New Non-replicative Vectors to Induce Protective Anti-viral and Anti-tumor Cytotoxic T Cell Responses

Claude Leclerc

Unité de Biologie des Régulations Immunitaires, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France.

Introduction

$CD8^+$ cytotoxic T lymphocytes (CTLs) play an important role in the elimination of cells infected by pathogens and in the regression of tumors. CTLs recognize antigen-derived peptides presented by major histocompatibility (MHC) class I molecules on the cell surface and are usually activated by peptides resulting from the processing of endogenous intracellular proteins. Because antigens have to gain access to the cytosol to enter the class I-restricted presentation pathway, exogenous soluble proteins are usually unable to stimulate CTL responses. Therefore, several strategies have been developed to deliver exogenous antigens into the cytosol. Protein or peptide antigens delivered in association with appropriate adjuvants efficiently stimulate CTL responses. However, alum is still the only adjuvant currently licensed for use in human vaccines. Several recombinant bacterial or viral live vectors have also been shown to sensitize CTLs *in vivo* but are risk-prone. DNA vaccination may also represent a powerful strategy to activate CTL responses, but the safety of this method remains to be determined. Therefore, the development of safe strategies to induce CTL responses with non-replicating antigens is still an important prerequisite for the design of new efficient vaccines.

Recently, we have developed new and efficient non-replicative recombinant vectors able to deliver antigens into the MHC class I pathway based either on the invasive property of a bacterial toxin or on the capacity of parvovirus-like particles to deliver foreign CTL epitopes into the cytosol of cells.

Recombinant adenylate cyclase toxins of *Bordetella pertussis*

The adenylate cyclase toxin (CyaA) of *B. pertussis* is able to invade a large number of eukaryotic cells and to deliver its *N*-terminal catalytic domain (400 amino acid residues) directly into the cytosol through the cytoplasmic membrane [1, 2]. Foreign peptides can be inserted into various permissive sites of the catalytic domain of CyaA without altering its main properties such as stability, and catalytic and invasive activities [3, 4]. Purified CyaA toxins carrying CTL epitopes from the nucleoprotein of LCMV or from the V3 region of HIV-1 gpl20 were shown to stimulate strong specific CTL responses, mediated by class I-restricted $CD8^+$ T cells and able to kill target cells coated with the relevant peptide [5].

Mice immunized with the recombinant toxin carrying the LCMV epitope developed strong CTL responses against LCMV-infected target cells. Moreover, these mice were protected against an intracerebral challenge with a virulent strain of LCMV that killed all non-immunized mice within 7 days [6]. This protection was abolished after *in vivo* elimination of $CD8^+$ T cells. A mutant toxin devoid of adenylate cyclase activity (i.e., cAMP synthesizing activity) was constructed by insertion of a dipeptide into the catalytic site of the molecule. This genetically detoxified invasive toxin carrying the LCMV epitope also stimulated a strong CTL response against both peptide-coated and virus-infected target cells. Mice immunized with this detoxified molecule were fully protected against a lethal intracerebral LCMV challenge [6].

The capacity of CyaA to also induce anti-tumor therapeutic T cell responses was recently demonstrated using melanoma cells transfected with ovalbumin. Mice immunized with a recombinant CyaA toxin carrying a CTL epitope derived from ovalbumin were able to reject tumors that express ovalbumin [7].

These results, which represent the first demonstration of the *in vivo* induction of protective CTL responses by a detoxified invasive toxin illustrate the strong potential of this strategy to develop new preventive or therapeutic vaccines against viral infections or cancers. Recent results will be also presented which further document the efficiency of recombinant adenylate cyclases to stimulate responses against various CTL epitopes. Mechanisms by which the epitopes carried by the recombinant toxin are delivered to MHC class I molecules have also been recently deciphered [8].

In particular, we have established that the recombinant CyaA is currently processed and presented like an endogenous cytosolic antigen by a mechanism requiring: 1) processing by the proteasome, 2) the TAP molecules, 3) the neosynthesis of MHC class I molecules [8].

