclusion chromatography, for example) and concentrate each group. Each resulting group is then tested separately. Another important consideration is the parasite stage utilized for the obtention of the extract. Resistance to

Toxocara canis, for example, is expressed against the infective larval stage. Unless one knows that the protective antigens are also present in other stages, the stage affected by immunity must be used to prepare the extracts. Ultimately, the investigator may have to try several possibilities to select the one that is successful. This method of identifying protective antigens obviously does not work with antigens that are not active in natural infections or infestations, as it is the case with the "occult antigens" being assayed for vaccination against ticks (WILLADSEN & KEMP, 1988). Because of the weak immunogenicity of the protective antigens, only the invention of very sensitive techniques for the detection of single antigen-antibody systems permitted the development of the method. Western blotting (RENART, REISER & STARK, 1979) is the technique of election when the number of antigen-antibody systems is moderate but two dimension western blotting (ANDERSON *et alii*, 1982) may be necessary when many different antibodies to parasite materials are present.

PURIFICATION OF THE TARGET ANTIGENS

Once a reduced number of putative protective antigens is identified, the next step consists of purifying each candidate and testing its protective capacity in vivo.

1. CONVENTIONAL TECHNIQUES

The traditional techniques of purification based on non-specific physicochemical properties of the molecules (such as salting-out, molecular sieving, ion-exchange, isoelectric focusing)are adequate with materials available in large quantities which is not the case with antigens of parasites. The introduction of high pressure liquid chromatography (HPLC) permitted to scale down the procedures and put them within the realm of immunoparasitology. Still these techniques are insufficient when dealing with microquantities of specific antigens that are diluted among a large number of contaminants with comparable physicochemical properties.

2. AFFINITY CHROMATOGRAPHY

A further advance was the introduction of affinity chromatography which became popular only in the second half of the 1970's (WIKCHEK & HEXTER, 1976). In this technique, a molecular species is separated from other molecules by adsorption with a substance (ligand) that specifically binds the molecule of interest. For example, an antigen can be separated from other antigens by combination with its specific antibody. In reverse, a specific antibody can be separeted from other antibodies by combination with its specific antigen. There are commercial kits (from Bio-Rad, Richmond, California, and Pharmacia, Uppsala, Sweden, for example) that permit the attachment of virtually any protein to an insoluble substrate so investigators can easily prepare an affinity chromatography column to their own specifications. A major limitation of this technique, however, is that it requires that one of the reagents (antigen or specific antibody) is pure beforehand. The development of the hybridoma technology by KHOLER & MILSTEIN (1975) provided a solution to this problem.

3. HYBRIDOMA TECHNOLOGY

Hybridoma technology consists of fusing an antibody-producing lymphocyte with a myeloma tumor cell. The resulting hybrid cell (hybridoma) shares the ability of the lymphocyte to secrete a specific antibody and the ability of the tumor cell to grow and multiply vigorously in vitro. Because each lymphocyte produces antibodies of a single specificity, a mouse injected with a parasitic extract will develop many antibodiy-producing lymphocytes each secreting antibodies to a single antigenic determinant (or epitope) of a single antigen. Fused to myeloma cells, these lymphocytes will form hybridomas that will continue secreting the same antibodies in a test tube. Through a tedious routine of cell dilutions and culture followed by testing of the antibodies secreted, it is possible to select a population of lymphocytes that produces antibodies to the exact antigen that the investigator desires. Because these antibodies come from a single cell or clone that replicated into identical units, they were called monoclonal antibodies. The monoclonal antibodies are thus the ligand to be used on an affinity chromatography column to purify the corresponding antigen. Laborious and complicated, these techniques, nevertheless, provided the investigator for the first time with the possibility of obtaining a single molecular species of antibody or antigen.

4. DETERMINATION OF EPITOPE COMPOSITION

A patient and dedicated investigator can still go a step further. The most probable amino acid composition of the reactive epitope can be determined by digesting the antigen with different enzymes, testing the binding of the resulting fragments to the corresponding monoclonal antibodies, and investigating the amino acid sequence of the fragments that best fit the antibody. In theory at least, it should be possible to synthesize the epitopes that generate protection to different parasites and put them on a single carrier to create polyvalent vaccines (ROTHBARD, 1992). We still do not understand completely the characteristics that made a carrier a good inducer of immunity but research is being conducted in this area.

