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INTERNATIONAL SYMPOSIUM
SYNTHETIC AND GENETIC ENGINEERING VACCINES

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1988

INTERNATIONAL SYMPOSIUM SYNTHETIC AND GENETIC ENGINEERING VACCINES

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INTERNATIONAL SYMPOSIUM SYNTHETIC AND GENETIC ENGINEERING VACCINES

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Mem. Inst. Butantan, 50 (Supl.), 1988

PRESENTATION

As Director of the Instituto Butantan I have the pleasure to open the International Symposium - Synthetic and Genetic Engineering Vaccines.

This is a very gratifying and rewarding occasion. In first place because we consider that a Symposium on a relevant and topical interest, with the participation of such high level speakers is a significant contribution to our program of preparation and training of human resources. Since 1984 our staff in the Instituto Butantan established a successful program, in which human resources constitute the supporting pillar. With this purpose many scientific events, courses, workshops and symposia were organized, including the one we are starting today.

In second place because in this opportunity we initiate formally a program of scientific and technological cooperation with the Weizmann Institute of Israel. As in other programs of scientific cooperation we already established with distinguished institutions in our country and abroad, we intend to strenghten the ties with the Weizmann Institute, a leading and internationally well known institution, for its scientific contributions in several fields of knowledge, with emphasis in medical and biological siences.

In third place, because this Symposium due to its content is very important for the human being and in particular to our people. Brasil is a country of contrasts, a permanent challenge to its scientists. On the one hand its extensive territorial boundaries, its huge natural and mineral resources, great exporter of agricultural and industrial products, the eight world largest economy. On the other hand, underdevelopment, high infantil mortality and morbidity due to several problems as malnutrition, dehydration and measles. The incidence of parasitic and infeccious diseases is high; schistosomiasis affects 12 million brazilians, Chagas disease reaches 8 million, dengue contaminated already 1.5 million, malaria is rising and several other diseases undermine the health and welfare of the population.

Therefore, for public health programs the development of modern biotechnology is very important. It leads to the improvement of existing vaccines as well as development of new ones that can only be foreseen by the use of the new technology of recombinant DNA and synthetic proteins. Through genetic engineering a new world discloses offering a wide range de possibilities for the development of immunobiologicals. These should allow the control of diseases affecting our people, through the use of precise diagnostic tools, and adequate vaccines and serums. The subject of this symposium is extremely opportune for the present moment, specially for the Instituto Butantan, which following the tradition established about a century ago by is founder Vital Brazil, is also today pioneering scientific and technological development regarding public health.

My acknowledgments to all brazilian and foreign participants of this Symposium, I am deslighted to see that this event is being followed with great interest by many investigators, health professionais and by a great number of graduate and postgraduate students. Welcome to everyboody and best wishes of a successful meeting.

WILLY BEÇAK

OPENING LECTURE

To begin with I want to express my gratitude to Dr. Willy Beçak and Dr. Isaias Raw for inviting me to come here. I am deeply honoured and thankful to be present at the inauguration of the First Symposium on Synthetic Vaccines and Vaccines Produced by Genetic Engineering.

This invitation came to me in a very suitable occasion because the subject of the last meeting I organized at the Pontifical Academy of Sciences was the same that will be discussed here. I believe that the initiative taken by the Instituto Butantan and the Weizmann Institute is of extreme importance for it deals with one of the most vivid aspects of modern scientific life, not only for its scientific interest, but also because these vaccines open new possibilities for the prophylaxis of some of the most serious diseases which affect human kind nowadays. The satisfaction of being in São Paulo is very great. São Paulo is, undoubtedly, the scientific capital of Brazil. From here the first pioneers advances were made with strength and courage, for the sake of the "Sanitary redemption of the Brazilians", a sentence I heard from my father many times.

Adolfo Lutz, Emilio Ribas and Vital Brazil, in whose house we are congregated now, were the first pioneers who allowed the creation of the Instituto Oswaldo Cruz, where I was raised and scientifically educated, the Institution where I belong and to which I am tied up with idealism and affection. These were the same feelings of my father and Oswaldo Cruz when they directed it for 17 years, each.

The first Institute for the production of vaccines in Brazil, the Instituto Vacinogenico built by the Baron of Pedro Afonso under the direction and financial support of Pedro II, the Emperor, became the "Instituto Oswaldo Cruz". It was at the Butantan Institute and at the Oswaldo Cruz Institute that the first attempts to introduce the pasteurian age in our country were made.

Research on the vaccine against the yellow fever was mostly done at the end of last century and the beginning of this century. Even today, if we visit the old bookshops, in Rio, we can find many monographies and thesis of great Brazilian scientists of that period. Among them I want to cite Domingos Freire and João Batista de Lacerda, who had vainly attempted to find the causal agent of the yellow fever, the great scourge of the Brazilian population. Their attempt to find the pathogenic agent to produce the vaccine was vain because those scientists were looking for a bacteria and the yellow fever is caused by a virus.

The first vaccines that appeared in our country were the vaccines against rabies, a fact that motivated the Emperor establishing the "Instituto Vacinogenico". It is curious to point out the work of a young man from Minas Gerais, Francisco de Mello Franco, who studied medicine at the University of Coimbra. He was the first scientist to utilize the "jennerian vaccine" against smallpox in a laboratory out of Great Britain.

The meeting that we will attend here represents a unique event in the history of Biology in Brazil. We will see the admirable advances obtained in the production of vaccines and, much more thant this, the understanding of both the structure and the elements that the organism mobilizes against the patogenic aggression.

We will also see how far we are now from the time when, in China, before the Christian age, doctors used to place fragments of crusts - taken from people affected by small-pox - in contact with the skin of healthy people immunizing them against this disease.

If Jenner was the first to immunize against smallpox utilizing a virus similar to the virus of smallpox, but non-patogenic for the man (1976), the great step was taken by Pasteur, with his vaccines of atenuated patogenic strains "chicken cholera", in 1789, the "anthrax", 1881, and "rabies" in 1885, being this last one the first utilized in man.

In 1892, Wright produced a vaccine against "typhoid fever" with atenuated strains.

Following this event, Ramon came up with his sensational discovery which opened a new era for vaccines. Utilizing a fraction of the tetanic bacillus, Ramon

produced an anatoxine with great immunologic power, that modified the panorama of inoculations.

Other vaccines have been produced since then and I was a witness, in the fourties, of the vaccination against yellow fever of 20 million Brazilians, a number only overpassed by the massive vaccination against meningytes, some years ago.

Speaking of vaccines by atenuated virus, we should quote the extraordinary work of Albert Sabin.

The evolution of Modern Biology is observed not only in the field of immunology but also in immuno-genetics, which gives new overtures to medicine.

The works of Macfarlane-Burnett and Peter Medawar have shown the importance of lymphocytes in the cellular immuno-defense.

The recombination of DNA and the knowledge of the molecular aspects of genetics has also opened new fields and has brought to immunology a completely new approach.

The knowledge of the immuno-system has opened new fields for the production of vaccines. Future vaccines may be of different categories and they will certainly be discussed by the great experts who are participating in this Symposium.

Some of the new vaccines are synthetic and I would say that they are a bit delayed in relation to the possibility they may offer. Others are vaccines in which the antibodies provoked by the epitopes, mimethyze the epitopes themselves.

I would also like to mention that under certain aspects we should call "synthetic" the vaccines obtained from the recombination of DNA which, in my opinion, present great perspectives.

Another point to be focused is that the epitopes may be inserted in one antigene or one transported. This will give preventive medicine an enormous possibility because, with a single introduction in the organism of various epitopes we can obtain the immunization against many diseases.

From the vaccines obtained by the recombinant DNA, certainly the one to begin with the strugle is the vaccine against "Hepatites B". Not only for the extension of the problem - and even if vaccines obtained from the plasma of carriers of the virus are available in the mark -, the vaccine thus obtained is necessary to substitute the limitations imposed by shortness of the cited serun. This all indicates that humanity certainly will be free, very soon, from some of the scourges of humanity - AIDS, for example.

A Symposium like this, with its international characteristics, will give strength to the progress in the knowledge of vaccines, not only to our nation but also to ali countries. It will bring extraordinary results. I would only question about the time for these benefits to get to our country - certainly, a question difficult to be answered. Doctor Beçak has said, with strong reasons, of the high investments as well as of the human resources needed to give a conclusive solution to the problem. This is the focal point of the question. In many occasions, however, I have spoken of the "patents" for the "living beings" produced by Genetic Engineering. What has been done and what will be done for vaccines produced by Genetic Engineering must not be property of groups economicaly strong, or political parties, but Genetic Engineering ust be used for the benefit of the whole humanity. This knowledge has to be largely diffused in order hat all people can face the future and use the extraordinary benefits that science and technology can bring.

CARLOS CHAGAS

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PURIFICATION AND PROPERTIES OF GENETICALLY ENGINEERED PROTEIN CONTAINING THE REPEATING SEQUENCE IN THE GLYCOPHORIN-BINDING PROTEIN OF MALARIA MEROZOITE

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SUMMARY: A protein containing the glycophorin-binding sequence, M3R, has been genetically engineered in **E. coli**, and a method of its purification from the bacterial source has been established. The method involves: a) extraction, b) heat treatment at 80° for 3 min, c) concentration of M3R by acid precipitation, d) HPLC on a reverse-phase C8 column, and e) purification by reverse-phase C4 chromatography. The purified protein migrates as a single band of Mw = 22000. M3R has been assayed by the rabbit antibody raised against a synthetic peptide containing a partial sequence of the protein. The overall yield has been approximately 20 mg of pure protein from 100 gm of bacterial paste. Automated sequence analysis has confirmed the purity as well as the identity of the protein as M3R; its sequence of 15 residues from the NH_2-terminus agrees with that predicted from the gene sequence. Structural analyses of its peptide fragments have further confirmed correctness of its sequence. Rabbit antibody prepared against M3R has been found to react with the merozoite of **P. falcipurm** merozoite.

Malaria parasite at its merozoite stage of development contains a glycophorin-binding protein in its envelope. This protein is considered to be responsible for the attachment of merozoite to red blood cells prior to its invasion. The structure of the glycophorin-binding protein is characterized by the presence of 13 repeating sequences of 50 amino acids (1). For the protein to exhibit the glycophorin-binding activity, the presence of at least three tandem repeats has been found necessary.

In order to obtain a potential vaccine for malaria at the merozoite stage, a protein containing three repeats of the 50-amino acid sequence, M3R, has been genetically engineered in E. coli, and its purification has been achieved. For the assay of M3R during purification, a 16-residue peptide containing part of the repeating sequence has been synthesized. The anti-peptide antibody raised in rabbit reacted strongly with M3R in immunoblot assays. The purified protein has been found to contain the correct sequence as predicted from the gene structure. Rabbit antibody prepared against M3R has been found to react with merozoites in culture.

Material and Methods

The gene containing 3 repeating sequence from the glycophorin binding protein had been expressed in **E. coli** by Dr. J. Kochan of Molecular Genetics according to the published procedure (1).

The peptide (NH_2-R-N-A-D-N-K-E-D-L-T-S-A-D-P-E-G-COOH) was synthesized and used to raise antibodies in rabbits. The antiserum was used for the assay of M3R by Western blotting (2).

Amino-terminal sequence determination was done by the automated Edman degradation using a ABI model 479A sequencer.

High Performance Liquid Chromatography (HPLC) were performed on C4, C8 or C18 alkyl-bonded silica with either ethanol or acetonitril gradient as indicated. The solvent gradients were generated by the ChromaTrol Model II gradient maker (Eldex Labs., CA). The column effluent was monitored with a on-line UV monitor.

Results and Discussion

Purification procedure

Extraction: Five hundred grams of cells were suspended in four liters of 50mM ammonium acetate, pH 7.5, 1.0 mM PMSF, 0.05mM EDTA. The cell suspension was stirred at 4°C for one hour, filtered through cheesecloth and then passed through a French Press equipped with a cooling coil. The resulting suspension was centrifuged at 8500 rpm for one hour and the pellet discarded. The supernatant was stored at -80° until further processing.

Heat treatment: Five hundred ml of thawed material in a 3 litter Erlenmeyer flask was rapidly heated to 80°C in a boiling water bath and held there for 3 minutes. The material was rapidly cooled in an ice bath and then centrifuged for 20 min at 8500 rpm. The pellet was discarded.

Acid treatment: The pH of the heat supernatant was lowered to 4.5 by dropwise addition of glacial acetic acid at 4°, then to 3.5 by dropwise addition of 6N HCl. Stirring was continued at 4°C for 30 minutes before it was centrifuged at 10000 rpm for 15 minutes. The supernatant is discarded.

HPLC on C8-alkyl bonded silica: The pellet from above was suspended in 75 ml of 50mM ammonium acetate, pH 6.5 containing 1 M NaCl. The pH was adjusted to 6.5 by the dropwise addition of concentrated NH_4OH at 4°. After stirring for one hour, the sample is centrifuged at 10,000 rpm for 15 min to remove any insoluble material. This was then applied at 2 ml/min. to a C8 column (1.5x30cm., 40-60 u particle size from Separation Industries). The column was washed with 90 ml of 50 mM ammonium acetate, pH 6.5, 1.0M NaCl, then eluted with a ethanol gradient as indicated in Fig. 1.

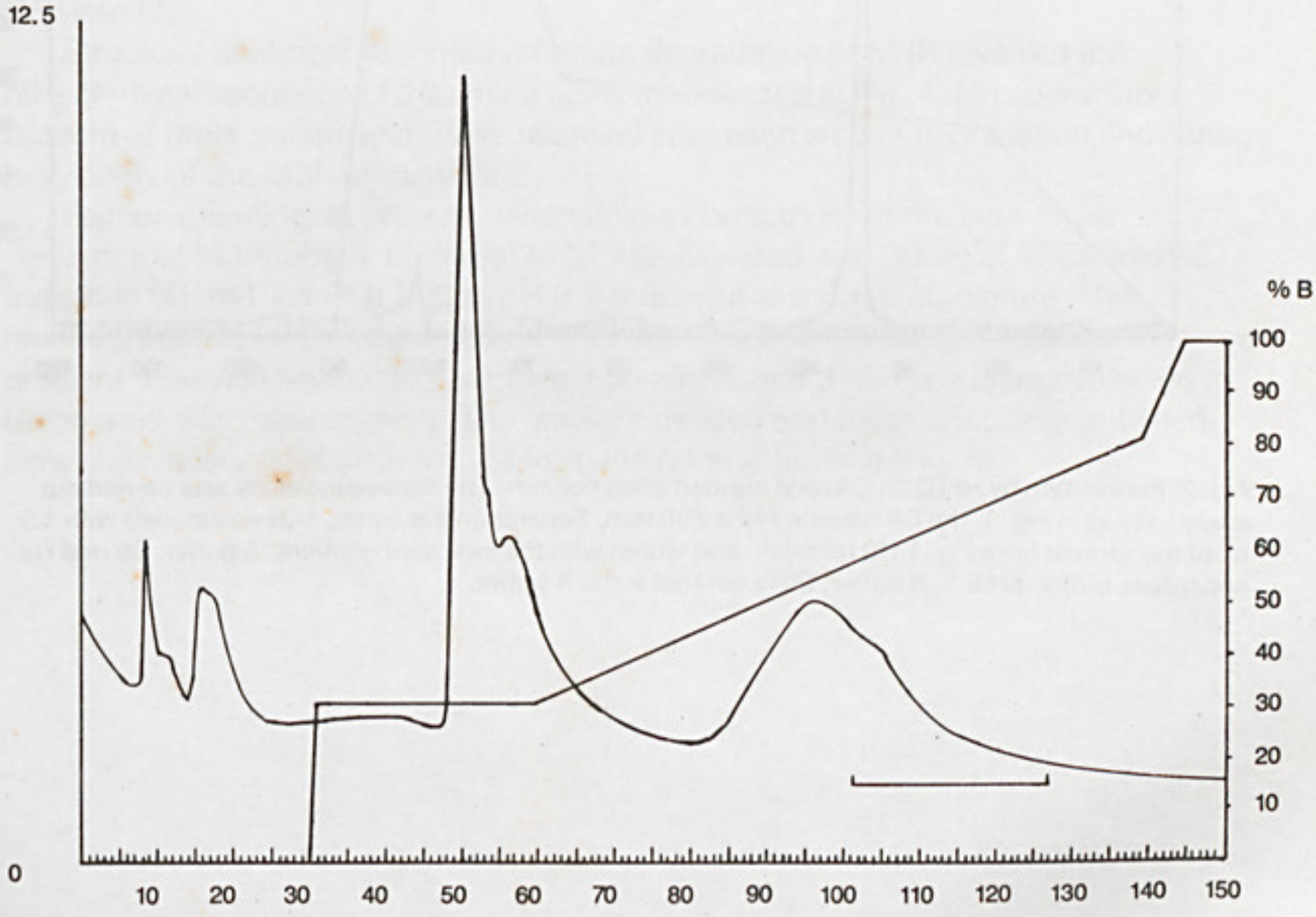

Fig. 1. Chromatography of acid precipitate on C8 alkyl-bonded silica: The sample (80 ml) was pumped into the column (1 x 30 cm) at 2 ml/min, followed by a 90 ml wash with 50 mM ammonium acetate buffer, pH 6.5 containing 1M NaCl. The column was then eluted (O min) with the gradient of ethanol as indicated in the figure. The effluent was monitored for absorbance at 280 nm. The fractions indicated with a bar were combined and purifed on a C4 column (Fig. 2). A buffer: 50 mM ammonium acetate. pH 6.5, B buffer; 100% ethanol.

HPLC on C4-alkyl bonded silica: This was carried out essentially as descrived above except that a prepacked C4 column (Separation Industries) was used. The fractions from C8 HPLC containing M3R by immunoblot assay were combined, flash-evaporated to dryness and dissolved with 10 ml of 50 mM ammonyum acetate buffer, ph 6.5 containing 1 M NaCl. It was then applied to the column as described above. M3R was eluted as a single sharp peak (Fig. 2).