Recombinant parvovirus-like particles

Hybrid recombinant parvovirus-like particles (PPV:VLP) are formed by the self-assembly of the VP2 capsid protein of PPV carrying a foreign epitope at its N-terminus. Chimeric PPV:VLPs expressing a $CD4^+$ T cell epitope from the VP1 protein of poliovirus type 1 or from the PreS2 region of hepatitis B virus stimulated $CD4^+$ T cell proliferative responses and cytokine production [9, 10].

Hybrid PPV:VLP carrying a $CD8^+$ T cell epitope from the LCMV nucleoprotein induced in mice strong CTL responses against both peptide-coated or virus-infected target cells [11]. These CTL responses were obtained in the absence of any adjuvant but immunization of mice with hybrid PPV:VLP in the presence of alum did not prevent CTL activation. lnterestingly, the $CD8^+$ class I-restricted cytotoxic activity induced by these recombinant particles persisted *in vivo* for at least 9 months.

Furthermore, the hybrid parvovirus-like particles carrying the LCMV epitope were able to induce a complete protection of mice against a lethal LCMV

Trabajos seleccionados del Congreso Biotecnología Habana`99. Noviembre 28 a diciembre 3 de 1999.

Selection of papers from Biotecnología Habana`99 Congress. November 28-December 3, 1999.

1. E Hanski. TIBS 1989;14:459–63.

2. M Mock, A Ullmann. Trends Microbiol 1993;1:187–95.

3. D Ladant, P Glaser, A Ullmann. J Biol Chem 1992;267:2244–50.

4. P Sebo, *et al.* Inf Immun 1995;63:3851–7.

5. C Fayolle P, *et al*. J Immunol 1996;156: 4697–06.

6. MF Saron C, *et al*. Proc Natl Acad Sci USA 1997;94:3314–9.

7. C Fayolle, *et al.* J Immunol 1999;4157–62

8. P Guermonprez, *et al.* J Immunol 1999; 162:1910–6.

9. C Sedlik, *et al.* J Gen Virol 1995;76: 2361–8.

10. R Lo-Man, *et al.* Eur J Immunol 1998; 28:1401–7.

11. C Sedlik, *et al.* Proc Nati Acad Sci USA 1997;94:7503–8.

infection. These results represent the first demonstration that hybrid non-replicative VLP carrying a single viral CTL epitope can induce protection against a viral lethal challenge in the absence of any adjuvant [11].

The capacity of these pseudo-particles to stimulate cellular or humoral mucosal responses has also been recently investigated. Intranasal immunization of mice by PPV:VLP expressing the LCMV epitope induces CTL responses. Moreover, anti-PPV IgA antibody responses can also be evidenced in mucosal fluids of intranasally immunized animals [12].

Altogether, these results illustrate the strong potential of these alternative new strategies to develop vaccines able to stimulate cellular immunity. Moreover, these new vectors represent important new tools to dissect the various MHC class I presentation pathways.

12. C Sedlik, *et al.* J Virol 1999;73:2739–44.

Intranasal Immunization Using HBsAg-Acemannan Formulations

Aguilar JC, Pichardo D, Leal MJ, Crombet L, Pentón E, Muzio V, Guillén G

División de Vacunas. Centro de Ingeniería Genética y Biotecnología.
AP 6162, CP 10600, Ciudad de La Habana, Cuba.

*I*ntroduction

At present, the search for new adjuvants and immunological stimulators, as well as the development of new ways of delivering antigens and pharmaceuticals, is one of the lines of world research in the pharmaceutical field, especially in vaccines [1].

Acemannan is a polysaccharide composed of mannose with O-acetylations, in approximately 8 out of every 10 mannoses. lt is extracted as a major component of the mucilaginous substance or leaf gel of *Aloe barbadensis* Miller, a medicinal plant used throughout history.

Different *in vitro* tests indicate that mannans activate monocytes and macrophages inducing the production of IFN-γ, TNF-α, GM-CSF, IL-1β and IL-6 [2]. The purposes of this work are: a) to demonstrate the enhancing activity of acemannan on the humoral immune response against hepatitis B surface antigen (HBsAg) through intranasal inoculation (IN), and b) to select the optimal adjuvant concentration range of acemannan.