Another major problem remained, however. At their best, these techniques generate barely enough materials for laboratory assay but preclude production of the reagents in commercial quantities. As it turned, the solution for industrial production also solved the problem of purification.

PRODUCTION OF TARGET ANTIGENS

Natural production of protective antigens is impossible for practical purpose; 40,000 *Boophilus microplus* ticks (about 1,000 g) were needed to obtain 0,1 mg of a purified antigen which would be barely enough to vaccinate one calf under field conditions (WILLADSEN, MCKENNA & RIDING, 1988). Evidently, a more efficient method to produce the target antigen must be found.

Chemical synthesis is inefficient for proteins above about 50 amino acids or 5,000 molecular weight.
For proteins of the size necessary to be effective antigens (>15,000 molecular weight), the only efficient current method of production is gene cloning.

1. GENE CLONING

The bases of protein synthesis in the cell are simple. Chromosomes are single molecules of DNA formed by a linear arrangement of millions of nucleotides. Three consecutive nucleotides code for an amino acid. The DNA segment that codes for a single protein is called a gene. A gene coding for a medium size protein (40,000 molecular weight) contains some 1,200 nucleotides. When protein synthesis begins, the respective gene is copied (or transcribed) onto a messenger RNA molecule (mRNA). With the assistance of the cell machinery, the mRNA puts together (or translates) the actual protein, one amino acid at a time. The many regulations that operate during this process do not retract form its essential simplicity. Gene cloning consists simply of replicating this process in bacterial cells growing *in vitro*. Recent techniques permit the use of eukaryotic cells instead of bacteria (SAMBROOK, FRITSCH & MANIATIS, 1989).

2. GENE PRODUCTION

Gene cloning begins with the selection of a parasite stage, organ, or tissue that produces large amounts of the desired antigen. This guarantees that the respective gene is active (or expressed) in the selected cells. Identifying the precise fragment of DNA that codes for the desired antigen and extracting it undamaged is virtually impossible so researchers prefer to collect the corresponding mRNA. Because the mRNA is a complementary copy of the gene, one can copy the mRNA in reverse and end up with the original gene. A cell, however, has many different molecules of mRNA (at least one for each protein produced) and it is impossible to select the correct mRNA at this time. For this reason, researchers commonly retrieve all the RNA from the parasites cells and leave the selection of the appropriate molecule for later. Commercial kits and straightforward procedures permit to purify the mRNA molecules and assay their functionality. Once active mRNAs are obtained, one can use these molecules as templates to synthesize the complementary DNAs (cDNA) with the enzyme, reverse transcriptase. Appropriate enzymes are them used to degrade the mRNAs templates (RNase H) and synthesize the second strands of DNA (DNA polymerase I). The genes so constructed must now be inserted into a DNA sequence able to replicate inside a bacterium. Plasmids are commonly used for this.

3. INSERTION OF THE GENE INTO A REPLICATING UNIT

Plasmids are circular minichromosomes of many bacteria. Many plasmids carry natural genes that code for resistance to some antibiotics. As we will see, this has been put to good use in cloning. Many plasmids have been mapped extensively and we have many restriction enzymes that can cut them open at very precise locations along their nucleotide sequence. The selected plasmid is cut open with a specific restriction enzyme so the ends of the cut have a specific nucleotide composition. Short sequences of nucleotides (or linkers) similar to the sequences cut by the restriction enzyme on the plasmid are artificially attached to the ends of synthesized genes and subsequently cut with the same enzyme. This makes the nucleotide sequence at the ends of the genes complementary to the nucleotide sequences at the ends of the cut on the plasmid. Because of this complementarity, the ends of the genes will spontaneously attach to the ends of the cut on the plasmid when mixed together. The plasmid returns to its circular configuration then but this time carrying the parasite gene inserted in it. By fairly simple procedures, the plasmids are inserted into bacterial cells (*Escherichia coli* is a common candidate) and these are grown *in vitro*. The next step is to identify those bacterial cells that carry the plasmid with the inserted gene (recombinant plasmid) and eliminate the others. This is rather simple because one of the criteria for the selection of the plasmid its resistance to some antibiotic. Growing the bacteria with adequate concentration of that antibiotic will kill all the susceptible cells and will leave only the bacteria that carry the recombinant plasmid.