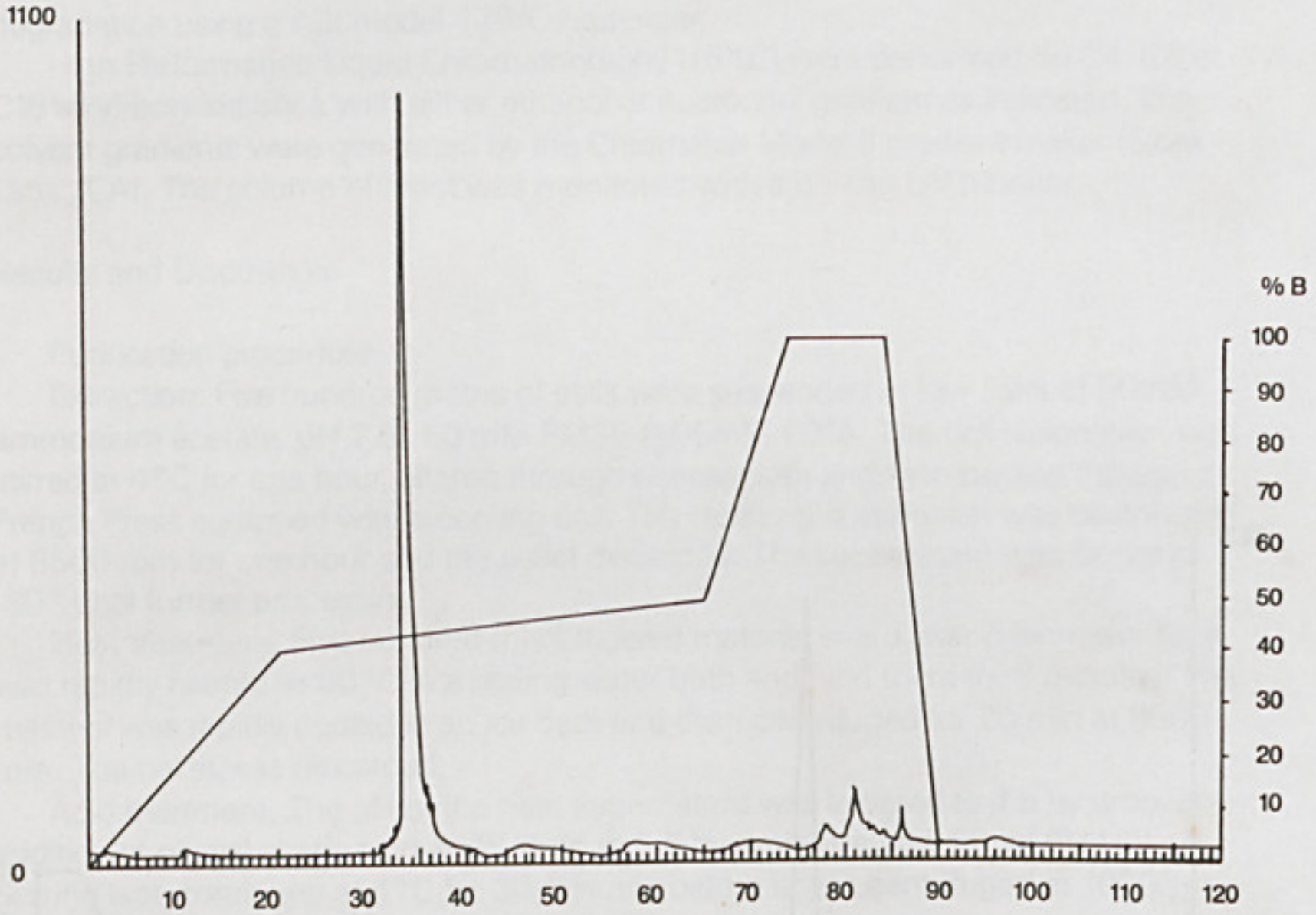

Fig. 2. Purification by HPLC on C4 alkyl bonded silica column: The chromatography was carried out essentially as in Fig. 1 The C4 column (45 x 250 mm, Separation Industries, NJ) was loaded with 1.5 ml of the sample from Fig. 1 (10 ml total), and eluted with the indicated gradient. A buffer: 25 mM Na-phosphate buffer, pH 6.5, B buffer; 80% ethanol in the A buffer.

Results of purification are summarized in Table I. Overall purification of 60 fold has been achieved with 60% recovery from the cell extract.

Table I. Purification of M3R from E. coli extract

	Volume ml	Protein mg	Activity*	Purification fold	Yield %
Extract	115	696	248	1	100
Heat supernatant	100	76	184	7.4	80
pH 3.5 precipitate dissolved	16	61	184	9.1	80
C8 fraction	5	6.3	124	60.	54
C4 fraction	2.4	5.0	99	60.	43

* Activity: After electrophoresis and Western blotting, the blot was reacted with anti-peptide antibody, and M3R bands detected by reaction with ^{125}I-protein A. The radioctive bands were then cut out and counted. 1 unit=10^6 cpm.

Characterization of M3R

SDS-PAGE: Purified M3R showed a single band of M =22000 in a SDS-polyacrylamide electrophoresis (Fig. 3). It reacts strongly with anti-peptide serum on Western blot.

Structural analyses: Automated Edman degradation of M3R revealed the NH_2-terminal sequence of 26 amino acids as indicated in Fig. 4. No appreciable amount of other amino acids were released after each step of degradation, indicating high purity of the M3R preparation.

Peptide mapping: In order to ascertain the correctness of the intra-chain sequence of M3R, about 1.5 mg of M3R was digested with 30 ug of TPCK-treated trypsin in 50 mM Tris-HC1 buffer, pH 8.5 overnight at room temperature. The resulting peptides were separated on a reverse phase HPLC using a acetonitril gradient. Five well separated peaks were obtained, and 3 of these were subjected to automated sequence analysis. The results indicated that these peptides contained intra-chain sequences as predicted from the gene sequence (Fig. 4).

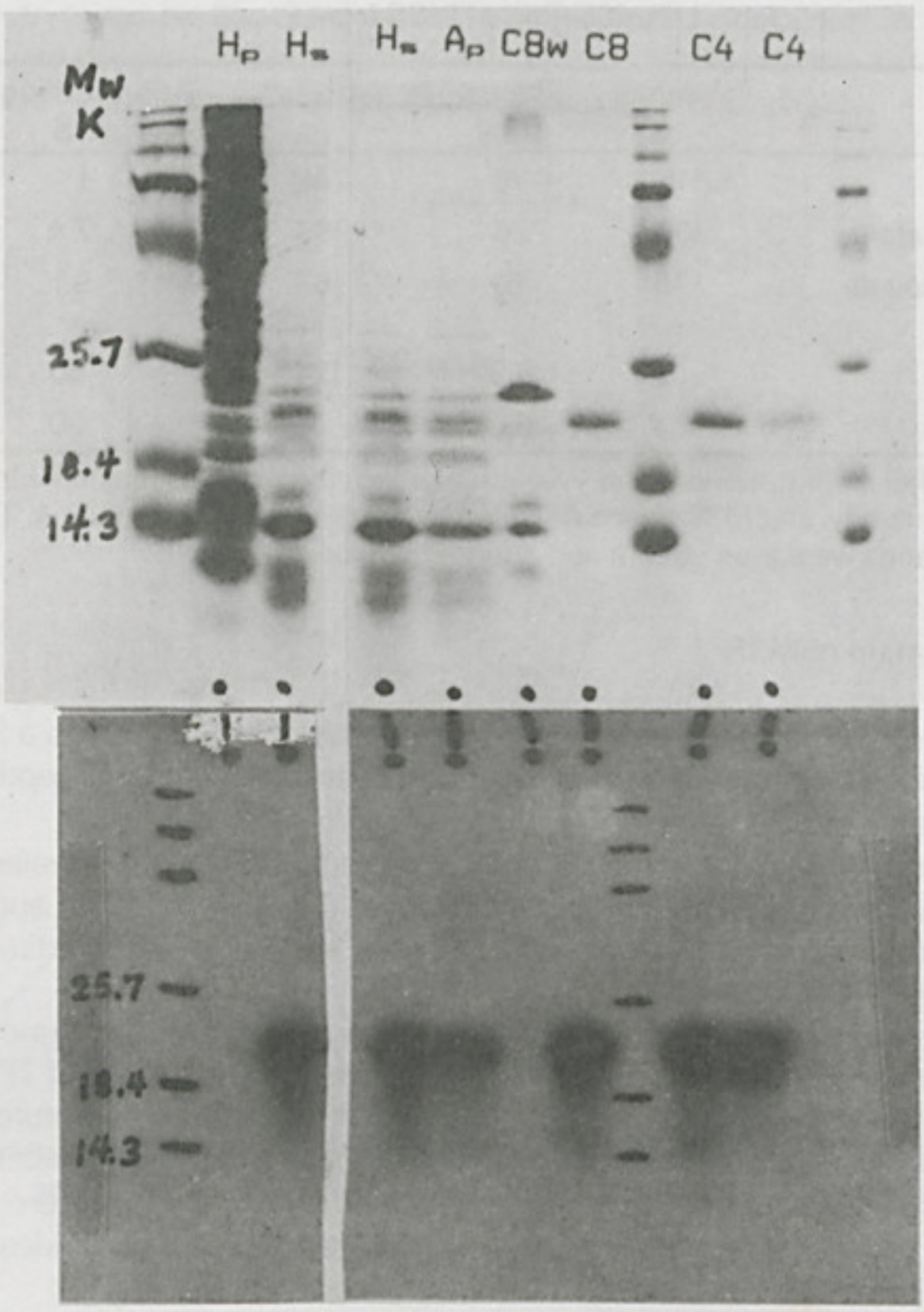

Fig. 3. SDS-Polyacrylamide electrophoresis of fractions from purification steps: A. Gel stained with Coomassie Blue, B. Western blot of the duplicate gel, reacted with anti-peptide antibody and visualized with ^{125}I-Protein A. H_p = Heat precipitate, H_s = Heat supernate, A_p = Acid precipitate, $C8_w$ = Wash after sample application to C8 column, C8 = M3R fraction from C8 column, C4 = Peak from C4 column

AMINO ACID SEQUENCE OF M3R

Met-Asn-Lys-Asn-Ser-Asp-Pro-Leu-Glu-Ser-Phe-Ile-Phe-His-lys-Ile-Leu-Thr-Asn-Thr-Asp-Pro-Asn-Asp-Glu-Val-Glu-Arg-Arg-Asn-Ala-Asp-Asn-Lys-Glu-Asp-Leu-Thr-Ser-Ala-Asp-Pro-Glu-Gly-Gln-Ile-Met-Arg-Glu-Tyr-Ala-Ala-Asp-Pro-Glu-Tyr-Arg-Lys-His-Leu-Glu-Ile-Phe-Tyr-Lys-Ile-Leu-Thr-Asn-Thr-Asp-Pro-Asn-Asp-Glu-Val-Glu-Arg-Arg-Asn-Ala-Asp-Asn-Lys-Glu-Asp-Leu-Thr-Ser-Ala-Asp-Pro-Glu-Gly-Gln-Ile-Met-Arg-Glu-Tyr-Ala-Ser-Asp-Pro-Glu-Tyr-Arg-Lys-His-Leu-Glu-Ile-Phe-Tyr-Lys-Ile-Leu-Thr-Asn-Thr-Asp-Pro-Asn-Asp-Glu-Val-Glu-Arg-Arg-Asn-Ala-Asp-Asn-Lys-Glu-Asp-Leu-Thr-Ser-Ala-Asp-Pro-Glu-Gly-Gln-Ile-Met-Arg-Glu-Tyr-Ala-Ala-Asp-Pro-Glu-Tyr-Arg-Lys-His-Leu-Glu-Ile

Fig. 4. Amino acid sequence of M3R; The structure as predicted from cDNA sequence was confirmed by the automated Edman degradation of purified M3R for 26 residues from the NH_2-terminus, and by sequencing of 3 isolated tryptic peptides (indicated with bars, 1, 2 and 3)

Immunological property

The preparation of M3R was found to be strongly immunogenic to rabbits, and antisera of very high titers were obtained.

Specific binding of the anti-M3R antibody to **P. falciparum**: A drop of a blood culture of P falciparum merozoite was air dried on a microscope slide and fixed in cold acetone. It was then reacted with 100 fold diluted rabbit anti-M3R serum for 20 min at 37°, with or without addition of M3R or the synthetic peptide. After washing with PBS, fluorescein-conjugated goat anti-rabbit IgG was added and the samples incubated for another 20 min. The slides were then washed and viewed under microscope (3).

Rabbit anti-M3R was found to react with all isolates of **P. falciparum**. The reaction was blocked by the addition of M3R, but not by the 16-mer synthetic peptides (data not shown). These results indicated that malaria parasites at merozoite stage contain a protein with the structure analogous or partially identical to that of M3R.

While binding of anti-M3R may not necessarily inactivate the parasites, the results indicate possible use of M3R as a anti-malarial vaccine. Binding of antibody to glycophorin-binding protein on merozoites will likely prevent the parasites from invading the red blood cells.

ACKNOWLEDGEMENT The authors thank Dr. J. Kochan and Dale Mueller for cloning and expressing M3R in E. coli, Jeff Hulmes for peptide sequencing, and Dr. Richard Pink for performing the anti-M3R binding assay on **P. falciparum**. blood cultures.

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DEVELOPMENT OF A SYNTHETIC VACCINE AGAINST MALARIA SPOROZOITES

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Protetive immunity against rodent, simian and human malaria sporozoites is acquired by immunization with irradiated sporozoites (1). Passive transfer of antibodies specific for the repeat domain of the immunodominant surface protein of sporozoite, the curcumsporozoite (CS) protein, confers protection against challenge with viable sporozoite (2). Furthermore, active immunization of mice with synthetic peptides representing the repeated B-cell epitope induces a high degree of protective immunity (3).

To obtain antibody mediated protection high antibody titers are needed. This has been achieved by coupling B-cell epitopes to foreign protein carriers such as tetanus toxoid (3). However, this procedure has serious limitations. First, large quantities of conjugates are required for immunization, which limits its use in man due to carrier toxicity, and second, individuals previously immunized with the carrier protein could have an impaired antibody response to the conjugated B-cell epitope (epitope supression).

A synthetic subunit vaccine would be more efficient if it contained parasite-derived B and T epitopes. This vaccine would prime sporozoite-specific T-helper cells, which would induce a secondary antibody response upon exposure to sporozoites injected by the bite of infected mosquitoes. It would also boost pre-existing anti-sporozoite immunity in individuals living in malaria endemic areas and hopefully also induce cell-mediated mechanisms of protection.

To achieve these goals, it is essential to identify functional T helper epitopes which occur either within the CS protein or in a closely related sporozoite antigen.

By using synthetic peptides representing the CS protein of **P. berghei**, we have identified several T helper epitopes. They are located in the amino terminal as well as carboxi-terminal end. The repeat domain though recognized by antibodies, is not recognized by T cells. Immunization with the synthetic peptide which contains both a T- and a B-cell epitopes, induces antibodies against the repeat sequence. The T epitope sequence is therefore capable of priming T-helper cells, overcoming the unresponsiveness to the repeat sequence.

Finally, we observed that sporozoite immunization primes specific helper T-cells which proliferate *in vitro* with the synthetic peptides representing these T epitopes. This immunization protocol mimics the conditions which will be encountered when applying a vaccine in a malaria-endemic area. Most of individuals to be vaccinated in

such an area would have been primed by the bite of sporozoite infected mosquitoes. The vaccine would, therefore, be expected to induce a secondary, anamnestic response, as we observed in mice.

The **P. berghei** model is uniquely suited for the comparison of the efficacy of different constructs and to determine the best chemical linkage, the optimal type of association of T- and B-cell epitopes and the most favorable molar ratio between these epitopes within the synthetic polymers. In view of the considerable structural and functional similarities of the CS protein of the malaria parasites of rodent and human malaria, the assay of candidate vaccine in the rodent model should provide valuable clues for the preparation of malaria vaccines for human use.

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APPROACHES TOWARDS VACCINATION AGAINST LEISHMANIASIS

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Leishmaniasis is a catch-all name encompassing a spectrum of different human diseases caused by protozoan parasites belonging to the genus **Leishmania**. Though a correlation generally exists between the clinical picture of disease and the species of parasite present, exceptions are not uncommon. Basically, cutaneous, mucocutaneous and visceral leishmaniasis are the three major types of disease encountered. The first disease usually results in a localized ulceration of limited duration at the site of the sandfly vector's bite. Mucocutaneous and visceral leishmaniasis are more serious in nature and rarely spontaneously resolve. Visceral leishmaniasis is generally fatal if untreated (1-3).

Even so, interest in "vaccination" against leishmaniasis has a long history. Throughout the Middle East virulent material from active sores (**L. major** or **L. tropica**) was inoculated into an arm or a leg, thus sparing the person a disfiguring lesion on their face. This was predicated on the folk knowledge that such ulcers are generally mild and frequently resulted in life long protection (4). Modern usage of virulent parasites in a similar, but standardized and controlled manner has been employed in Israel, U.S.S.R. and presently, Iran (5).

While no similar experience exists with visceral leishmaniasis, upwards to 20% of the persons infected with this disease have only transitory asymptomatic infections (1,6). This finding taken together with occasional reports of spontaneous healing, suggests that vaccination against visceral leishmaniasis may be possible by manipulating host immunological responses.

Several stratagems are in use for development of vaccine against leishmaniasis. Obviously a pan-leishmanial vaccine for all the diseases is preferred, and evidence exists in the literature to support cross-protection between different species of the parasite (7). However, a vaccine for cutaneous leishmaniasis is thought to be more easily achieved and most research has been directed to this end. Studies by Howard *et al*. and others (8-11) in a mouse model system with crude parasites as antigen demonstrated that protection can be achieved with a such preparations. Even the highly susceptible Balb/c mice could be protected against **L. major** provided the antigen was administered by intravenous or intraperitoneal route. Subcutaneous

injection of antigen led to the exacerbation of the disease and the induction of T cells which could transfer disease susceptibility to naive animals (8, 9, 12). Resistance was likewise transferable from the protected animals to naive mice by T cells, but not hyperimmune sera (8). Wild populations are unlikely to show a similarly sensitivity to the route by which crude antigen is injected, however vaccine trials in humans with leishmanin, a crude parasite mixture, have not been promising to date (13).

Parasites are complex organisms. They contain a mixture of components with different immunogenic properties and potentials. During one life cycle they pass through several stages of development, each displaying a set of new antigens. Frequently the host may be exposed to more than one stage of the parasite and multiple sets of antigens, antigenic variation. Immunization with the whole organism may lead to undesirable responses, such as immune suppression, sensitization or exacerbation of the disease. One approach to this problem has been the development of defined vaccines which utilize unique parasite antigens appropriately packaged to induce the designed host response, protection or the elimination of pathology. This minimalistic approach attains its ultimate goal in the design of multicomponent vaccines, built up from modules, each containing a separate parasite epitope coding for different T and B cell responses. In the case of leishmaniasis efforts are only now underway to identify such antigens and their appropriate epitopes.

Almost every leishmanial antigen which has been purified so far is under examination as a potential vaccine candidate. Best studied are the parasite surface protease (PSP) and the lipidophosphoglycan (LPG). The PSP is a major surface glycoprotein present on promastigotes of all leishmanial species (15). It is thought to be an acceptor for complement and is involved in the adherence of parasites to macrophages (15). Recently this protein was incorporated into liposomes and shown to retard progression of disease caused by **L. mexicana** in Balb/c mice (16). Several other groups were unable to demonstrate similar properties using alternative adjuvants (Kahl, personal communication), suggesting that antigen presentation may be important for obtaining protection with this antigen. Antibodies against this protein have been described in both human visceral and mucocutaneous leishmaniasis (15), however we have been unable to demonstrate antigen-specific T-cell proliferation to the pure protein in patients with cutaneous leishmaniasis (Jaffe and Passwell, unpublished data). The recent cloning of the gene for this protein should allow the identification of the relevant protective epitopes (16).

LPG and its secreted phosphoglycan form (PG), also known as excreted factor - EF, have been postulated to play important roles in parasite uptake and survival (17-19). Several laboratories have shown that immunization of both susceptible and resistant mice with LPG results in protection. Patients with cutaneous leishmaniasis appear to produce lymphoproliferative responses to this molecule (Jaffe and Passwell, unpublished data). However, disconcertingly, immunization with the PG form of the molecule, which lacks the lipid anchor, caused exacerbation of the disease in resistant mice (20). This may be due to differences in macrophage presentation of the amphiphilic and soluble form of the molecule. This finding that the LPG and PG-forms have antagonistic properties will complicate utilization of LPG as an immunogen. Since PG can be produced as a degradation product from the LPG by cleavage with phospholipase C (21), exacting controls will be necessary to demonstrate that no alipidoglycan is formed during purification or *in vivo*. However, it may be possible circumvent this problem and prepare a suitable immunnogen for vaccination by covalently attaching lipid moeities to PG which are not easily degraded by resident host enzymes. Since it should be possible to produce large quantities of

the secreted PG through fermentation, covalent modification of PG may be a viable alternative to the isolation of LPG from parasites.

Several other parasite components, gp10/20 from **Leishmania mexicana** and dp72 and gp70-2 from **Leishmania donovani**, have been examined. The small molecular weight proteins, gp10/20, only modified the course of the disease when administered in large quantities. Immunization with these glycoproteins resulted in exacerbation of the disease (22).

The two **L. donovani** proteins mention above were originally purified in our laboratory for use as diagnostic reagents, because monoclonal antibodies against them were specifically inhibited by sera from patients with visceral leishmaniasis (23-25). These same monoclonal antibodies, when pooled and given by passive transfer to Balb/c mice reduced **L. donovani** parasitemia upon subsequent challenge. Preliminary experiments using the pure proteins look very promising. Following a challenge with **L. donovani** amastigotes, mice immunized with one of these proteins had parasitemias 70% lower than the controls, which only received adjuvant, or than mice immunized with the second protein (Jaffe, unpublished data). The mechanism of protection is currently under investigation.

Other approaches have also been used to identify candidate antigens for vaccination. Monoclonal antibodies against leishmanial components have been screen for protective activity by passive transfer and Winn type assays in mice. This procedure has identified several parasite antigens for further study. These include antibodies: M2 against a 46kd **L. mexicana** glycoprotein (26); T2 and T3 against multiple **L. major** components (5); D2, D10 and D13 against **L. donovani** proteins (Jaffe and McMahon-Pratt, unpublished data) and several crossreactive antibodies (27,28). Purified p-M2 has proved promising in initial protection assays with mice (McMahon-Pratt, personal communication).

One method which also appears to be rewarding is the dissection, using biochemical techniques, of complex antigenic mixtures which demonstrate protection in animal model systems. Rather than screening purified parasite antigens as an after thought, mixtures already shown to induce protection are scrutinized while systematically separated into their component parts. Such an approach has already unearth several promising leads (19,29).

Finally leishmanial antigens are now beginning to be examined in the light of findings from studies on the immunology of human leishmaniasis. T cell western blots are helping to define the range and nature of cellular responses to defined antigens during the course of disease (30). Understanding human immunological responses to the parasite and to purified parasite antigens will be important in bringing a vaccination for leishmaniasis to fruition.

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MOLECULAR BIOLOGY OF SCHISTOSOME - A STEP TOWARDS RECOMBINANT VACCINES

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The *objectives* of our research are to study the regulation of gene expression at different stages of the life cycle of schistosome, to understand the molecular basis of host-parasite relationship, and use this information to develop protective vaccines against Bilharzia.

cDNA libraries of cercaria and worm **(S.mansoni)**, constructed in λ gt11 were screened with antibody probes revealing stage specific gene expression (precipitating many proteins from worm mRNA translation products and a few proteins from cercaria, or vice-versa). Several stage specific clones were isolated. Two clones encoding the homologues of a heat-shock protein (HSP70) and a calcium-binding protein (CaBP) are decribed below.

The HSP70 cDNA (1.05 Kb long) was cloned from a worm library and its nucleotide sequence determined. The predicted amino acid sequence shows 76% homology with the carboxyl-terminal portion of human HSP70. Quantitative Southern blots revealed about three gene copies per haploid genome. From nuclear DNA libraries construted in EMBL4 we isolated genomic clones, some of which have two HSP70 related genes, in complete agreement with the gene dosage estimate. Northen blots showed selective expression of HSP70 in worm mRNA but not in cercaria mRNA. Newport and colleagues recently reported that with anti-HSP70 antibodies they detected the HSP70 protein in both worm and cercaria. This apparent inconsistency was resolved by looking into the hepatopancreas of infected snails. We found that sporocysts synthesize HSP70 mRNA, but the level of expression probably depends on their maturation stage. It seems that high expression occurs in embryos and it decreases to nearly zero levels in mature cercaria. It is conceivable that the HSP70 protein in the free swimming cercaria was programmed by mRNA in the sporocyst, and the protein has a longer life time than the mRNA. These experiments raise several important topics. 1) The fact that snail maintenance and shedding of cercaria (triggered by light) occur at the same room temperature indicate that at least one of the HSP70 genes is developmentally regulated. 2) Schistosome exhibit a complex pattern of HSP70 gene expression: High in sporocyst, practically zero in cercaria and again high in worm. 3) One HSP70 gene may be developmentally regulated during sporocyst-cercaria transformation (actually it may regulate this process via HnRNA splicing), and another gene may be triggered by heat during cercaria-worm transformation.

The putative CaBP cDNA (0.36 Kb long) was cloned from a cercaria library, and we determined its nucleotide and derived amino acid sequences. Northern blots

showed selective expression in cercaria but not in sporocyst or worm. To understand the nature of the encoded protein we used computer programs that brought up significant (but not striking) homology (~ 30%) to members of the calcium binding protein family. However, further analyses revealed clear resemblance to the domain structure and organization of CaBP molecules: 1) The schistosome CaBP contains two calcium binding loops of correct size (12 residues) and composition (six residues carry side chain oxygen to bind the calcium ion), 2) The distance between the loops (24 residues apart) is identical to the spacer length conserved in CaBP molecules. In addition, the schistosome CaBP programmed by mRNA shows Ca^{++} — dependent electrophoritic mobility (increased with Ca^{++} —ions and decreased with EGTA), like other CaBP molecules.

The CaBP gene was cloned and sequenced. The gene and cDNA sequences were compared and primer extension experiments were performed. The results established that the cDNA contains the complete coding sequence (69 codons), a portion of the 5' untranslated region (34 nucleotides upstream to the initiator-Met codon), the entire 3' untranslated region (56 nucleotides), and the poly-(A) tail. The structure of the gene is similar to that of eukaryotes. The promotor is composed of CAAT and TATA boxes as well as a cap site; one short intron (91 nucleotides long) interrupts the coding sequence. To our knowledge this is the first cDNA with defined 5' terminus and complete gene structure determined for helminth parasites. The CaBP is interesting because: 1) Most of the metabolic (activation of regulatory enzymes like Kinases) and physiological (contraction, secretion, etc) events triggered by calcium ions are mediated via CaBP molecules, 2) The preferential expression of this CaBP in cercaria raises questions as to what function(s) specific to cercaria it regulates, and whether all or only a few cells express the CaBP molecule.

A general issue revealed by the CaBP is the rapid change in gene expression during schistosome metamorphosis. We found that the CaBP mRNA is missing in infected hepatopancreas just prior to shedding, but is readily detected in cercaria at one hour after shedding. Other genes which are turned on (like the CaBP) or shut off within the short time interval (~1hr) of transition from snail to free swimming cercaria were identified. This system is likely to provide information on the mechanism of stage - specific gene activation/inactivation during the life cycle of the parasite.

Clone 21LT isolated from a worm cDNA library annealed with the mRNA of sporocyst, cercaria and worm. The unique feature of this clone is that it also hybridized with *normal* snail mRNA. Thus, the 21LT cDNA is a potential candidate for studying molecular mimicry at the nucleotide and protein levels. So far biological mimicry between snail and schistosome is known for shared sugar epitopes defined by serology. This system can provide relevant information on the molecular basis of host-parasite relationship.

Surface antigens of the schistosome have been studied extensively to develop protective vaccines against Bilharzia. Recently it was shown that internal and excreted proteins of the parasite can elicite immunity levels comparable to those achieved by surface antigens. We isolated several clones encoding internal antigens differentially expressed in cercaria or worm. We plan to evaluate the immune-protection conferred by these proteins when administered alone or in a mixture. Immunity directed against both the invasive stage and the adult worm may be advantageous, and a mixture of antigens may provoke higher protection than each antigen alone.

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PERSPECTIVES OF GENETIC ENGINEERING VACCINES AGAINST FOOT-AND-MOUTH DISEASE

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of wild and domestic cloven-hooved animals. It is an acute disease characterized by vesicular lesions which lead to significant productivity losses as well as to indirect economic losses due to embargoes on trading.

FMD virus (FMDV) is an aphthovirus belonging to the family **picornaviridae**. The virion is icosahedral, without envelope, of about 25 nm of diameter and consists of 60 copies each of four coat proteins VP1, VP2, VP3 and VP4, one or two copies of VP0, the uncleaved precursor of VP2 and VP4 and 3D (the viral RNA polymerase). The viral structural polypeptides VP1, VP2 and VP3 are arranged in 12 pentameric subunits each of which forms one of the 12 vertices of the icosahedron. The intact virion sediments at 145S. By lowering the pH to 6,5 or heating at 45°C the virion dissociates into 12S particles which consist of five copies each of VP1, VP2 and VP3 and an aggregate of VP4 molecules. The 12S particles have low immunogenicity, approximately 1% compared to that of the entire viral particle. VP1 is the only structural polypeptide that purified and injected into cattle is capable of inducing neutralizing antibodies (1, 2, 3) and is located at the apices of the virion. VP4 is internally located. The viral genome consists of a single-stranded RNA of approximately 8000 nucleotides.

The viral RNA is infectious and serves as mRNA. Upon translation a polyprotein is produced which is subsequently cleaved into a series of intermediate precursors which are further processed to give the mature non-capsid and capsid viral proteins (VP).

Methods used to control FMD vary with the individual country. In FMD - free countries (Australia, the United States, Japan, etc.) control occurs by slaughtering of infected or exposed animals as well as by having strict restrictions on importation of animals and animal products from countries where FMD is still a problem. In countries where the disease is endemic regular vaccination programs take place. Control of the disease is highly dependent upon strict quality control of the vaccines

and good campaigns which require a deep understanding of the epidemiological situation of the region.

Overall considerations of FMD vaccines

It is evident that effective vaccines are highly dependent upon the quality of the antigens, inactivant, adjuvants and adequate handling of the antigens during production and distribution. In addition, knowledge of biochemical, antigenic and immunogenic properties of the virus are essential aspects which should be taken into consideration for designing an appropriate vaccine. Several techniques for the analysis of nucleic acids and proteins are being increasingly used for studying biochemical properties. Such techniques include nucleic acid analysis through RNA fingerprinting and RNA sequencing as well as viral protein analysis through SDS-polyacrylamide gel electrophoresis (PAGE), isoelectrofocusing, two-dimensional gel electrophoresis, tryptic peptide analysis, etc. These biochemical methods together with recently developed serological techniques (RIA and ELISA) and the development of monoclonal antibodies provide ideal tools for the precise biochemical and antigenic characterization of active, evolving, vaccine and laboratory strains.

Crucial for an adequate vaccine design is also the understanding of viral biological properties such as variability, persistence and host range, lability of virions, etc., enabled by the application of the above mentioned methods together with recently developed DNA recombinant techniques.

Antigenic variation

Vaccination is complicated by the occurrence of the virus in 7 serotypes: the European types O. A and C, also present in South America, the South African Territorie types Sat 1, Sat 2 and Sat 3 and the Asiatic type Asia 1. In addition over 60 known subtypes resulted from variation within each serotype, with little cross-reactivity among them.

Immunization against one subtype may not protect against another one. Antigenic evolution while the viruses are replicating may frequently give rise to new subtypes and is one of the most important causes contributing to the spread of the disease in endemic regions. Therefore in order to assure efficient vaccination campaigns it is essential to monitor emerging strains and to assess the degree of similarity between the viral vaccine and the strains circulating. In Argentina during the last 3 years, 2 viral strains were replaced in the vaccine, the strains C Argentina 84 and C Argentina 85 replaced the strain C_3 Resende (4) and the strain A Argentina 87 replaced the strain A Argentina 81. We are intensively involved in the biochemical and serological characterization of field and vaccine strains. Figure 1 shows the significant differences of the selected vaccine strain A Argentina 87 when compared to the previously used vaccine strain A Argentina 81.

Persistence

After infection or vaccination with live attenuated strains, FMDV is known to persist in oesophageal-pharyngeal regions of cattle and other ruminants for time periods of up to several years without signs of the disease. During this time, rapid evolution of viruses was found (5). We recently described a decreased reactivity of viruses persisting within the first 63 days post-infection to a set of neutralizing monoclonal antibodies (6). This demonstrates the high risk for the animal and for those susceptible hosts in the surroundings.

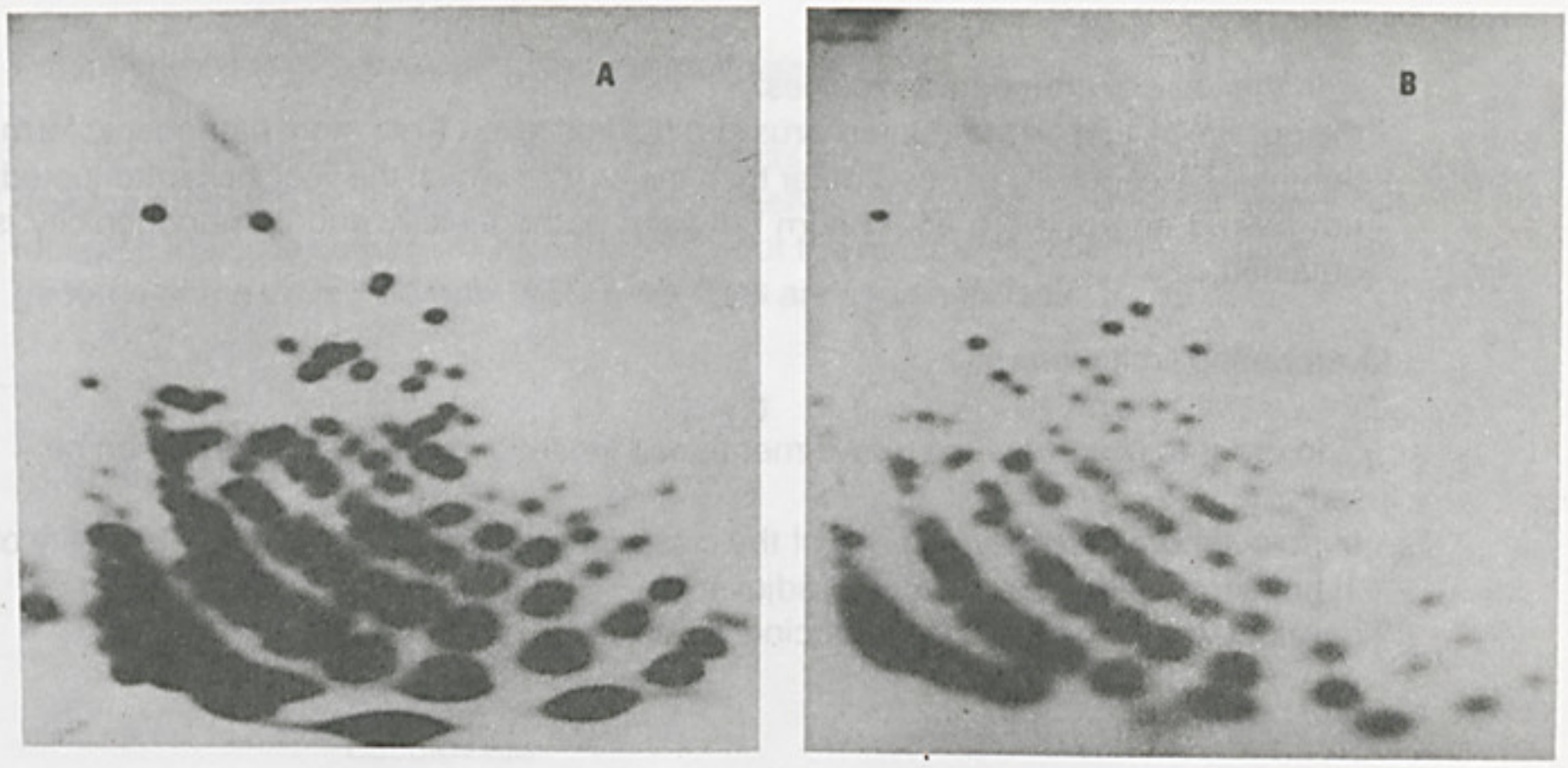

Fig. 1 - RNAse T_1 Two-D maps (Fingerprints) of (A) FMDV type A strain Argentina/81 and (B) FMDV type A strain Castellano-Argentina/87

Host range

The host-virus complexity of FMDV with more than 30 natural hosts allows the selection of viral populations with varying genetic and antigenic properties or with altered pathogenicity. This fact constitutes a risk when attenuated vaccines are used, since attenuation for one species (e.g., cattle) does not assure attenuation for others such as pigs.

Current FMD vaccine designs

At present, chemically inactivated whole-virus vaccines and to a much lesser extend live attenuated vaccines, obtained through serial passages of the virulent strains in non-susceptible hosts, are used for the control of FMD.

While the overall success of these vaccines in controlling the disease is widely recognized, there are a number of considerations which make it desirable to improve them.

In the case of killed vaccines:

- many viruses are difficult to grow in sufficient quantities to ensure enough antigenic mass per dose for effective immunization;
- handling large volumes of infected cultures constitutes a biological security risk;
- requirement for biological containment facilities;
- risk of incomplete inactivation (only a fraction of the production is tested for inocuity);
- risk of escape of viruses (in this regard, it has been demonstrated that in recent years some FMD outbreaks in Central Europe occurred near vaccine production units) (7);
- requirement of refrigeration during storage and distribution, especially in tropical countries;
- alterations of the antigenic structure due to inactivants;
- requirement for several periodical vaccinations;
- eventual bacterial contamination could degrade the antigen through proteolytic enzymes.

In the case of attenuated vaccines:
- the possibility that an attenuated virus strain could revert to its more pathogenic form.
- potential susceptibility of hosts other than the one for which the vaccine is attenuated;
- not always an optimum equilibrium between pathogenicity and immunogenicity is obtained;

Overcoming limitations

In order to overcome the above mentioned limitations, two approaches can be used:
- try to overcome some limitations of the classical vaccines by improving the quality of 1) antigens, 2) inactivants and 3) adjuvants.
- developing new generation of vaccines.

Improving classical vaccines

Antigens

In the case of FMDV, the quality of the antigen is highly dependent upon the conditions used to grow the virus.

In the last years, methods of tissue culture have advanced considerably which improved the industrial production of antigens. Vaccines have been produced successfully and without the need to concentrate the antigen from pig kidney cells growing on microcarriers (8). Spier and Whiteside (9) showed that the production of FMDV type O_1 from BHK cells grown on microcarriers gave higher infectivity and complement-fixing activity than suspension cultures.

However in all these cases, large volumes still had to be manipulated. Alternative methods of high density cell cultures need to be studied, involving standard perfused microcarriers (glass beads, gelatine, biosilon, polyacrylamide, cytodex I, II or III) ceramic matrix, microencapsulation, hollow fibers, collagen spongue, etc. in which densities varying from 10^7-10^9 cells/ml could be reached.

Inactivants

An improvement in vaccine production came with the introduction of virus inactivation by means of aziridine derivatives.

Formaldehyde has been the traditional agent used for the inactivation of virus infectivity and is still widely used. However, formaldehyde inactivation does not follow first order kinetcs and failures have occurred. The introduction of aziridine derivatives, in particular BEI (10, 11) together with more effective safety tests, have given great confidence in the safe application of these vaccines.

Another important alternative is the use of an enzymatic approach consisting on the activation of a FMD virion-associated ribonuclease at alkaline pH in the presence of monovalent ions such as K+, Cs+ or NH_4+. A first order kinetic of inactivation was obtained with maximal preservation of the antigen (12)

Adjuvants

Great interest is now focused on the optimal conditions to present the antigen to the immune systems. This is important not only for improvement of classical vaccines but also for the successful development of new vaccines. In the case of FMD, we showed that replacement of the classical hydroxide-saponin adjuvant by oil adjuvant gave longer lasting and enhanced immunity in young and adult cattle and swine (13, 14).

Prospectives for the development of new vaccines

During this decade, one of the prime objects in research on FMD has been to develop methods for the production of the essential immunizing components without recourse to tissue culture methods and without the need to handle the infective genome of the virus. Potential vaccine designs are shown in Table 1.

Table 1

I. **Subunit vaccines**
  a) Expression of VP1 in:
    Bacteria
    Eukaryotic systems:
    Yeast
    Poxvirus
    Baculovirus
  b) Synthetic peptides
II. **Attenuation** by direct gene manipulation
III. **Anti-idiotypes**
IV. **Antisense:** Virus resistant cells
V. **Complementation** of a virus defficient strain in cells constitutivelly producing the defficient protein
VI. **Anti-cell receptor:** antiviral

We have pursued approaches I and II and the rest of this work will be separated into two parts: one describing the potential of subunit vaccines and the other part describing the potential of attenuated vaccines.