4. SELECTING THE RELEVANT GENE

Because all the parasite mRNA was collected, until this moment the researcher has been working with many genes, only one being the gene that codes for the desired antigen. Now is the moment to select the bacteria that carry the relevant gene and discard the others. A second criteria for the selection of a plasmid is that the plasmid is able to produce (or express) the protein codified by the genes. The problem then becomes simply a matter of identifying which bacteria are producing the desired antigen and separate them form the others. A common way to do this is to plate and grow the bacterial cells under conditions that permit the production (or expression) of proteins. They are then covered wit a nitrocellulose paper to adsorb the bacterial products, and the paper is later treated with the antibody to the desired antigen (as is western blotting). From the position of the antigen-antibody reaction one locates the bacteria producing the antigen and transfer them to a fluid culture medium. An optimum growth may produce up to 40 mg of antigen per liter of culture. Once the bacteria are growing in the fluid medium, the medium is harvested periodically and the antigen purified by any appropriate conventional procedure.

DELIVERY OF THE ANTIGENS

The modest immunogenicity of parasite protective antigens in nature suggests that, to be effective, a vaccine should induce an immunity stronger that the corresponding infection. A number of substances that enhance the specific immune response to antigens have been reported. Traditionally, they are called adjuvants. Adjuvants can act by three mechanisms: improving antigen presentation enhancing antigen targeting, and causing a positive immune modulation. Antigen presentation is how the antigen is offered to the immune cell

(inside a lipid phase or on the surface of microspheres, for example). Antigen targeting refers to effects on the efficiency of the antigen-immune cell encounter (by attracting macrophages or facilitating phagocytosis, for example). Immune modulation includes any positive effect on the efficiency of the lymphoid cell response to the antigen (stimulation of specific subsets of lymphocytes, for example).

1. CLASSIC ADJUVANTS

The oldest and still probable the most common adjuvants in veterinary practice are aluminum salts, first used by GLENNY in 1926, and saponin, first used by RAMON in 1926. Water-in-oil emulsions were used even earlier but the current formulation, the complete or incomplete Freund's adjuvant (with or without the addition of killed *Mycobacterium* cells) was developed in the 1940's.

This has been reserved by laboratory use since it may cause intense inflammation. These and other empirically developed adjuvants act mostly by improving antigen targeting. They cause some degree of local inflammation that attracts macrophages, the antigen absorbed onto adjuvant particles is phagocytized more easily, and adsorption probably protects the antigen from degradation by serum enzymes so it reaches the satellite lymph nodes in larger quantities. They appear to have also some immune modulator effect but it is not well known yet.

2 - ARTIFICIAL ANTIGEN-CARRIERS

The first attempt to manufacture materials specifically designed to improve antigen targeting appears to have been the formulation of antigen-carrying liposomes (ALLISON & GREGORIADIS, 1974).

Liposomes are microvesicles (50 to 1000 nm) formed by a phospholipid film stabilized with cholesterol around a water core. Incorporation of an antigen into the water phase constitutes an economic and effective way to deliver the antigen to the antigen-processing cells (GREGORIADIS, 1990). Chemical manipulation of the antigen-carrying liposomes permit to maintain the antigen inside the vesicle or expose it on its surface which may influence that nature of the immune response. Subsequently, microspheres of hydrophobic proteins or biodegradable polymers (dextrans, polyesters, cyanoacrylates) have been used for the same purposes. ISCOMS (Immune Stimulating Complexes) are small particles composed of antigen, lipids, and Quil A (a purified fraction of saponin) that have been particularly effective for generating mucosal immunity (MOWAT & DONACHIE, 1991).

3. IMMUNOMODULATING SUBSTANCES

Starting in the 1970's, a number of peptides with immunomodulating effect began to be used as adjuvants, notably the synthetic muramyl dipeptide (MDP), N-acetyl-L-alanyl-D isoglutamine, and its analogues. These mimic the action of the peptidoglycan of *Mycobacterium* but do not required a water-in-oil emulsion. The lipopolysaccharide (LPS) of gram-negative bacteria also has a strong immunomodulating effect on B and effector T cells (SEPPALA & MAKELA, 1984).

Great hopes have been placed on the use of cytokines to manipulate immune responses. A number of cytokines (gamma interferon, interleukins, 1,2 and 4) (BLECHA, 1990) are known to have a direct action on the development of the immune response so their use as adjuvants appears justified. Unfortunately, many of these substances are species specific (gamma interferon), others are less effective on heterologous cells (interleukin 1), and all are too expensive nowadays.