I. Subunit vaccines

This approach became possible when some structural features necessary for eliciting a good immune response were identified:

- The neutralizing activity is largely confined to VP1; VP1 isolated and used as a vaccine elicits neutralizing antibody responses and protects cattle and swine from infections (1, 2).
- The neutralizing activity was generated by fragments obtained by cyanogen bromide or enzymatically spanning the regions corresponding to amino acid residues 145-154 and 201-213 (15).

a) Expression of VP1 in bacteria

The structural gene coding for VP1 of the prototype strain C_3 Resende was isolated and expressed in two different expression systems:

- the pPLc24 system which carries the pL promoter of phage lambda, inducible by temperature in cells containing a temperature sensitive mutant repressor protein (16);
- the pUR expression system consisting of a set of 3 vectors carrying the lac promoter induced by IPTG (17).

A significant level of expression was obtained after induction of both of these systems. Figure 2^A shows the expression of the fusion protein at different times post-induction in the pUR system. The induction of a 145 Kd VP1- β galactosidase fusion protein can be clearly observed and distinguished from the 116 Kd β galactosidase protein. The β galactosidase - VP1 nature of the induced protein was further confirmed by its reactivity with antibody against virus on a Western blot, figure 2^B. Part C of the same figure corresponds to the preparative gel used to establish whether the fusion protein is able to elicit an antibody response. Cattle were

immunized with partially purified preparations of the fusion protein. The vaccine was prepared by pulverizing a slice of the polyacrylamide gel containing the protein and emulsifying in complete Freund's adjuvant. The vaccine was given subcutaneously to 18 bovines on days 1 and 30 up to 500 ug were inoculated. Serum samples were taken every 7 days. No significant neutralizing antibody response was detected up to 30 days post revaccination.

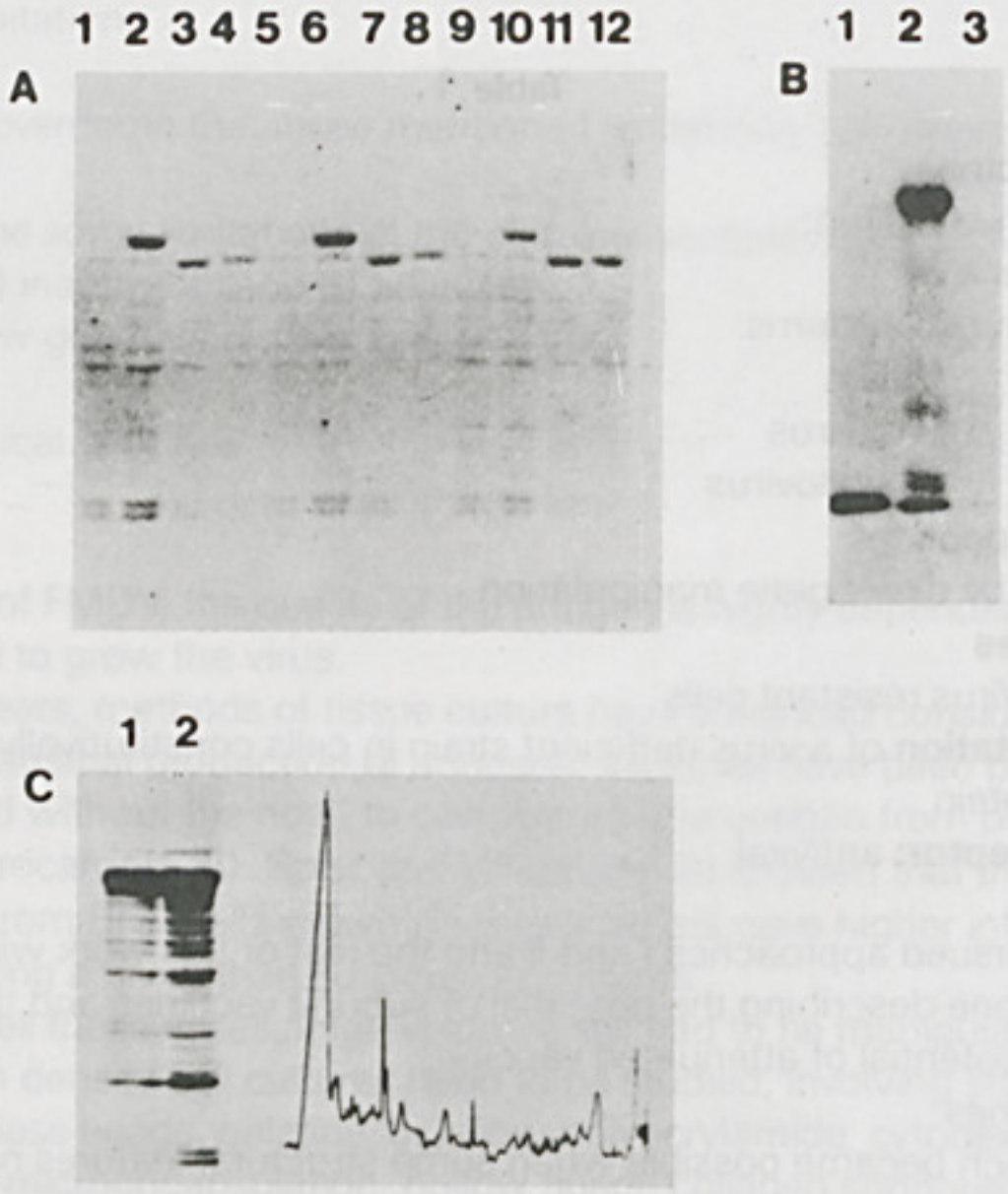

Fig. 2. Expression of cloned FMDV-VP1 gene in the pUR system.
SDS-polyacrylamide gel analysis of total cellular proteins stained with Coomassie brilliant blue.
a) Different times post-induction, lanes 1, 5 and 9, no induction; lanes 2, 3 and 4, 4 hours post-induction; lanes 6, 7 and 8,2 hours post-induction; lanes 10, 11 and 12, 1 hour post-induction, in the 3 different expression vectors lanes 1, 2, 5, 6, 9 and 10 correspond to the pUR 289 vector: lanes 3, 7 and 11 correspond to the pUR 288 vector and lanes 4, 8 and 12 correspond to pUR 278 vector.
b) Autoradiograms of proteins transferred to nitrocellulose and reacted with I^{125}-labelled VP1-specific antibodies, lane 1, isolated VP1, lane 2 expressed after 3 hours induction in the pUR 289 vector; lane 3, no induction.
c) Preparative polyacrilamide gel electrophoresis and corresponding scanning.

These results are not surprising considering that the isolated protein is weakly immunogenic possessing less than 0,1% of the activity of the virus particle (18). Meloen in 1982 demonstrated that neutralizing monoclonal antibodies raised against intact virus do not recognize isolated VP1 (19). It becomes always more evident that VP1 in the virion adopts a conformation which is highly dependent from a substantial interaction with the other structural proteins.

Other laboratories reported that vaccines produced with several A strains through VP1 specific fusion proteins were protective in cattle. However, significant amounts of polypeptide and revaccination regimes were required (20).

Alternative hosts as well as alternative genomic fragments should be pursued so that the surface epitopes reach the appropriate conformations required for protection *in vivo*. Moreover, more information is required on the importance of the immunological response to internal viral antigens in relation to T-cell immunity.

b. Synthetic peptides

The fact that the entire VP1 is not necessary for eliciting protective antibodies in animals, encouraged us to the use of this approach.

The prediction of the location of immunogenic sites within VP1 capable of eliciting a neutralizing antibody response was based on several approaches which include: immune response in mice of proteolytic and chemical fragments of VP1 (15); comparison of predicted amino acid sequences in different serotypes; hydrophilicity plots superimposed on amino acid sequence variation plots and identification of helical regions displaying hydrophilic and hydrophobic zones on opposite sides of the helix (21). These methods predicted amino acid residues 144-159 and to a lesser degree 200-213 of VP1 as good candidates for eliciting a neutralizing response. According to these predictions we synthesized a series of peptides, covering various regions of the polypeptide, corresponding to the sequence of serotype O_1 Kaufbeuren by using the solid phase Merriefield process. The peptides were linked to keyhole·limpet haemocyanin and tested for immunogenicity in guinea pigs. 200 ug of peptides were injected subcutaneously with complete Freund's adjuvant and revaccination was at 29 days with incomplete Freund's adjuvant.

As can be seen in Table 2 synthetic peptides representing each of the two potential antigenic regions of VP1 induced high levels of antibodies which recognized intact virus, but only residues 140-160 were protective. No cross neutralization activities were observed with other serotypes. Despite the optimal results obtained in guinea pigs with the peptide corresponding to residue 140-160, when this peptide was inoculated in cattle, very low levels of neutralizing antibodies were obtained even after revaccination.

Table 2

Protection of guinea pigs vaccinated with synthetic peptides corresponding to the VP1 region of FMD virus O_1 Kaufbeuren against challenge with the homologous virulent virus

Sample N.º	Challenge of guinea pigs with virus O_1 Kaufbeuren	Neutralization of viruses by anti-peptide serum[b] microneutralization test performed in tissue culture				
		O_1 Kaufbeuren	O_1 Campos[c]	A_{24} Cruzeiro	A Venceslau	C_3 Indaial
89-34 - 1	0/5[a]	<1.2	ND[d]	ND	ND	ND
89-34 - 2[e]	6/6	3.9	2.4	≤1.2	≤1.2	≤1.2
89-34 - 3	1/8	≤1.2	ND	ND	ND	ND
89-34 - 5	0/2	≤1.2	ND	ND	ND	ND
89-34 - 6[f]	0/6	2.4	2.25	≤1.2	≤1.2	≤1.35
89-34 - 8	0/5	≤1.2	ND	ND	ND	ND
89-34 - 9[f]	1/6	3.3	3.3	≤1.2	≤1.2	1.50
89-34 - 10[e]	6/6	4.35	3.3	≤1.2	≤1.2	1.35

[a] Protected/challenged (challenged with $10^4 ID_{50}$ of guinea pigs adapted O_1 K FMDV)
[b] Values are expressed as $-\log_{10}$ of serum dilution that protects 50% against 100 $IDTC_{50}$
[c] By fingerprinting O_1 K and O_1 Campos show a high degree of homology (better than 95%).
[d] Not done.
[e] Oligopeptide corresponding to amino acid residues 140-160 of VP_1
[f] Oligopeptide corresponding to amino acid residues 200-213 of VP_1.

It is evident that additional work is required which should establish:
- the importance of additional peptides of another genomic regions;
- optimal carrier proteins;
- alternative methods of coupling;
- use of immunological potentiators;
- use of mixtures of peptides as multivalent vaccines;
- studies of immunological memory;
- significance of a priming effect.

Moreover additional information on the 3-D structure of the virion through X-ray crystallography is essential. The poor performance in cattle compared to the promising results in guinea pigs would be indicative that attempts with peptide containing domains which react with helper T-cells should be pursued. Recently, the potential importance of sequences 200-213 in enhancing the response of sequences 141-160 was suggested by the serological evidence that a peptide containing both sequences was more reactive with neutralizing monoclonal antibodies against the intact virus than either sequence alone (22). Moreover a vaccine prepared from two peptides 141-158 and 200-213, linked by a diproline spacer, in order to promote adequate folding, and having cysteine residues at each end of the linked peptide, for the purpose of polymerization of the molecule as a means of eliminating the need of a carrier, elicited protection in cattle even when no revaccination was applied. However, it required significant amounts of peptide and complete Freund's adjuvant (23). In addition, it was shown that tandem peptide sequences fused to bacterial proteins elicited high levels of neutralizing antibodies and protected pigs against challenge. However, only limited animals were used on the trial (24).

II. *Live attenuated vaccines*

Live viral vaccines offer several significant advantages over inactivated or subunit viral vaccines, namely induction of more effective local immunity and greater duration of immunity. Such vaccines, however, are at present only of limited use due to the considerations mentioned above, which could not be overcome in the late fifties when these attenuated vaccines were developed (25).

The observation for several picornaviruses that viable virus can be rescued from cloned cDNA was an essential breakthrough to study the genetic determinants of attenuation and to construct safe attenuated vaccines (26, 27) Infectious DNA can be specifically mutagenize to obtain modified strains which can replicate and retain antigenic identity without propensity for virulence. Moreover, once a stable attenuated strain is identified or generated through adequate alterations of an infectious clone, and provided that the genetic determinants of attenuation are not located in the immunogenic regions, one can extend the attenuated phenotype to other serotypes, by introducing through recombination via cDNA *in vitro* the genes for immunogenicity from a new strain into the genome of an ideal avirulent strain. To make such an approach feasible indentification of viral genes that specify virulence is of critical importance. Therefore, the biological and biochemical characterization of several attenuated strains of different serotypes was undertaken.

As expected due to the high variability of RNA viruses and to the multiple passages used to generate these attenuated strains, molecular weight and charge differences between the polypeptides of the attenuated strains and their corresponding wild type strains were observed randomly throughout the entire genome (28, 29). A remarkable feature, however, was a common increased electrophoretic mobility of the precursor polypeptide P3 in all attenuated strains when

compared to their wild type strains. Preliminary data identifies a genomic deletion in the region of polypeptide 3A of the attenuated strains (Figure 3).

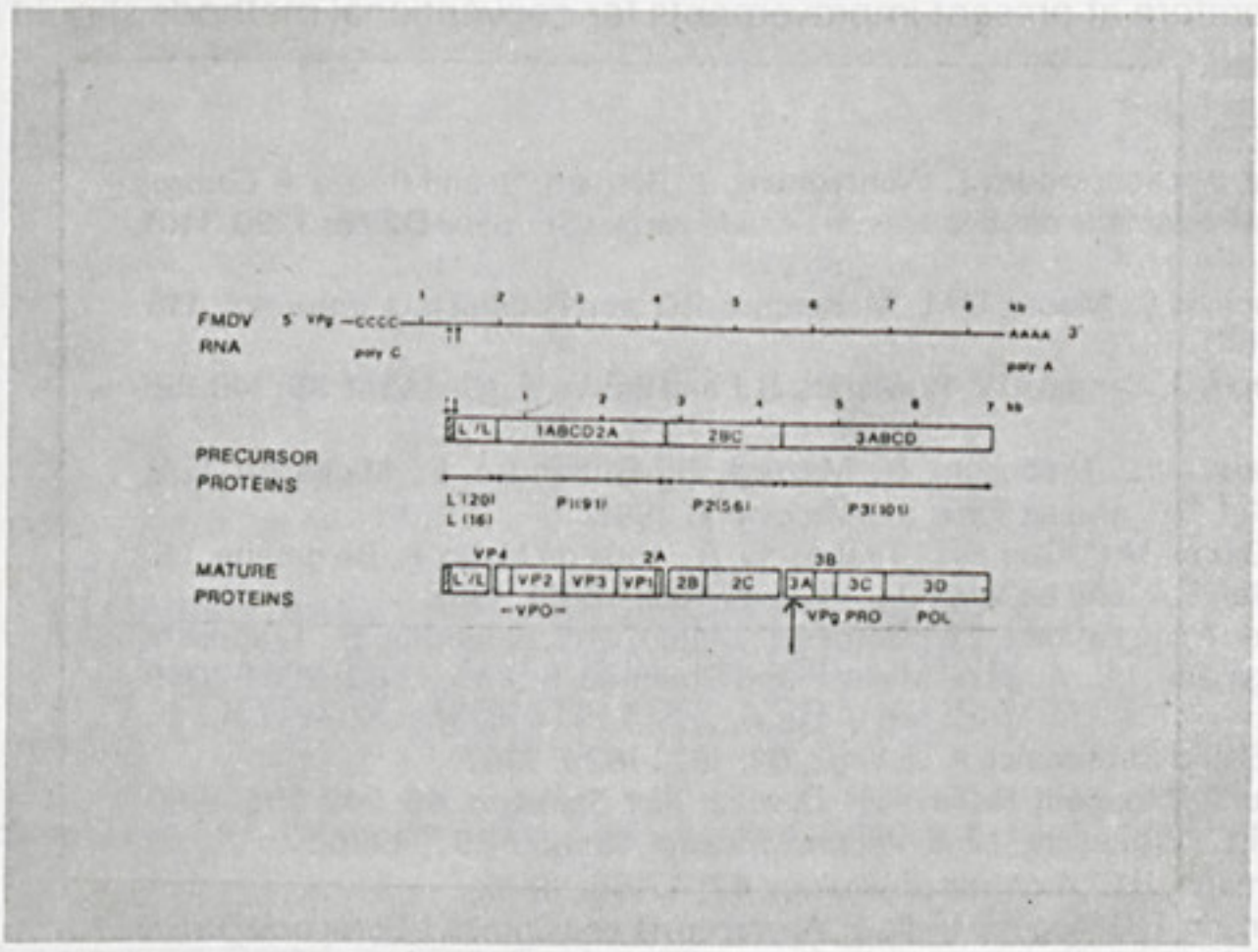

Fig. 3 - Biochemical map of FMDV

The potential relevance of this genomic region for the attenuated phenotype was indicated by the following facts:

- The biochemical and biological properties of intermediate strains isolated during the process of attenuation indicated a direct relationship between the appearance of the attenuated phenotype and the alteration in P3.
- A genomic deletion is in agreement with the fact that no revertants could be isolated for the strains studied.
- The biological properties of recombinant viruses between wild type and attenuated strains located the major genetic determinants of attenuation in the 3' half of the genome (30).

The fact that viable recombinant viruses were obtained constituted a crucial prerequisite for the development of attenuated strains for new emerging serotypes based on other stable attenuated strains.

As more becomes known on the functional and non-functional regions of the FMD genome, it may be possible to make other specific deletions to ensure attenuation for example for other hosts such as swine.

All these approaches are feasible provided that an infectious DNA clone is available. We are working in this direction but so far, we and no other laboratory could obtain the cloning of the entire FMDV genome as a contiguous cDNA sequence. It seems that the poly (C) tract or sequences 5' to the poly (C) tract are extremely difficult to clone since they were not observed in any clone reported until now by any laboratory. Uncoventional methods will need to be pursued in order to clarify whether the development of new safe live vaccines can be taken into consideration for FMD.

Concluding remarks

At present, the potential of biotechnological methods for improving FMDV vaccines is unquestionable. It should be noted, however, that the developments are

only beginning and much more needs to be known about immunological mechanisms and the molecular biology of FMDV before an overall success can be seen. Therefore at present improvements for conventional methods should not be disregarded.

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CLONING AND EXPRESSION IN *ESCHERICHIA COLI* OF cDNA SEQUENCES ENCODING FOOT-AND-MOUTH DISEASE VIRUS VP1

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Foot-and-Mouth Disease (FMD) is highly contagious, affecting cattle, sheep, pigs and goats. The disease is of economical importance since it causes productivity losses estimated to be around 25%. An aphtovirus belonging to the picornaviridae family is the causative agent of the FMD. Several serotypes of the virus have been isolated from infected animals, being A, O and C found in Brazil. The viral particle is composed by 60 copies of each of the four structural proteins VP1, VP2, VP3 and VP4, and a single-stranded RNA of about 8,300 nucleotides.

VP1 is the only viral protein able to elicit the production of neutralizing antibodies (Laporte et al., 1973, Bachrach et al., 1975). Two regions, corresponding to aminoacid sequences 138-154 and 200-213 were identified within the VP1 protein, which are important to induce the production of neutralizing antibodies (Strohmaier et al., 1982). Synthetic peptides corresponding to aminoacids 141-160 have been shown to elicit neutralizing antibodies. However, the immunogenic activity in this case was 10 to 100 fold lower than when an equivalent amount of virus particles was used to immunize the animals (Bittle et al., 1982).

Sequences encoding the entire or part of the VP1 protein have been isolated from different virus serotypes and expressed in **E. coli.** Kleid et al. (1981) have shown that the VP1 of FMDV A12 can be expressed as a trpLE fusion protein under the control of trp promoter-operator. The immunogenicity of this fusion-protein has been shown to be low if compared to the intact virus particle. This low immunogenicity probably reflects a different conformation adopted by the VP1-trpLE fusion-protein compared to the VP1 native conformation in the virus particle. Recently, Broekhuijsen et al. (1986) have constructed expression plasmids containing up to 8 repeats of the antigenic determinant of FMDV corresponding to aminoacids 137-162 of the VP1 protein. The fusion proteins obtained consisting of one, two or four copies of the aminoacid sequence 137-162 attached to the N-terminus of β -galactosidase were inoculated into guinea pigs and pigs. Proteins containing two or four copies of the

sequence 137-162 were highly immunogenic and protected pigs against challenge infection (Broekhuijsen et al., 1987).

In the present work cDNA was prepared using RNA extracted from FMDV strains AVenceslau, A24Cruzeiro and C3Indaial and inserted into either plasmid pACYC184 or phage λgt10. The synthesis of the first strand of the cDNA was primed with a synthetic oligonucleotide complementary to a conserved sequence localized 33 nucleotides from the 3' end of the VP1 RNA sequence (Beck et al., 1983; Beck and Strohmaier, 1987). The double stranded cDNA was linked to EcoRI adapters and ligated to either pACYC184 or λgt10, cleaved with EcoRI. Several recombinants were obtained containing cDNA inserts ranging from 200 to 2000 base pairs in size. cDNA from one of the recombinants was partially sequenced and shown to contain the VP1 sequence corresponding to aminoacids 117 to 210. This DNA segment has been cloned into the EcoRI site of the expression vector pUR291 (Rüther and Müller-Hill, 1983) to produce a fusion protein where the 3' end of the β -galactosidase gene is lost. Moreover, in another type of construction the EcoRI site of the cDNA has been filled in using Klenow polymerase and ligated to BamHI linker. After digestion with BamHI the cDNA was cloned into the BamHI site of the pBR322 vector. Thereafter the cDNA was purified and cloned into the BamHI site of the pUR vectors (Rüther and Müller-Hill, 1983). Some of the clones containing the puR292 recombinant with the cDNA insert have expressed a β -galactosidase-VP1 fusion protein with β -galactosidase activity. The fusion proteins synthesized by *E. coli* containing the recombinant plasmids derived from both types of construction were recognized by antibodies of antisera from rabbits immunized with viral VP1 in a western blot. These same fusion proteins were also recognized by a monoclonal antibody which reacted specifically with FMDV C3Indaial VP1 protein. The same cDNA sequence was also cloned into the HpaI site of the plasmid pCB20, a derivative of the vector pAT153, which contains the HindIII-BamHI fragment corresponding to nucleotides 34449 to 36895 of phage. In this system the VP1 sequence was expressed under the control of the phage P_L promoter and is synthesized as a fusion protein, N protein-VP1. This fusion protein reacted to the same monoclonal antibody which recognized the β -galactosidase-VP1 protein.

The fusion proteins (with β -galactosidase or N protein) were purified by electroelution after preparative polyacrylamide-SDS gel electrophoresis or by affinity chromatography (Ulmann, 1984). To assay the ability of the fusion proteins to elicit a specific response, mice were injected with 15 to 100 μg of the proteins emulsified with complete Freund's adjuvant. After ten days the mice were injected with the same amounts of antigen emulsified in incomplete Freund's adjuvant. Eight days after the second injection the sera were taken and analyzed by ELISA using either viral VP1, intact virus particle, β -galactosidase-VP1 or β -galactosidase as antigens. No detectable level of antibody was observed which recognized the viral VP1 or intact virus particle. Only the β -galactosidase-VP1 and β -galactosidase were recognized by the antibodies. These results indicate that the β -galactosidase-VP1 protein assumes a conformation which does not allow the recognition of the VP1 sequence by the immune system of the animals assayed.

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SYNTHETIC MACROMOLECULAR ANTIGENS, DRUGS AND VACCINES

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After it was shown that synthetic polypeptides may be antigenic in experimental animals (1), their investigation led to the elucidation of molecular parameters of antigenicity, including aspects such as composition, size, shape, electrical charge, steric conformation, accessibility of the immunologically important portion of the antigen, and the optical configuration of the component amino acids (2). It also permitted the clear distinction between immunogenicity and antigenic specificity (3)

The synthesis of a peptide ''loop'' derived from hen eggwhite lysozyme, and its subsequent attachment to a multichain synthetic polypeptide, led to a synthetic conjugate which provoked antibodies reacting with a unique region within the native enzyme, which was conformation-dependent (4). This led us to distinguish between ''sequential'' and ''conformational'' antibodies (5). Based on this observation we speculated that if this could be done in the case of lysozyme, it should be possible to do it with other native proteins, including viral coat proteins and bacterial toxins, leading to a new approach to vaccination (6).

Synthetic vaccines of the future, prepared either by chemical synthesis or by genetic engineering, should contain the right epitopes for recognition by B cells (to form antibodies) and by T cells (in conjunction with class II or class I antigens), a built-in adjuvant and the right carrier (or a way of substituting for it through covalent attachment of lipids, cross-linking or some other method). It should also take in consideration the genetic make up and the danger of antigenic competition (when several different peptides are administered at the same time, possibly attached to the same molecule).

Successful studies on peptides derived from viruses such as MS2 bacteriophage (7) and influenza (8) are described in this volume by Ruth Arnon. We have also shown that it is possible to prepare a totally synthetic molecule containing a synthetic peptide epitope derived from diphtheria toxin and a synthetic adjuvant (muramyl dipeptide) attached to a synthetic carrier, which can provoke the formation of antibodies neutralizing the dermonecrotic activity of diphtheria toxin (9). Similarly, in a series of studies (10-15), we have prepared synthetic antigens provoking antibodies capable of neutralizing the cholera toxin, as followed by several methods.

Another series of studies, which has preoccupied us for the last 21 years, has been the development of a candidate drug against the exacerbating - remitting stage of multiple sclerosis (16). This drug, denoted Cop 1 (17), may be considered an immunomodulatory vaccine, switching the response to specific suppressor T cells, as shown for the model disease, experimental allergic encephalomyelitis. This is due to immunological cross-reaction between Cop 1 and the basic encephalitogen of the myelin sheath of the brain (18,19). At the same time it is of interest to stress the fact that Cop 1 is a macromolecular drug, which after hydrolysis gives amino acids which are the usual components of proteins. As such, it is an harbinger of a family that - I am sure - will increase enormously in the years to come.

And last, I would like to mention here another family of macromolecular drugs, and I am referring to the topic of immunotargeting of drugs and toxins. In this case attachment of drugs or toxins to polyclonal or monoclonal antibodies yields conjugates that will bring the attached molecule to the desired site thanks to the "guided missile" which is the antibody. Our pioneering work on adriamycin (doxorubicin) and daunomycin (daunorubicin) attached to various anti-tumor antibodies (20,21) has been later extended to liver hepatomas and anti-alpha fetoprotein antibodies (22,23). The conjugates keep their antibody and drug properties and prolong the life of the experimental animals better than all the controls. In rats, we could start the therapy, in vivo, several days after the hepatomas were transferred, and more than half the animals survived during the whole course of the experiment (23).

It seems not too far-fetched to assume that in the not too distant future there will be many examples of successful use of synthetic vaccines, macromolecular drugs and immunotargeted therapy.

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THE EVOLUTION OF THE SPLIT GENE STRATEGY AND DIVERSIFICATION OF ANTIBODY MOLECULES

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The objectives of our research are to understand the evolution of the split gene strategy of Ig genes and of the germline and somatic mechanisms, in relation to the generation of antibody diversity. Studies in mammals have shown that both germline and somatic mechanisms contribute to the generation of antibody diversity. The germline element refers to the inherited repertoire of the V, D and J gene segments which encode the variable regions of Ig polypeptide chains. The somatic element operates during B cell ontogenesis, involving combinatorial joining of V, D and J in a flexible manner and somatic mutations of the rearranged genes. The balance between the germline and somatic elements is ill defined. Nonetheless, we notice already in mammals that this balance may vary considerably among species, as mouse has many more V_k genes (~ 1000) than human (~ 50). Our studies on gene expansion in the J_k cluster of rat and on the limited repertoire of germline V genes in chicken, are summarized below.

It is generally thought that gene duplication is the process by which a small number of primordial DNA segments expanded to form the large multi-gene family of Ig. However, the unit and mechanism of duplication are not easily defined due to noncoding DNA segments with extensive sequence divergence which flank the coding regions. The J_k cluster is a convenient system to study evolutionary processes because the number of J_k genes is small (< 7) and the noncoding DNA spacers between J's is short (~ 0.3 kb). On the other hand, the number of V_k genes is large (~ 1000) and the noncoding DNA between V's is long (> 5 kb). We cloned and sequenced the J_k cluster of rat and found it to contain seven J_k genes as compared to five J_k in mouse. The expansion of the rat J_k genes occurred so recently on evolutionary time scale that time was too short to allow extensive genetic drift of the duplicated noncoding DNA which retained 98-99% homology. This enabled us to determine the unit of duplication as the J_k coding region plus 5' noncoding DNA (34 5pb), the mechanism of gene duplication as two consecutive events of unequal crossing over involving the 3' ends of the J1 and J2 coding regions, and the time of duplication within the last 1 to 2 million years (rat/mouse divergence occurred before 10×10^6 years). Mutations have occured in codon 96 of both duplicated genes, the only position along 345 bp where J2A, J2B (the new genes) and J2 differ from each other. This results in three different amino acids (Asp, Asn and Tyr not present in any other J_k at position 96) which are physiologically

significant because they increase the diversity of CDR3. Although the occurrence of these mutations may be a coincidence, it seems to reflect germline diversification of codon 96. We suggest that the mutations are random and rare as elsewhere, but once they yield a new amino acid at position 96, they are fixed by selective pressure to increase antibody diversity.

We have shown that a few germline V genes encodes the bulk of chicken L-and H-chains, based on the following findings. cDNA libraries of chicken spleen and Harder gland (a gland near the eye enriched with plasma cells) constructed in pBR322 were screened by differential hybridization and by the mRNA hybrid-selection translation-immunoprecipitation protocol. Eleven L-chain cDNA clones were identified from which VL probes were prepared and each was annealed with kidney DNA restriction digests. Surprisingly, all VL probes revealed the same set of bands, corresponding to about 25 Germline VL genes of one subgroup. Nucleotide sequence analyses of five VL cDNA clone showed > 90% homology. The amino acid sequences derived from the nucleotide sequences were either identical or nearly identical to the major N-terminal sequence of L-chains in chicken serum. These findings, and the fact that the VL probes were randomly selected from normal lymphoid tissues, strongly indicate that the bulk of chicken L-chains is encoded by a few germline VL genes, probably much less than 25, because a few VL genes cloned were found to be pseudogenes. Analyses of the CL locus (Southern blots, cloning and sequencing of the CL gene and cDNAs) indicate CL allotypes, two of which were identified in the homozygote and heterozygote forms. To study the H-chain locus, spleen cDNA was cloned in the λgt11 expression vector and the library screened with anti-H-chain antibodies. Clones encoding the constant ($C\mu$, $C\gamma$) and variable regions were isolated and characterized. Here again all VH clones (8 independent isolates) yielded the same pattern in Southern blot analyses of kidney DNA. The hybridizing bands corresponded to about 30 VH genes of one subgroup, indicating that the VH gene dosage is also small (the presence of pseudo-VH genes is not yet known). The nucleotide sequences of two VH segments showed 83% homology, as expected for VH of the same subgroup. These findings provide strong evidence that one subgroup of VL and one subgroup of VH genes encode the bulk of (>95%) chicken light and heavy chains, i.e., the inherited repertoire of chicken V genes is rather limited and it did not expand to generate the multiple subgroups of V genes observed in mammals. The immune potential of chicken is comparable to that of mammals. Therefore somatic mechanisms should play a major role in the generation of antibody diversity in chicken.

The L-chain locus contains only one functional VL gene (other members of the VL subgroup are pseudogenes) and one JL. This organization rules out the possibility of L-chain diversification by combinatorial joining of gene segments since it requires at least multiple Vs or multiple Js. In the H-chain cDNAs we found two markedly different diversity segments (DHs of 10 or 20 amino acids showing only 20% homology) joined to similar VH and JH sequences (83% and 93% homology). These findings strongly indicate that chicken genome has multiple (at least two) DH gene segments that diversify the H-chains by the combinatorial joining mechanism.

The information available so far demonstrates that elements of the germline and somatic mechanisms had evolved in a stepwise manner, and at the same evolutionary stage the exploitation of these elements differ in the L- and H-chain loci. Diversification by the formation of a large pool of germline V genes evolved in mammals but not yet in chicken. Combinatorial joining of gene segments that diversify both L- and H-chains in mammals is operative in chicken only for H-chains and not for L-chains.

POLIOVIRUS SEQUENCES CLONING INTO VACCINIA VIRUS

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Vaccinia virus is the prototypic member of the poxvirus family. Due to its biological properties and its successful history as an immunizing agent in smallpox vaccination this virus became a strong candidate for generating live recombinant vaccines (Panicali & Paoletti, 1982; Mackett et al., 1982). The progress in molecular biology study of this virus permited the use of genetic engineering technics to express foreign antigens (Brown et al., 1986).

Some biological properties of the virus contributed to its use as an expression vector. The large genome of the virus (187 kb) allows stable integration of foreign DNA sequences as large as 25 kb. The virus DNA replicates within the cytoplasm of infected cells and the infectious cycle shows distinct stages from uncoating to assembly. The virions have all enzymatic machinery necessary for transcription, modification and viral genome replication (Brown et al., 1986). The DNA promoter sequences are different from those sequences found in both pro and eukariotic cell (Hanggi et al., 1986). Those sequences are recognized only by the viral RNA polymerase. As a consequence, the key to obtain foreign gene expression is to put the sequence to be expressed under the control of a viral promoter.

The purified vaccinia DNA is not self infectious and therefore the "in vitro" manipulation of vaccinia genome is not possible. The approach used to obtain the recombinant virus includes the construction of a bacterial plasmid that contains the vaccinia promoter sequence followed by the foreign gene and flanked by non essential vaccinia virus DNA sequences. A cell culture is then co-infected with intact vaccinia virus and the recombinant plasmid. "In vivo" recombination event occurs, at a low frequency, between the homologous sequences present in the hybrid bacterial plasmid and the viral genome. This results in the formation of stable recombinant infectious particles that can be easily selected if the foreign sequences interrupts a marker gene in the vaccinia genome. Many recombinant vaccinia virus have already been obtained expressing antigens of hepatitis B, rabies, influenza, malaria and HIV-1 (Tartaglia & Paoletti, 1988).

Our purpose is the study of poliovirus antigens expression by using vaccinia virus as a vector. The causative agent of poliomyelitis is one of the best known animal viruses as respect to its molecular biology and immunochemical properties (Koch & Koch, 1985). Therefore it is interesting to verify how vaccinia viruses express poliovirus sequences.

Poliovirus is a virus of the Picornaviridae family whose RNA genome has already been cloned by the cDNA strategy (Koch & Koch, 1985). The viral particle contains four structural proteins (VP1, VP2, VP3 and VP4) arranged into an icosahedral symmetry. The fine structure of purified virions was obtained by X-ray crystallography (Hogle et al., 1985). Those results were related to the immunochemical studies and the main epitopes of the virus surface could be visualized. Van der Werf et al. (1983) showed by genetic engineering manipulation that aminoacids comprised between positions 92 and 105 of VP1 polypeptide were important for the immune response.

CLONING OF VACCINIA SEQUENCES INTO BACTERIAL PLASMID: A bacterial plasmid was constructed by cloning the *Hind* III-J fragment of vaccinia genome. The thymidine kinase (TK) gene is localized into this fragment and is a very useful marker because viruses with foreign insertions can be selected for TK-phenotype (Weir et al., 1982).

Vaccinia virus particles, strain Lister 180, were purified from vaccinal scars on sheep skin. The virus DNA was digested with *Hind* III-J and the J fragment was isolated from 0.7% agarose gel electrophoresis by electroelution and then ligated to *Hind* III cleaved pBR322 derivative in which the *Eco* RI and *Cla* I sites had been eliminated. The restriction map of this new plasmid named pIB 038 showed that the unique sites for *Eco* RI and *Cla* I are inside the TK gene. This plasmid was used to insert polio sequences in the correct reading frame.

CLONING OF SABIN-PV1 UP1 IMMUNODOMINANT EPITOPE SEQUENCE: By analyzing the hydrophobicity profile of VP1 polypeptide from poliovirus type 1 (Sabin strain) we were able to detect a hydrophylic region which was significantly different from the Mahoney strain. This region was comprised between aminoacids 93 to 102 and must be responsible for the immunodominant epitope of this particular strain. The DNA sequence that codes for this region was chemically synthesized. The final sequence contains, besides the nucleotides that code for the epitope aminoacids, a *Cla* I site at the 5' end and a *Eco* RI site at the 3' end. At the 3' end there is also an additional sequence coding for a *Bam* HI site. Therefore, the complete sequence can be inserted into pIB 038 in only one orientation and in the correct reading frame. The extra *Bam* HI site permited the rapid screening of a clone that contained the new plasmid designated pEP 072.

CLONING OF MAHONEY VP1 SEQUENCE: We used the VP1 sequence of Mahoney poliovirus that was cloned as a cDNA in a bacterial plasmid. This plasmid designated pCW 18Δ0 contains the DNA sequence coding for the COOH terminus of VP3, the whole VP1 and the NH2 terminus of 2A, a non structural protein (Wychowski el al., 1986). This fragment was rescued with a double digestion *Bal* I - *Bgl* II and *Eco* RI linkers were put at both ends. The fragment was then inserted into the *Eco* RI site of pIB 038. The resulting molecules were used to

transform competent **E. coli** cells. One of the positive clones (pBP 012) has the insert in the correct orientation. The DNA of this plasmid was partially sequenced by the dideoxy method and it was shown that the insertion was in the correct reading frame.

DISCUSSION: We have constructed two bacterial plasmids containing one the main epitope of VP1 from Sabin type 1 poliovirus (pEP 072), and other with the complete VP1 Mahoney sequence (pBP 012). In these plasmids, the polio sequences were inserted into the vaccinia TK coding sequence and flanked with vaccinia sequences. The homologous recombination of this plasmid with wild vaccinia virus will provide the opportunity to analyze the expression of hybrids Tk-polio polypeptides under the TK promoter control in a recombinant vaccinia virus.

The recombinant viruses so obtained could be probed with monoclonal or polyclonal antibodies. The results obtained could be related to the immunochemical studies made in the whole poliovirus.

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PERSPECTIVES FOR THE DEVELOPMENT OF DENGUE VIRUS VACCINES

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Introduction

The **Flaviviridae** is a family of about 60 viruses many of which cause diseases in man. Based on serological reactivities these viruses have been classified (De Madrid and Porterfield, 1974) in 3 main groups: yellow fever, dengue and encephalites. The encephalites group is further subdivided according to the insect vector (mosquitoes or ticks).

The mosquito-borne viruses yellow fever, dengue and encephalites account for millions of cases annually in tropical areas world-wide. Yellow fever is endemic in Africa (with a recent outbreak in Nigeria, De Cock et al, 1988) where transmission cycle is more complex than in the Amazonian Basin and Central western part of Brazil, both endemic areas (Pan American Health Organization, 1984). Here a few dozen cases/year are reported but this is clearly an underestimate being all classified as jungle yellow fever. The reinfestation of urban centers with the mosquito vector **Aedes aegypti** and the ease of transportation in and out of endemic areas have raised considerably the risk of reurbanization of yellow fever (see Coimbra et al, 1987). That this is possible is further corroborated by the recent outbreaks of dengue virus, transmitted by the same insect vector, in the last couple years in Brazil (Schatzmayr et al, 1986). The availability of a safe attenuated yellow fever virus vaccine and its large production in the country, together with vector control programme have so far avoided the return of urban outbreaks.