4. REPLICATING ANTIGENS

None of theses advances, however, addressed the problem of the inherent short life of the antigen in the vaccinated host. Without a replicating antigen, the immune response will always be transient and bound to disappear with time. It is true that infection with the homologous organism should trigger a secondary response but massive infections may cause irreparable damage before immunity takes over. A replicating vaccine, however, is within the realm of our current technology (WHO, 1990). Apathogenic viruses that are host specific any of moderate distribution in the target species could be inserted with the gene that codes for the parasite antigen. Judicious selection of the place of insertion should allow stable replication of the modified virus in its normal host and optimal expression of the parasite antigen. Once in the host, this *recombinant* virus will produce the parasite antigen along with the other proteins coded by its genome. Infection of the host with this virus will be the equivalent of vaccinating with a replicating antigen. *Recombinant* pox viruses could be used to deliver antigens that require a systemic immune response (ESPOSITO & MURPHY, 1989) whereas *recombinant* adenoviruses or enteroviruses may be used to elicit immunity in the digestive tract (GRAHAM & PREVEC, 1992). Because some pox viruses can receive the DNA equivalent to 10 to 20 genes, administration of replicating polyvalent vaccines should be possible with this method.

Actually, any bacterium or protozoan that is used for vaccination could be modified in order to express proteins from heterologous parasites (DOUGAN, SMITH & HEFFRON, 1989).

CONCLUSIONS

The brief account above shows how the traditional methodology of immunoparasitology has changed toward a very sophisticated technology in only a few years. Many of us are still struggling to understand its intricacies. Because of the power and complexity of biotechnology, we common mortals tend to watch it and its high priests with reverence. But it is a young and naive science that made grandiose claims in the 1970's that it could not maintain. A writer predicted in 1984 that "in 5 to 10 years, Saudi Arabia may look like the wheat fields of Kansas". Actually, the 1989 drought in Kansas brought its fields to resemble the Saudi Arabian desert (BROWN & YOUNG, 1990) As we use this powerful tool, we discover new obstacles and new challenges. Some of theses will be conquered by biotechnology itself but

others will necessitate older and well proven methods. Still an exact knowledge of the biology of the parasite and the epidemiology of the infection are necessary to provide biotechnology with its required foundation. We have acquired the power to do things that were unthinkable only a decade ago. Let's hope we have the wisdom to do the right things.

SUMÁRIO

Apesar da existência de parasiticidas eficazes, as enfermidades parasitárias dos animais para consumo ainda causam enormes prejuízos à agricultura e nutrição humana. O desenvolvimento de resistência aos parasiticidas, nos últimos anos, agravou o problema, forçando a busca de soluções alternativas para os métodos atuais de controle. Vacinas contra parasitas, aparentam ser uma opção possível. O desenvolvimento de uma vacina antiparasitária, requer porém, a identificação do(s) antígeno(s) protetor(es), sua purificação assim como a respectiva produção em quantidades suficientes. Só recentemente foi desenvolvida uma metodologia racional para identificar supostos antígenos. A purificação era inviável até que apareceram técnicas de cromatografia de afinidade e de produção de anticorpos monoclonais permitindo a faixa adequada de microescala e a obtenção de reagentes monoespecíficos. A utilização judiciosa dessas técnicas permite, agora, a obtenção de espécies moleculares puras e a pesquisa da composição de aminoácidos dos epitopos individuais. A produção de quantidades utilizáveis de antígeno depende da clonagem de genes. Começando com o tecido do parasito, que produz o antígeno desejado em abundância, é agora possível sintetizar o gene correspondente, incorporá-lo numa bactéria, e fazer a coleta do antígeno da cultura bacteriana. Devido à baixa imunidade produzida pelos antígenos de parasitas a vacinação deverá provocar uma imunidade maior do que a infecção natural. Novos métodos de administração de imunógenos em conjunto com agentes imunomoduladores, ou como parte de réplica de bactérias ou vírus, assegura que o antígeno irá gerar uma resposta alta e persistente. A biotecnologia é uma ciência cara e complexa, porém permite oportunidade de pesquisas, que seriam inimagináveis há poucos anos.

PALAVRAS CHAVE: antígenos, biotecnologia, parasitas, técnicas, vacinas.

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