Dengue viruses constitute an ever-increasing health problem throughout the tropical areas world-wide. The four serologically distinguishible types account for the very high morbidity observed. All four serotypes are endemic in southeast pacific, the caribbean region and some countries in Central America with outbreaks of or more serotypes at a time. Dengue viruses have shown an amazing spreading tendency which is probably due to the reinfestation of many countries with the insect vector and/or the control of yellow fever virus. The outbreaks in Brazil are a

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good example for that and it seems to be a matter of time to have the other serotypes besides serotype 1 introduced into the country. Despite the fact that classic dengue is a relatively mild febrile state not always accompanied by headache, joint pain and rash, there is a major need vaccines arising from the existence and persistent spread of dengue hemorrhagic fever (DHF) or and dengue shock syndrome (DSS) with high morbidity and significant mortality (Monath, 1986). Currently two hypothesis would explain the occurrence of these severe forms of dengue; a) the virulence of the serotype and its strain (Rosen, 1986); b) the sequential infection, where preexisting antibodies of a primary infection in subneutralizing titers would enhance a secondary infection, specially if the latter virus belongs to serotypes 2 and 4 (Halstead, 1988). Dengue vaccines, therefore, must be effective and safe when used in these regions where DHF/DSS occur and where exposure to multiple serotypes is likely. To date, no reliable dengue vaccine, for any serotype, is available for mass vaccination, although some candidates have been obtained by serial passages in tissue culture.

Regarding the encephalites group, Japanese encephalites (JE) is by far the most serious case, given its high morbidity and in some instances mortality and/or permanent damage to parts of the nervous system. It accounts for several hundreds of thousands cases/year in Asia and its vector, ***Aedes albopictus***, is now present in the Americas as well. There is a killed JE vaccine which requires periodic immunization (Kitano et al, 1986).

All in all, it is clear that flaviviruses in general and specially dengue viruses are likely to grow as major health problem. The classical technique for obtaining attenuated viruses, i.e. serial passage in cell culture, although promising has met with little success for dengue viruses. Alternatively, there is hope that recombinant DNA technology might help elucidate viral replication mechanisms and possibly other biological parameters such as virulence/attenuation. This knowledge would be fundamental for designing second generation dengue vaccines.

This paper reviews (not exhaustively) the studies of several aspects of flavivirus genome structure and sequence variability; gene expression and epitope mapping and finally the synthesis of viral proteins in heterologous systems; as related to dengue vaccine development.

Virion morphology and composition

Flaviviruses are spherical viruses about 40-50 nm in diameter with an isometric, probably icosaedral, nucleocapsid surrounded by a lipoprotein envelope containing host cell lipid and two virus-specific polypeptides, the envelope (E) and the membrane (M) proteins. A capsid (C) protein complexes the nucleic acid forming the capsid. The virions contain single-stranded RNA (3-4x10E6 d) which is infectious.

Viral replication.

Flavivirus entry into cells follows the endocytic pathway and the proccess of virus replication and virus replication take place in the cytoplasm. Budding occurs into cytoplasmic vesicles which are then transported out of the cell. Upon uncoating the positive stranded RNA is translated in the rough endoplasmic reticulum giving rise to a unique polyprotein precursor of more than 3000 aminoacids which is then cleaved off to generate the structural and the nonstructural proteins (Cleaves, 1985).

In the flavivirus genome structural proteins are coded by the first quarter of the RNA with the remaining encoding the nonstructural proteins (NS1, ns2a/2b, NS3, ns4a/4b, NS5; Rice et al, 1985; Coia et al, 1988). The cleavage sites are rather well conserved among several flaviviruses not only on their position but also the aminoacid sequences as deduced from cDNA and protein sequencing (Rice et al, 1985; Bell et al, 1986; Biedrzycka and Wright, 1987; Hahn et al, 1988). The proteases involved in each of these cleavages are as yet unknown with the existing hypothesis based on the specific aminoacid sequence cleaved. Assignment of specific function to each of the nonstructural proteins is also incomplete. In fact, only NS3 and NS5 have been suggested as the RNA replicase components based on the high degree of aminoacid sequence conservation among flaviviruses. Isolation of replication complexes is technically difficult but plus and minus strands can be purified, also partially hybridized as shown by RNase resistance (Brinton, 1986). It is possible that host proteins are involved in each plus and minus strands synthesis initiation and elongation and this could be of importance regarding permissiveness and genetic resistance to flaviviruses.

The establishment of persistent infections have been associated with the generation of defective interfering particles (DIs; Holland et al, 1982) during RNA replication. DIs have been described for JE virus (Schmaljohn and Blair, 1979) and were detected in yellow fever virus-infected mammalian cell cultures. (C. Rice, personal communication) but in the latter it did not compete the parental virus. So far no DI-like RNA has been observed in viral preparations from yellow fever 17DD virus - infected chick embryo extracts (Santos, Post, Cabral, Souza Lopes and Galler, unpublished). DI RNAs have been successfully used to establish some of the cis-acting sequences required for RNA replication in alphaviruses (Schlesinger and Weiss, 1986) but may be not that useful for flaviviruses.

Regarding the other nonstructural proteins, the NS1 has a structural character given its consistent glycosylation pattern in a number of cell lines (Galler, Post and Rice, unpublished) as shown for yellow fever 17D virus strains and other flaviviruses as well (Winckler et al, 1988) and the number and position of cysteine residues conserved throughout the flaviviruses. It will protect animals against homologous virus challenges if given as a subunit immunogen or as monoclonals against it (Gibson et al, 1988). It is not part of the mature virus and its function in the virus cycle and possibly pathogenesis, if any, is still unknown.

The last 4 nonstructural proteins, ns2a/2b and ns4a/4b have been identified in protein extracts of flavivirus-infected cells (Speight et al, 1988), but establishing their relevance to the virus cycle awaits further research.

Comparative analysis of Flavivirus genomes.

Detailed comparisons of flavivirus genomes has recently become possible due to the accumulation of nucleotide and aminoacid sequences (Rice et al, 1985; Wengler et al, 1985; Deubel et al, 1986; 1988; Zhao et al, 1986; Dalgarno et al, 1986; Trent et al, 1986, Mason et al, 1987; Hahn et al, 1988). A very recent paper (Hahn et al, 1988) gives an overall pattern emerging from the comparisons:

a) at the nucleotide level, strains of the same virus have about 90% homology but up to 96% aminoacid sequence homology.

b) in the same serogroup aminoacid sequence homology drops to 65-70% and necleotide sequences are hardly comparable.

c) members of different serogroups share about 40-50% of aminoacid sequences.

d) short conserved nucleotide sequences have been identified at the 3 end of flavivirus RNA as well as stable secondary structures (Rice et al, 1986; Brinton, 1986; Wengler and Castle, 1986: Hahn et al, 1987b) that could be involved in RNA replication and/or packaging.

e) Several stretches of aminoacids along the genome are perfectly conserved among all flaviviruses analysed so far. However, the nucleotide sequences which code for these strings are divergent as to randomize, but not completely, codon usage.

f) Number of transversions is smaller than transitions suggesting higher rate of misincorporation during replication. In addition transitions in the third codon position are less likely to lead to a change in codon assignment than are transversions and would more likely survive selection pressures.

g) Aminoacid sequences homologies as displayed in a homology plot of any two flaviviruses from different serogroups can vary from nearly 0 to 100% depending on the region of the genome compared. In extreme cases as the nonstructural proteins ns2a/2b and 4a/4b aminoacid sequence homology values are lowest, however, the hydrophobic character of these polypeptides is strikingly conserved.

h) Hidrophobicity/hidrophilicity plots of aminoacid sequences are remarkably similar among flaviviruses despite all the aminoacid sequence divergence.

All in all, it is clear that all the flaviviruses are closely related and have evolved from a common ancestor. There are conserved domains where the aminoacid sequence is important whereas in others the hydrophobic character seems to matter. It is expected that variable and conserved domains can be correlated with specific protein epitopes important for biological properties like cell penetration, interaction with the immune system and so on. These knowledge would be essential for designing second generation dengue vaccines.

Perspectives for the development of dengue vaccines.

The only candidate vaccines for dengue are the dengue 2 PR 159/S1 strain (Bancroft et al, 1984) and a dengue 4 strain (Eckels et al, 1984). In both cases attenuated viruses were obtained by serial passage in mammalian cell culture leading to small plaque temperature sensitive phenotypes. The former yielded low viremia, was not transmitted by mosquitoes and showed some degree of seroconversion specially if volunteers were previously given the yellow fever vaccine. The frequency of side reactions, however, was too high for mass immunization. The latter had low infectivity even for YF-immune volunteers and its genetic instability also led to the discontinuation of its use.

Another group in Thailand, however, has used primary dog kidney (PDK) cells to serially passage and try to attenuate the different dengue serotypes. They have succeeded with dengue 2 virus which is now entering clinical trials and dengue 1 and 4 are being animal tested. Dengue 3 was difficult to propagate in PDK cells but changes in the cell system have overcome this problem. Since different passages have been tested for a number of biological properties sequence analysis of virus pairs could lead to the molecular basis of virulence/attenuation for dengue viruses and possibly for flaviviruses (J.Strauss and R.Shope, personal communication).

The yellow fever system could also serve as a model for virulence/attenuation studies: the virulent strain (Asibi) which gave rise to attenuated (17D) strain and the number of passages in cell culture separating both are well characterized (Theiler and Smith, 1937). Nucleotide sequence analysis of genomic RNA from both viruses led to the identification of a number of aminoacid changes. These would be

responsible for tissue culture adaptation and/or attenuation (Rice et al, 1985; Hahn et al, 1987a). It is anticipated that obtaining an infectious clone for yellow fever virus would allow the identification of the relevant changes for attenuation. This is so because there is an animal system that reflects the infection in man (Monath, 1981), which could be used to test the phenotypes of viruses regenerated from the cloned cDNA bearing mutations made **in vitro.** This infectious clone has not yet been obtained but a full-length cDNA copy covering the whole genome for the YF 17D-204 virus has been constructed (C.Rice, pers.comm.).
It is reasonable to expect that such a methodology, if successful for the YF virus, could be easily applied to attenuating dengue viruses. Full-length infectious clone constructions are being attempted for different dengue virus serotypes in several laboratories. The most serious complication with this approach is the inexistence of an animal system other than man where the different virus phenotypes could be tested and that would reflect the most severe forms of dengue virus infection (DHF/DSS). In addition, the enhancement phenomena argue for a polyvalent vaccine where all 4 serotypes (attenuated or killed forms) are given at once.

It is also of importance to map, and later express, protective epitopes on the surface of flaviviruses. This approach could also help identify enhancing epitopes. There are several methodologies to do that: a) use of specific synthetic oligopeptides whose sequences are derived from cDNA or protein sequencing studies. These peptides are used to immunize animals which are then challenged with a flavivirus. Both envelope and NS1 proteins of Murray Valley (Roehrig, pers.comm.) and dengue 2 (J.Schlesinger, pers.commun.) are being characterized this way. The former has selected sites based on the hydrophilicity peaks whereas the latter is using a set of overlapping oligopeptides. b) monoclonal antibodies are a powerful tool to dissect viral antigens. Several groups have now developed monoclonals against different flaviviruses and this approach was recently reviewed (Heinz, 1986). A neutralization epitope as identified by a monoclonal antibody, has been identified for both YF Asibi and 17D strains by sequencing neutralization scape mutants (Lobigs et al, 1988). The same region was also shown to be a neutralization epitope for MVE virus using synthetic oligopeptides (Roehrig, pers.commun.).

It has long been speculative that the YF 17D virus strain if properly engineered could carry and express dengue virus epitopes. Again the lack of suitable experimental model for dengue is a limitation. In addition, the phenotype of a YF infectious clone when obtained would have to be determined. It remains as a possibility since epitopes of other viruses have now been introduced into a Sindbis virus infectious clone (Rice et al, 1987) with success (J.Strauss, pers.commum.).

It is also possible to map epitopes on flavivirus proteins using bacterial expression vectors. This approach has been used before for other viral proteins (keegan and Collet, 1986; Strebel et al, 1986; Spindler et al, 1984) but is limited to continuous epitopes. Usually a bacterial plasmid vector (or phage as lambda gt11, Young and Davis, 1983) contains a promoter (lambda pL/pR; trpE) and the recombinant protein is obtained as a fusion product between a bacterial protein (betagalactosidase; protein A; triptophan oxidase or the phage MS2 polymerase) with the viral protein domain after appropriate cloning. Screening with antisera allows the identification of recombinants expressing the viral proteins and deletion-expression mapping, which includes DNA sequencing displays the viral epitopes. Using the trpE vectors (Spindler et al, 1984) we have expressed the carboxi terminal half of a dengue 2 virus envelope protein and detected by western blotting common epitope(s) with a dengue 1 virus after probing the filter with an antidengue 1 hyperimmune serum (Muylaert and Galler, unpublished). The identification of such

epitope(s) is of importance in understanding the enhancement phenomena and fusion proteins as well as antibodies against it would be valuable tools to attempt that.

Eukaryotic cell expression of flavivirus proteins has been pursued mainly using vaccina vectors given the potencial of recombinant vaccinia as new live vaccines. The flavivirus proteins expressed include the structurals from dengue 2 S1 strain (J.Strauss, pers.commum.), from yellow fever virus (Rice, Lenches, Galler, Dalrymple and Strauss, in preparation), from dengue 2 Jamaica strain (Deubel, pers.commun.) and from a dengue 4 (C.J.Lai, pers.commum.).

In all cases, synthesis, processing and modification were correct for the structurals but the YF-NS1 protein was not stable or correctly processed since was present in VV-YF recombinant-infected cell extracts in very low levels as compared to the envelope protein (Rice, Lenches, Galler, Dalrymple and Strauss, in preparation). The immunogenicity of all constructs was poor failing to elicit high low levels of neutralizing antibodies, low HI titers and there was no protection upon challenge. Different hosts and inocculation routes were tested with negative results. It was concluded that live recombinant vaccina virus is not an appropriate system to obtain new vaccine candidates.

On the other hand, baculovirus expression of the structural proteins of dengue 4 virus and of the envelope and NS1 separately was satisfactory. Proteins were correctly processed and were, in fact, immunogenic (C. Lai, pers.commun.). Mice were given whole baculovirus-infected insect cell-extracts, recombinant or not. Recovery from insect cell hemolympha would be preferable than whole cell extracts (Maeda et al, 1985). Further experimentation is required before baculovirus-made flavivirus proteins can be established as bona-fide vaccine candidates.

Last but not least, is the possibility of using the purified nonstructural protein 1 (NS1) as a subunit vaccine. This glycoprotein has been shown to protect mice and monkeys against yellow fever and dengue 2 viruses (see Gibson et al, 1988 for a review) after immunization with affinity-purification of NS1 from YF or dengue-infected animal cells. Very low levels of neutralizing antibodies were present in some animals indicating some envelope protein was copurified. A recent observation might help avoiding this problem: there is a secreted form for NS1 in yellow fever infected cell lines (Galler, Post, Santos, Souza Lopes and Rice) in preparation and this has also been shown for dengue and SLE viruses (Winckler et al, 1988). It remains to be shown whether this secreted form will be protective or not. The structural differences between the two NS1 forms lie in the content of sugar residues (Galler and Rice, unpublished) and the role of carbohidrates in the immunogenicity of this protein is not clear. This aspect is under investigation.

Conclusion.

Dengue viruses represent an ever-increasing health problem throughout the world. Safe and effective vaccines have been pursued,but are not yet available. Several approaches to the development of dengue vaccines are being undertaken which include the identification and expression of protective epitopes and the molecular definition of virulence. The construction of infectious clones for different flaviviruses are of importance for the **in vitro** manipulation of pathogenicity and engineering live attenuated dengue vaccines. Subunit vaccines are also considered and adjuvants might turn out to be important in this respect. Most of the current approaches to developing dengue vaccines, and possibly any flavivirus vaccine, were summarized. Altogether, it should provide us with potential candidate

vaccines, be it a live engineered virus or a subunit vaccine or even a recombinat virus vector expressing a flavivirus protein or an epitope thereof. This is in accordance with the recently published World Health Organization Strategic Plan for Dengue (Brandt et al, 1988).

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HEPATITIS B VIRUS PROTEINS ELICITING PROTECTIVE IMMUNITY

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The immune response to current hepatitis B vaccines appears to be qualitatively different from the response elicited during recovery from natural infection. Such disparate responses can probably be explained by the absence or under-representation of preS-and the nucleoprotein core-specific determinants in the vaccines. The incorporation of these determinants into future vaccines may improve their efficacy.

Introduction

Viral structural proteins essential in the strategy of virus replication and of host-to-host transmission are also targets for the host's immune response. The host's defence mechanisms can be directed against the virus itself as well as against virus-infected cells, and may be mediated by antibodies and by lymphocytes, i.e. specific cytotoxic T (T_c) lymphocytes.

It is generally accepted that antibodies neutralizing the infectivity of viruses are directed against components exposed on the surface of the virus. The mechanism of antibody-dependent virus neutralization is, for most viruses, unclear at present. A single virus protein may exhibit different classes of antigenic determinants. Antibodies bound to some of these determinants may have little or no effect on the virus life cycle, while antibodies bound to other sites on the same protein may neutralize virus infectivity[1]. Different viruses usually have different mechanisms of neutralization and the outcome of the process of neutralization of infectivity may depend on the host cell. Since neutralizing antibodies are one of the main arms of protective immunity against viruses, a rational design of optimal vaccines for any particular virus requires sufficient knowledge about the virus-neutralization process.

Recent results indicate that both the surface proteins of viruses, and internal viral components contribute to the host's protective immune response. Viral proteins which are inaccessible on the surface of intact virus particles can be exposed on the membrane of infected cells and can thus become targets of either specific antibodies or of T_c lymphocytes, which will contribute ultimately to the elimination of cells in which the virus replicates.

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The elicitation of virus-neutralizing antibodies requires the collaboration of three distinct cell types, all of which must recognize specific sites on the virus proteins. Only one of them, the B lymphocyte, produces antibodies and recognizes the same sites on the virus protein (B-cell epitopes) as do the antibodies. The other two cell types are: (i) accessory (A) cells; and (ii) helper T(T_h) lymphocytes. When A cells present virus antigens associated with cell membrane proteins (class II histocompatibility antigens) to $T_h$cells, the cells become activated to produce lymphokines essential for replication and differentiation of various cell lineages involved in the immune response. Sites on virus proteins recognized by T_h cells (T-cell epitopes) are usually distinct from B-cell epitopes. T_h cells may react with the protein containing the virus-neutralizing epitope(s) or may recognize another, possibly an internal virus protein which becomes exposed *in vivo* as the result of partial virus uncoating. Thus, the T/B-cell cooperation may be based on recognition, by the respective cells, of distinct viral proteins on the same virus particle. Such interaction is termed intermolecular/intrastructural[2,3] to be distinguished from T/B-cell collaboration based on recognition of T- and B-cell epitopes localized on the same protein molecule (intramolecular help).

The exact features of protective immunity against many medically important viruses remain largely undefined. This review will summarize the emerging knowledge concerning the role of distinct hepatits B virus (HBV) structural proteins in eliciting protective immunity.

Hepatitis B is one of the major viral diseases throughout the world. There are about 280 million chronic carriers of HBV, and an estimated 20 million new infections occur annually. A causal link between HBV infection and primary hepatocellular carcinoma has been established and about 1 million new cases of hepatoma occur throughout the world each year. The number of deaths resulting from hepatocellular carcinoma is estimated to be about 350 000 each year. The late sequelae of HBV infection, including hepatocellular carcinoma, are one of the major causes of death in countries with a high prevalence of HBV carriers. Therefore, the prevention of HBV infections and the possible eradication of hepatitis B are of essential importance.

A possible means of providing protective immunization was discovered, much before knowledge on structural features of HBV was attained, when it was found that sera from HBV carriers (rendered substantially less infectious by heating) protected recipients against HBV infection.[4] Subsequent studies revealed that the immunogen present in these sera corresponded to subviral particles (HBsAg) containing virus envelope proteins (mostly S-protein) (see Centrespread illustration IV; see below). HBsAg particles purified from sera of HBV carriers by different methods became the basis of first-generation hepatitis B vaccines. These vaccines are efficacious in preventing HBV infections in most healthy recipients and in a proportion (depending on the manufacturing procedure) of immunosuppressed recipients (i.e. haemodialysis patients).[5,6] There is a proportion of non-responders to the vaccines (depending on the type of population and the source of vaccine), [5,7] and substantial differences in the levels of antibody response between individuals have been observed. Many non-responders to the vaccine who later became infected, and subsequently recovered, developed a good antibody response to HBV env proteins, including S-protein (representing the major protein component of the vaccines).[5,7,8] To quote Hadler et al.[7]: '... the disparate non-response to vaccine, as compared with the normal response to natural infection, remains a mystery...'. The shrouds of mystery can be dissipated by scrutinizing the immune response to the immunogen present in the vaccine in comparison with the immune response elicited

by natural infection. Such scrutiny necessitates precise information concerning the antigenic composition of viral subunits used for vaccination, and of intact virus particles.

HBV proteins

A computer search for initiation and termination codons on putative RNA transcripts from both the L- and the S-strands of HBV DNA species belonging to different serological subtypes of the virus, read in the three possible reading frames, identified four open reading frames (ORFs) on the DNA L-strand (see Centrespread illustration I). Two of the ORFs were unequivocally assigned to two distinct structural components of HBV: the envelope (env) proteins, and the internal nucleo-capsid core protein (HBcAg), respectively. There are two initiation codons at which the translation of the core (C) gene product could start, and three initiation codons at which the translation of the env gene product could start. Therefore, gene C is subdivided into the pre-core and core regions and the env gene into the preS1, preS2 and S regions. Region X of the HBV DNA is also transcribed and translated. However, the presence, location and possible function of X-protein within the HBV virion has not been established. Region P codes for the DNA polymerase/reverse transcriptase present in HBV particles. Assignment of these gene products to specific sequences within region P has not been accomplished. There are additional structural proteins in the virion: a protein covalently-linked to the 5' end of the L-strand of HBV DNA; a protein kinase found within the nucleocapsid core; and possibly additional non-structural proteins found in HBV-infected cells,[8,9]. The coding sequences for these proteins have not been established yet.

The DNA polymerase/reverse transcriptase, the protein linked to HBV DNA, and the protein kinase are minor components of the virus and are therefore unlikely to play a prominent role in eliciting defence mechanisms in infected hosts. Therefore, the major immunogens expected to play a role in protective immunity correspond to products of the env gene and of gene C. To date, there is no evidence for a possible role for the product of region X in the immune response, although it cannot be excluded. In order to understand the roles of the env and C gene products in protective immunity, it is necessary to discuss their properties in greater detail.

Several distinct mRNAs (S-, M- and L-RNA; see below) hybridizing with the env gene have been isolated from HBV-infected cells or from cells transfected with recombinant HBV DNAs (or portions thereof). The 5' - and 3' ends of these mRNAs were determined by S1 nuclease mapping and by primer extension analysis.[8] All the mRNAs have co-terminal 3' ends, but differ in their 5' ends (Centrespread illustratior II). These mRNAs can encode three distinct protein products (S-, M- and L- protein) showing variations at their N-termini but having a common C-terminus (Centrespread illustration III, upper right).

Greater than genome-length RNA species with the same 3' - terminal end as the mRNAs mentioned before, appear to serve as messages for the C-gene products. However, much smaller subgenomic fragments of HBV DNA, upon transfection, can direct the synthesis of proteins encoded by the C-gene.[10, 11]Proteins corresponding to the entire ORF or to the ORF with the pre-core sequence deleted were both synthesized in the transfected cells. The protein with the deleted pre-core sequence corresponds to the major component of the HBV nucleocapsid.

HBV envelope proteins

Translation products of each of the S-, M- and L-m RNAs (Centrespread illustration II) were identified in HBV. Because of differences in glycosylation, a total of six distinct proteins (glycoproteins) were discerned (Centrespread illustration III).[8, 9, 12–14] The relative proportions of these distinct protein species differ between virus particles and subviral particles (~20 nm spherical forms and tubules (Centrespread illustration IV and cover, section A). P25/GP29 (S-protein) are the major components of HBsAg particles, GP33/GP36 (M-protein) are much less abundant, while P39/GP42 (L-protein) are the least prevalent. The higher molecular-weight components are usually more abundant in tubules than in the ~ 20 nm spherical particles. The composition of subviral particles may differ at distinct stages of HBV infection.[8, 12] Although present in low amounts in subviral particles, P39/GP42 are essential components of HBV, about 40-80 copies being present in a single virus particle.[12].

Primary sequence analyses of S-, M-, and L-protein, indicated that preS sequences, compared with S sequences, are highly hydrophilic, and have a high content of charged amino acid residues. [8,9] These properties indicated that preS 1 and preS2 sequences are exposed on the surface of HBV and play an important role in immunological recognition and in reactions with cell receptors. The absence of cysteine residues (involved in disulphide-bond formation) in the preS region also suggested that these sequences would be much easier to mimic with synthetic peptide analogues as compared with sequences corresponding to S-protein. Some of these predicted properties are also evident from the antigenic index algorithm (Centrespread illustration III), indicating a much higher frequency of potential antigenic sites within the preS1 + preS2 sequence than within the S sequence.

These predictions are in agreement with the experimental finding that the frequency of contiguous B-cell epitopes on the preS1 + preS2 sequences is higher than that on the S sequence (Centrespread illustration V). Similar conclusions apply to the occurrence of T-cell epitopes. However, it has to be recognized that the antigenicity and the immunogenicity of S-protein depend on the maintenance of disulphide bonds. Therefore, some of the S-protein antigenic determinants would not be mimicked easily by synthetic peptides. Interestingly, the B-cell epitopes are localized on segments of the sequence with predicted beta-turns. T-cell epitopes encompassed regions containing some predicted alpha-helices, in agreement with the postulated importance of amphiphilic helices in T-cell epitopes.[8].

The HBV binding site for hepatocyte receptors was localized between residues preS(21-47) within the preS1 sequence. Antisera to the synthetic peptide preS(21—47) inhibited the reaction between HBV and HepG2 hepatoma cells. Less efficient inhibition was also observed with the preS 2-specific antiserum anti-preS2(120-145) (anti-preS(120-153)), probably because of steric inhibition or conformational changes within the preS1 sequence elicited by this antibody (Centrespread illustration V). The location of the binding site for the hepatocyte receptor was further confirmed by experiments in which the uptake of gold-labelled HBV by HepG2 hepatoma cells was followed (cover, section F). The uptake of the particles was completely inhibited by a mixture of synthetic peptides (preS(21-47) and preS(120-153)).

Hepatitis B core antigen (HBcAg)

Recent results show that expression of the pre-core region is not required for synthesis of HBcAg.[10, 11] However, the pre-core region allows the core protein to

become associated with cytoplasmic membranes, the endoplasmic reticulum and probably with the cell membrane. Thus, the pre-core sequence is instrumental in the targeting of the core proteins to cellular membranes. This is in full agreement with earlier results indicating that HBV-specific T_c-lymphocytes from HBV-infected individuals are directed against HBcAg expressed on the surface of infected hepatocytes.[20]

Lessons for hepatitis B vaccine design

HBsAg spherical subviral particles (Centrespread illustration IV; cover, section A) isolated from serum of HBV carriers or prepared by recombinant DNA techniques represent the only immunogens present in hepatitis B vaccines. Such vaccines, as discussed before, have a lower content of preS1 and preS2 sequences than HBV particles have, and completely lack HBcAg. Some manufacturing procedures completely remove preS1 and preS2 sequences from the immunogen (Centre spread illustration IV).[8,14] Although S-protein alone is known to elicit protective immunity in a high proportion of healthy recipients,[5-7] and monoclonal antibodies directed against an appropriate segment of the S-protein are virus neutralizing,[21] other epitopes present in HBV, and eliciting protective immunity, are absent or under-represented in such vaccines. These include three types of epitopes.

Epitopes on the preS2 sequence

These epitopes (i) evoke an immune response in humans recovering from hepatitis B;[8, 14] (ii) elicit virus neutralizing antibodies; [15] (iii) elicit protective immunity (in chimpanzees immunized with preS2-specific synthetic peptides);[22, 23] (iv) are immunodominant; [8,13,14,16] and (v) have the potential to provide T_h-cell help for an anti-protein-S-antibody response and thus can circumvent the non-responsive status to S-protein through preS2-specific T_h-cell helper functions.[8,16,17]

Epitopes on the preS1 sequence

These epitopes (i) elicit antibodies preventing the interaction between HBV and hepatocytes[19] and are expected to be virus-neutralizing by means of virus attachment blockade by antibodies; (ii) evoke an immune response in humans recovering from hepatitis B;[8, 14] (iii) contain epitopes which are more immunogenic at the B-cell and T-cell levels than epitopes on the S-region;[8, 18] and (iv) can circumvent immunological non-responsiveness to both S-protein and the preS2 region through preS1-specific T_h-cell functions.[18].

Epitopes on HBcAg

These epitopes elicit protective immunity in chimpanzees.[24,25] It is known that antibodies to HBcAg (anti-HBc) are probably not protective since such antibodies are present in most HBV carriers, and do not prevent HBV infection in babies who acquired anti-HBc transplacentally from HBV carrier mothers. The protective immunity elicited by active immunization with HBcAg may be explained by elicitation of HBcAg-specific T_c-limphocytes contributing to the elimination of HBV infected cells; and by intermolecular/intrastructural help[2,3] provided by anti-HBc-specific T_h - cells to anti-env protein-specific B lymphocytes.

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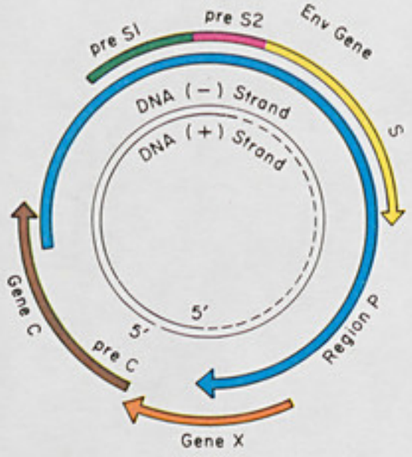
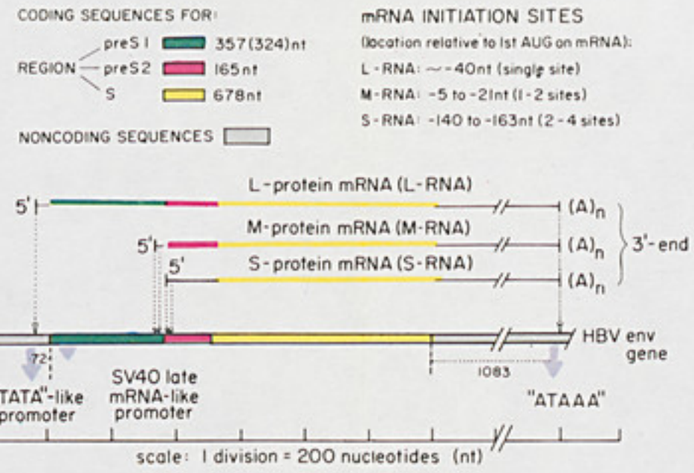


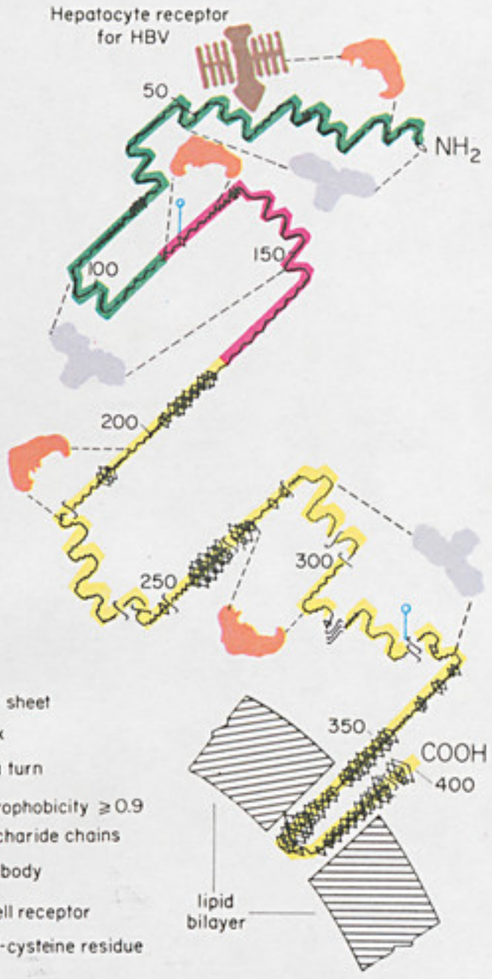
I

DIAGRAM I. Genetic map of HBV. The partially double-stranded DNA genome is drawn as the inner double circle; the innermost circle with a deleted region (indicated as a broken line) represents the DNA S- (small or plus) strand; the DNA L-(large or minus) strand has a nick located near the start of the open reading frame (ORF) for the core antigen (HBcAg) (gene C). The L-strand is the template for RNA transcripts. The ORFs are shown as broad arrows surrounding the genome.



II

DIAGRAM II. Schematic linear transcriptional map of the HBV envelope (env) gene. The positions of putative promoters controlling the transcription of the HBV env gene: (i) the 'TATA'-like promoter and (ii) a sequence resembling the late promoter of simian virus 40 (SV40), and the putative polyadenylation signal 'ATAAA' are indicated. The map is derived from results reviewed recently.[8]



V

DIAGRAM V. Predicted secondary structure of the HBV env gene product and identified regions containing B- and T-cell epitopes and the hepatocyte-receptor binding site. Locations of highly hydrophobic regions, of cysteine residues and of glycosylation sites are indicated. Based on studies with antisera to synthetic peptide analogues[8,9] encompassing the entire sequence, the only stretches of more than 10 AA not accessible to antibodies are between residues 256–268 and 360–385. The latter region is assumed to be embedded within a lipid bilayer, as shown. The other hydrophobic regions may also be associated with lipid components of the HBV envelope. The identification of B-cell epitopes was based on the recognition by human, rabbit or mouse anti-HBV (anti-HBs) of synthetic peptide analogues corresponding to segments of the L-protein sequence.[8,9,13–15] Epitopes made up of residues that are not contiguous in sequence may not be detected by this approach. The recognition of T-cell epitopes was based on identification of synthetic peptide analogues which induced proliferation of T-lymphocytes isolated from mice immunized with the native protein.[8,9,16–18] The hepatocyte-receptor binding sites were located by competitive binding assays using synthetic peptides and antisera to them as inhibitors of the HBV-hepatocyte attachment reaction.[19] The computer studies on the secondary structure were carried out by Dr John Devereux, University of Wisconsin.

IV

DIAGRAM IV. Schematic representation of individual structural subunits containing: (A) S-protein only; (B) M-protein, and (C) L-protein and of assembled HBsAg and HBV particles. The latter contain an internal nucleocapsid shown in the bottom split-open section.

III

DIAGRAM III. *Upper right: Schematic representation of HBV env proteins and their relatedness.* The open reading frame (ORF) on HBV DNA coding for HBV env proteins (see Diagram I) has the capacity to code for a protein consisting of 389-400 amino acids (AA), depending on the antigenic subtype of HBV. The 25 kD S-protein is derived from the *C*-terminal of the ORF and consists of 226 amino acids (AA). It exists in non-glycosylated (P25) and glycosylated (GP29) forms. The middle (M) protein (281 AA) contains the sequence of the S-protein with 55 additional *N*-terminal AA encoded by the preS2 region of HBV DNA, and occurs in two distinct glycosylated forms, GP33 and GP36. The large (L) protein (389 or 400 AA) contains the sequence of M-protein with 108 or 119 additional *N*-terminal AA encoded by the preS1 region of HBV DNA, and exists in a non-glycosylated (P39) and a glycosylaed (GP42) form.
Upper left: Schematic representation of results describing the separation of HBV env proteins by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. The proteins of HBV or of subviral HBsAg particles were detected either by a silver stain (the composition of the separated proteins is indicated by colour shading; for example GP42/P39 consists of preS1, preS2 and S sequences, indicated by green, red and yellow lines, respectively; the presence of the major protein of the viral nucleocapsid (HBcAg – the product of gene C; see Diagram I) is indicated by the brown band) or transferred to nitrocellulose membranes and subsequently detected by labelled antibodies specific for preS1 (green), preS2 (red) or S (yellow) sequences, respectively (only results with HBV are shown). Width and shading of bands is related to the quantitative proportions of the distinct env proteins.
Bottom: Antigenic index algorithm for L-protein.[8] This algorithm was designed to predict the location of antigenic sites (indicated by peaks above zero lines) from primary amino acid sequence data (Jameson and Wolf, in press).

Synthetic peptide analogues

Since it is currently impossible to obtain HBV in high yields, either from the plasma of HBV carriers or by cultivating the virus in tissue culture, the approaches for preparing vaccines containing all immunogens important in protective immunity have to rely either on recombinant DNA techniques or on synthetic peptides. Vaccines containing HBsAg particles rich in preS2 sequences, in addition to S-protein, are being developed.[8] However, constraints in the assembly of particles containing high levels of preS1 sequences[8] will probably make the recombinant DNA approach impracticable for the development of immunogens rich in preS1 sequences. For this reason, and also because of economic considerations, peptide synthesis might offer an attractive alternative approach to the design of hepatitis B immunogens.[8,26]

The preS2-specific peptides preS2(120-145) and preS(120-153), and the preS1-specific peptides preS(21-47) and preS(15-47), elicit high levels of antibodies recognizing native HBV particles (cover, sections B and C), and specifically recognize GP33/36 and P39/GP42 in Western blots (cover, sections D and E). As mentioned before, the anti-preS2-specific antibodies are virus neutralizing and the corresponding synthetic peptides elicit protective immunity. Similar experiments concerning synthetic analogues of the preS1 sequence are in progress. The role of an HBcAg-specific immune response in protective immunity, and the fine-specificity of this antibody response, is also amenable to experimental exploration using synthetic peptide analogues.

Conclusions

The disparate non-response to a hepatitis B vaccine, when compared with a good response to natural infection in some individuals, is less mysterious than it appears[7] and can probably be ascribed to the absence of essential epitopes in the vaccine described.[7] . Vaccination strategies considering preS-determinants, and possibly also HBcAg-determinants, should help to eradicate hepatitis B and the primary liver cancer associated with this disease.

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DEVELOPMENT OF A RECOMBINANT yHBs VACCINE AND ITS FIELD TRIAL

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Hepatitis B virus (HBV) infection has been one of the most important worldwide problem to be solved. It has been estimated that nearly 200 million people are suffering from this viral infection in the world. In Japan the persistent HBV carrier rate of this virus is estimated to be 2 to 3 percent of the population. Twenty to forty percent of these carriers develop chronic hepatitis and their several percent progress to liver cirrhosis and primary liver cancer. Therefore, intensive efforts have been required to solve this infection, particularly to develop effective vaccines.

Currently, two types of HBV-vaccine, plasma-derived and recombinant ones, are available. However, there are some problems in the plasma-derived vaccine as noted by many authors (1, 2, 3). One of these problems can be unknown contaminants and the other is a limited supply of the carrier plasma, for the production of plasma-derived vaccine. Therefore the recombinant HBV vaccines are promising in these points.

We have developed a yeast recombinant HBV vaccine (BR-HB), and some data regarding purification procedures, physicochemical characterization and immunogenicity of this vaccine will be presented. In addition, preliminary results of our clinical trial in healthy adults will be described in this paper.

Construction of the expression plasmid

The fully double-stranded, complete HBV DNA of HBs antigen subtype adr was prepared by the endogenous DNA polymerase, which was then cloned into charon 28 phage DNA at the XhoI site. Re-cloning of this cloned HBV DNA was performed using E. coli plasmid pBR322 DNA at the BamHI site. The cloned HBV DNA (clone M1B11) consists of 3195 nucleotide pairs which has only one site for both of BamHI and XhoI but has no EcoRI site.

As shown in the Figure 1, we constructed an expression plasmid for HBs gene. DNA fragments containing HBs gene were isolated from the cloned HBV DNA and ligated to the down-stream of yeast repressible acid phosphatase gene promoter region which was isolated from plasmid pPHO5 DNA (4). The resulting DNA was cloned into plasmid pBR325 DNA at the BamHI site and opened with KpnI enzyme

to eliminate the translation initiation codon, ATG, in the PH05 structure gene by digestion with the exonuclease Bal31. After treatment with T4 DNA polymerase and ligase, the plasmid was introduced into **E. coli** and a clone, ME5 was prepared. The BamHI fragment of this clone ME5 DNA was inserted into the BamHI site of a shuttle vector YEp13. The cloned plasmid, pBH103-ME5 DNA was used for transformation of **Saccharomyces cerevisiae.**

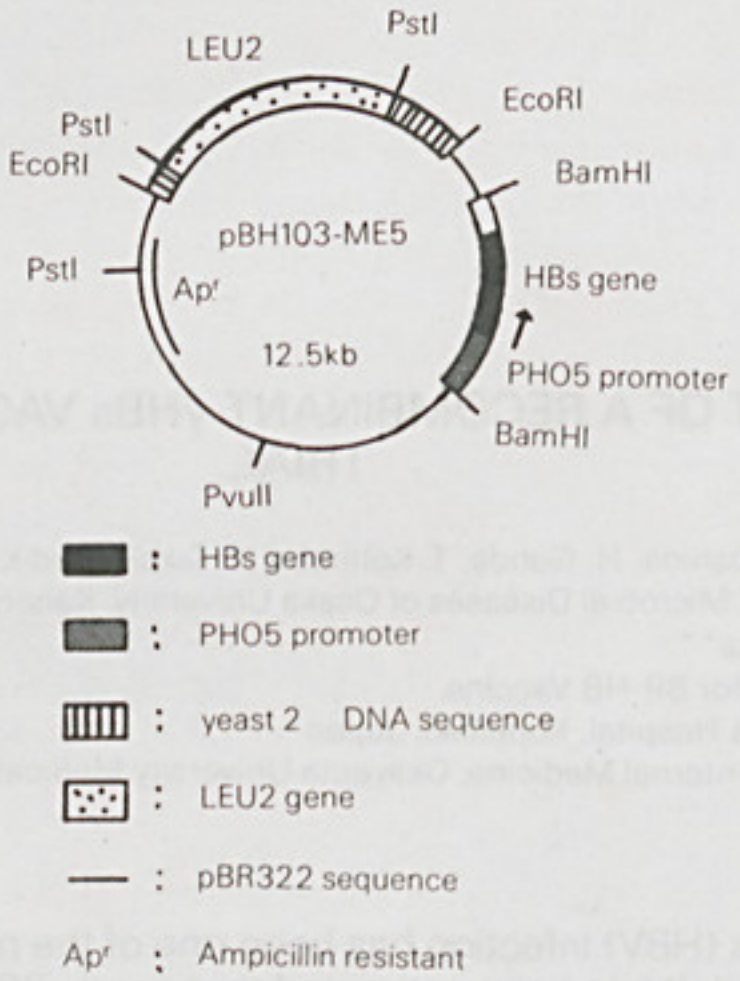

Figure 1 Structure of pBH103-ME5

Nucleotide sequence analysis of this gene indicates that the translation initiation site for HBs gene on this plasmid is presumed to be 82 bp down-stream from Hogness box in the PHO5 promoter region. The methionin codon at this site is estimated to be followed by 9 amino acids of the preS2 peptide and subsequently by 226 amino acids of the HBs antigen. Therefore, 236 amino acids in total were assumed to be synthesized in the yeast transformant.

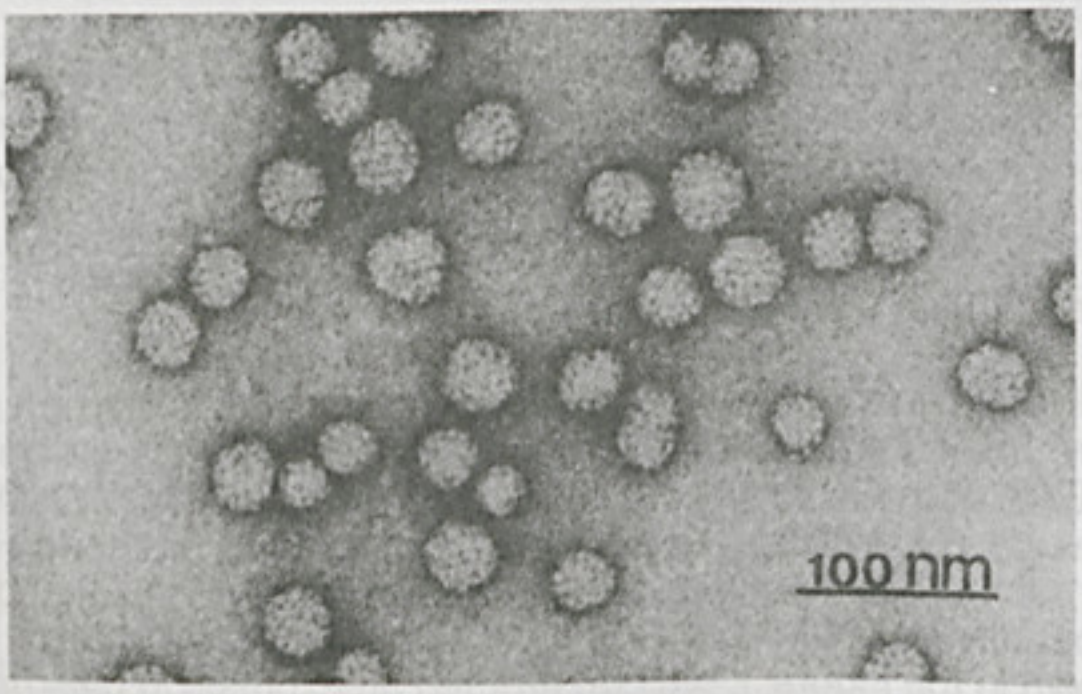

Figure 2 Electron micrograph of yHBsAg particles from recombinant yeast

Preparation of yeast-derived HBs antigen (yHBs antigen) and its physicochemical characterization.

The transformant yeast was cultured in Burkholder medium (5) and the synthesis of yHBs antigen was induced in a low phosphate content medium. The yeast cells were disrupted by a high pressure homogenizer and the crude extract was clarified with silica and charcoal treatments. Those steps were followed by isopycnic banding in KBr and rate zonal centrifugations in sucrose gradients by which the yHBs antigen was highly purified. As shown in Figure 2, the yHBs antigen had a spherical form 24 nm to 34 nm in diameters in electromicrographs which was similar to the HBs antigen particles seen in the patients serum during HBV infection.

An immunodiffusion analysis for the yHBs antigen showed that epitopes on the yHBs antigen were identified by a anti HBs goat serum against human native HBs antigen. The yHBs antigen banded at a density of 1.2 in CsC1 gradient and its molecular weight and isoelectronic point were about 24K and pI4.5, respectively.

The amino acid composition of yHBs antigen was almost identical with the one expected from the nucleotide sequence of the HBV DNA in the yeast transformant.

Circular dichroism analysis of this antigen showed a typical CD-spectrum for - helix protein structure and it did not change in the presence of 0.1% SDS or 7.2 M urea. This results suggested that the yHBs antigen was rigid and stable in the secondary structure.

Chemical composition of the yHBs antigen particles is summarized as follows. The contents of protein, lipid and carbohydrate were 53%, 36%, and 11%, respectively. The protein in the yHBs preparation consists of greater than 99% of HBs antigen determined by ELISA, HPLC, PCA in rats and SDS-PAGE. Carbohydrate was mainly mannose probably originated from host cell. However, any sugar units covalently bound to the yHBs peptide could not be detected. Yeast DNA in the preparation was determined to be less than 10 pg/20 g of yHBs antigen.

Immunogenicity of yHBs vaccine in animals

The immunological potency of the yHBs antigen was tested in animals. The antigens were adsorbed to aluminum hydroxide gel and given subcutaneously to the animals. Five weeks old BALB/C mice were injected once with 1 ml of serially diluted antigen and 5 weeks after immunization, anti-HBs titers were determined by using a commercial kit (PHA, Fujirebio., co., Ltd.) High titer antibodies against yHBs antigen were raised in the mice and our yHBs antigen preparation was determined to be more immunogenic than the plasma-derived HBs antigen.

Figure 3 showed the result of immunization of cynomologus monkeys with yHBs antigen. The monkeys were immunized subcutaneously with 10 μg yHBs antigen adsorbed to aluminum hydroxide 250 μg or 125 μg per dose at 4 weeks interval. The additional inoculum was given at 6 months after the first injection. The monkeys also responsed well.

Clinical trials of yHBs vaccine in healthy adults

The vaccine was prepared for the clinical trial to see side effects, and efficacy in differences of dose, administration route, sex and age in healthy adults who were negative for hepatitis B serological markers. The purified yHBs antigen was treated with formalin to stabilize the immunogenicity of antigen and adsorbed to aluminum hydroxide gel. Each dose in 0.5 ml contained either 10 μg or 20 μg of yHB antigen as well as 0.25 mg of aluminum hydroxide and 0.01% thimerosal as a preservative in phosphate buffer, pH 7.2. The vaccine was given thrice subcutaneously or intramuscularly at 0, 1 month, and 6 months.

None of the 453 vaccinees were reported to have a serious symptom attributable to the vaccine. Mild pain at the injection site was informed in 6% to 22% of the vaccinees. Only mild urticaria noted on the day of injection was reported in a single

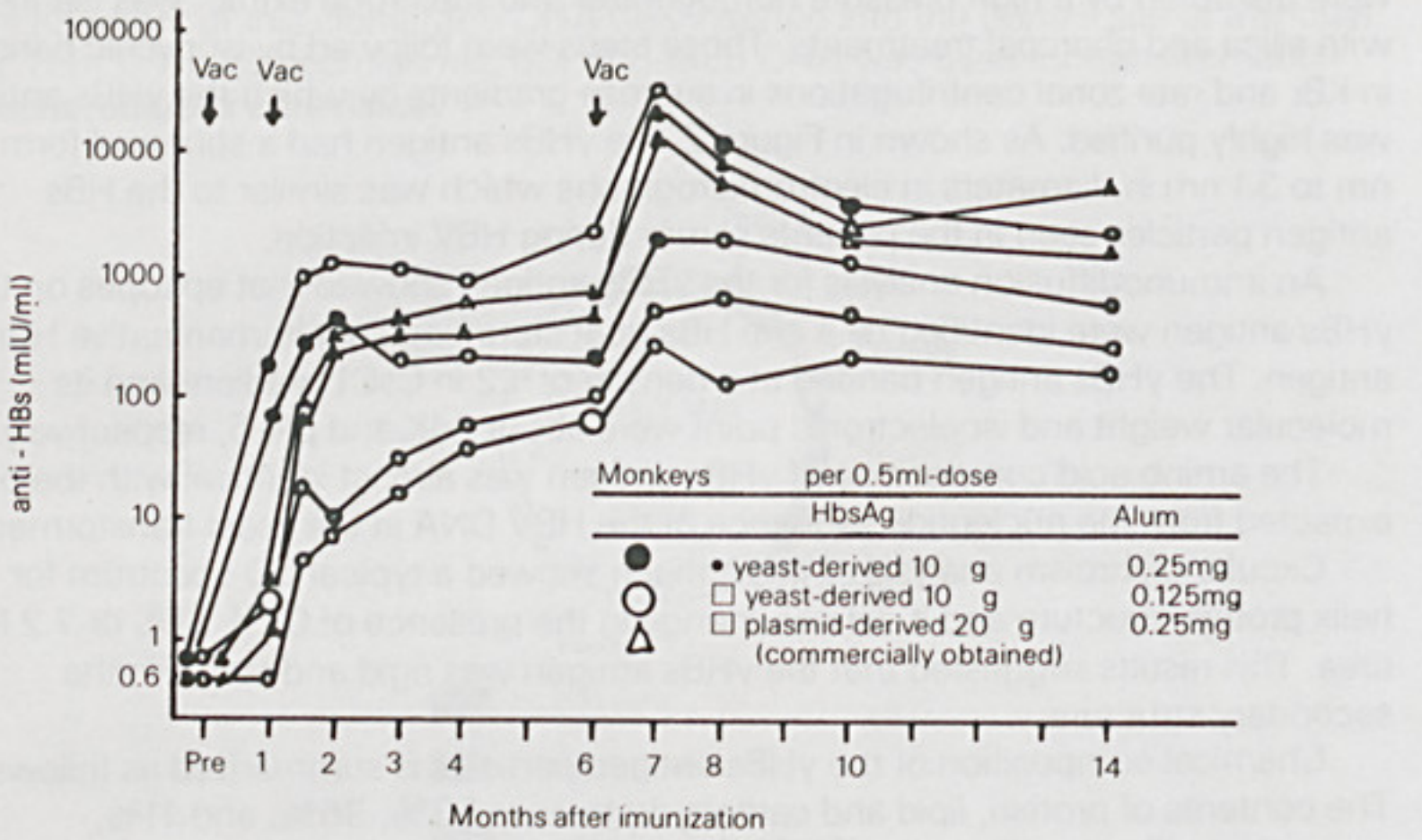

Figure 3 Immune response of cynomolgus monkeys injected with yeast-or human plasma-derived HB vaccine

case. In only several cases, local redness, headache and fatigue were described but these were quite mild and transient. There was no dose-dependency in these side effects, and their incidence did not increase in the time course of vaccination. Furthermore, there is no difference in side effects among the route of administration.

Analysis of serum sample before and after vaccination revealed no increases in yeast antibody titers as measured by ELISA and Western blot.

Table 1 shows the seroconversion rates and anti-HBs titer in the 453 subjects. The seroconversion rates at one month after the second dose were 60% to 80% and were raised to 86% to 94% at 4 months after the second dose. Finally one month after the third dose, 94% to 98% vaccinees responsed to have detectable amount of anti-HBs. When 20 μg of yHBs vaccine was given subcutaneously, at one month and two months, the seroconversion rate was slighly higher than the other groups given 10 μg of the antigen. However, at 6 and 7 months, there is no significant difference of seroconversion rates among these 4 groups.

The geometric mean anti-HBs antibody titer among responders was 141 or 200 mIU/ml at 7 months after subcutaneous injection of 10 ug or 20 ug of the yHBs vaccine, respectively (Table 1). In the group given 10 ug or 20 ug of the vaccine intramuscularly, the antibody titer was 281 or 363 mIU/ml at 7 months, respectively which tended to be higher than the titers determined in subcutaneous injection groups. The female and younger vaccinees responsed better than man or older vaccinees to the yHBs vaccine.

By giving the third dose at 4 months despite of 6 months, anti-HBs seroconversion rate in females one month after the final inoculation was 95.3% which is equivalent to the values observed in the standard inoculation schedule. However in male vaccinees, the seroconversion rates was 80.3% which is slightly lower than these. Again anti-HBs titer in female is higher than male.

Table 1 Immune responses in healthy adults vaccinated with recombinant yHBs vaccine(BR-HB)

Post.vaccination		1 month		2 months		6 months		7 months	
Route	Dose	seroconversion rate (%)	GMT*	seroconversion rate (%)	GMT	seroconversion rate (%)	GMT	seroconversion rate (%)	GMT
S.C	10µg	24/217(11.1)	5	132/214(62.1)	10	201/225(89.3)	25	188/200(94.0)	141
	20µg	11/53 (20.8)	3	42/52 (80.8)	5	46/49 (93.9)	40	49/50 (98.0)	200
i.m	10µg	7/67 (10.4)	3	44/65 (67.7)	13	56/65 (86.2)	32	60/64 (93.8)	281
	20µg	5/44 (11.4)	23	30/42 (71.4)	11	40/43 (93.0)	22	41/42 (97.6)	363

‡ Geometric mean titer of anti-HBs (mIU/ml)

Conclusion

A fragment of a cloned HBV-DNA (subtype adr) containing whole coding region for the HBs Ag was expressed in **S. cerevisiae**.

yHBs Ag thus produced was highly purified and characterized with physicochemical techniques. Amino acid and sugar analysis for the antigen peptides suggested their composition of about 236 residues certaining 9 additional residues of the C-terminus of preS2 region and lacking the carbohydrate units. The morphological appearance and physicochemical properties were similar to those seen in human plasma during HBV infection. HB vaccine was prepared for the clinical trial from purified formalin treated yHBs antigen adsorbed to aluminum hydroxide and given subcutaneously at 0,1 and 6 months to healthy adults volunteers to see side effects and immunogenicity.

It was concluded that our recombinant yHBs vaccine is safe and highly immunogenic. A clinical trial is still continuing to evaluate the efficacy of this recombinant vaccine in general population and babies born to HBV carrier mothers.

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SYNTHETIC PEPTIDES AS THE BASIS FOR ANTI-VIRAL VACCINES

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One of the exciting lines of investigation in immunology today is to establish whether synthetic peptides or their conjugates might replace the presently existing vaccines for providing protection against infectious diseases, including viral disease. In our early studies this approach was demonstrated to be feasible in the case of a model system, namely the bacteriophage MS-2. We demonstrated that a synthetic conjugate, containing a 20 amino acid residue peptide corresponding to a part of the phage's coat protein, elicited in rabbits antibodies with efficient anti-viral neutralizing activity (1). Furthermore, attachment of the synthetic adjuvant muramyl dipeptide to the above conjugate resulted in a completely synthetic anti-MS-2 vaccine with built-in adjuvanticity (2). Similar results were subsequently achieved in various laboratories with synthetic peptides corresponding to an antigenic determinant of diphtheria toxin (3), or to the immunizing fragment of M protein of Streptococcus pyogenes (4). The synthetic approach has been applied in the case of several animal viruses as well. Studies with hepatitis B, conducted by five different groups, all led to synthetic peptides which elicited antibodies reactive with the intact virus, in one case even inducing partial protective immunity in chimpanzees (5). In the case of foot and mouth disease virus, a 20 amino acid residue peptide of the VP_1 protein elicited neutralizing antibodies in guinea pigs that led to their protection against infection with the virus (6).

In our laboratory we have studied the system of the influenza virus. We have synthesized an 18-amino acid residues peptide corresponding to sequence 91-108 of the hemagglutinin of H3 influenza strains, and conjugated it to tetanus toxoid. This conjugate elicited in rabbits and mice anti-peptide antibodies that reacted also with the intact influenza virus of several A type H3 strains. Moreover, these antibodies were capable of inhibiting the hemagglutination activity of the relevant strains and to interfere with the *in vitro* growth of the virus in tissue culture. More importantly, mice immunized with the peptide-toxoid conjugate were partially protected against further challenge infection with several strains of influenza H3 virus (7). It should be noted that this particular sequence, which is common to at least twelve strains of influenza, comprises a folded region in the hemagglutinin peptide chain, which is adjacent to a proposed antigenic site. This could explain its cross-

strain protective effect. In this case as well MDP attached to the conjugate served as a built-in adjuvant (8).

More recent data are indicative of two other hemagglutinin synthetic peptides corresponding to the sequences 138-164 and 181-200 of the molecule, respectively, that also elicit protective immunity. One of these peptides comprises the ''loop'' region of the hemagglutinin (140-146) which is considered to form the entire antigenic site ''A'', and in addition a part of antigenic site ''B'' of the molecule. Conjugate of this peptide with tetanus toxoid induced in rabbits antibodies that cross-reacted with the intact virus. Furthermore, immunization of mice with this synthetic vaccine also resulted in partial protection against challenge infection (9). Conjugates containing the octapeptide 139-146 induced anti-peptide antibodies which did not recognize the virus even though anti-viral antibody reacted with the free peptides. The peptide 181-200 also elicited anti-influenza immune response with partial protection against infection. In the case of this peptide we could also demonstrate cellular immune response (10), which could be a significant factor in the future design of synthetic vaccines.

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