*M*aterials and Methods

The first experiment was conducted in 5 groups of 8 Balb/c mice, 7 to 10 weeks old. Mice were inoculated twice, 2 weeks apart, through the nasopharyngeal route using volumes of 50 μL. All mice were ketamine-anaesthetized and inoculated as shown in figures A-D. Extraction was performed through retroorbital punction 28 days after the start of the immunization schedule. Titers were determined by an anti-total HBsAg ELISA using an international standard (mIU/mL).

In the second and third experiments, different dose levels of HBsAg were tested and the systemic inoculation controls were: intramuscular (IM) (Exp. 2) and subcutaneous (SC) (Exp. 3), both adsorbed to alum. Mice were inoculated three times. Titers were determined by ELISA to detect total IgG in sera (Exps. 2 and 3) and total IgA in vaginal washes (Exp. 3).

The concentration of acemannan was determined by the Antrona colorimetric method and was referred as mg of total hexoses/mL.

The statistical analysis was performed using the Student's *t* test and the F test to determine variance homogeneity ($p < 0.05$ was considered a significant difference).

*R*esults and Discussion

A strong enhancing activity was evidenced in the groups in which acemannan was added (groups 2-5). Antibody titers in all groups were significantly higher than that of the control of HBsAg in PBS (group l). The excessive increase of polysaccharide concentration generated an inhibitory effect (group 5). This was probably due to an increase of the resulting viscosity. The optimal adjuvant concentration ranged between 0.150 and 0.450 mg/mL.

Experiments 2 and 3 demonstrate the strong effect of acemannan when mixed with HBsAg. In both cases, the intensity of IgG titers generated in sera after alum-adjuvated and acemannan-adjuvated groups was similar (Exp. 2, groups 2 and 3, and Exp. 3, groups A and B). No difference was observed for serum IgG titers after IM and SC inoculations, compared with the corresponding dose level of HBsAg inoculated nasally in acemannan.

In Exp. 2, the serum IgG response for the acemannan group was higher than that obtained with HBsAg in phosphate-buffered saline (Exp. 2, groups 1 and 2). In Exp. 3, only group A (nasally-immunized mice using acemannan) generated a strong vaginal IgA response.

*C*onclusions

1. We have demonstrated that the nasal route can be as efficient as systemic routes in the induction of anti-HBsAg antibody responses in serum, with the advantage of inducing strong mucosal responses.
2. The acemannan optimal adjuvant concentration range is 150 to 450 μg/mL when used nasally along with HBsAg.

1. Report of the Expert Panel VI: Concerted efforts in the field of mucosal immunology. Vaccine 1996;14:644–64.

2. Peng SY, *et al.* Mol. Bio-ther 1991; 3:79–87.

New Technical Developments of Automates for High Sensitive Analytical Biotechnology

Christian Wurzel, Brigitte Wittmann-Liebold, WITA GmbH

Warthestr. 21, D-14513 Teltow, Germany. E-mail: wurzel@wita.de

The research field of proteomics shifts the focus of biochemical interest to functional analysis. Whereas genome analysis reflects only the static state of the inherited information, the visualization and protein identification of entire cell extracts at different cell stages (proteomics) yields information on dynamic situations and hence, on the function of proteins. At least in parts protein separation and analysis is highly sensitive and automated, and concepts of high throughtput proteomics become visible due to new technical developments. Applications of the micro system technology make these systems smaller and faster. In opposite to DNA analysis, where PCR technique enables sample reproduction, protein analysis has to fight sensitivity problems. This is especially the case when limited material obtained by cell culturing or from biopsies and body fluids shall be analyzed. In the past, due to many technical innovations, the sensitivity of the Edman degradation of proteins and peptides could be increased from micromolar amounts requested in the first amino acid sequencer constructed by Edman and Begg [1] to low pico molar protein quantities necessary in the present day's automates. However, yet, the sensitivity has to be further enhanced in order to enable the analysis of proteins and peptides which are expressed in extremely low concentrations. With the use of high resolution 2-dimensional gel electrophoresis, which allows to resolve up to 10,000 proteins of a complex protein mixture from entire cells or tissue extracts [2], differences in the protein expression during differentiation and embryonal development can be traced [3]. Other proteome studies are concerned with antibiotic resistance, drug screening, and tumor development when apoptosis is inhibited [4]. In the last few years, new technical developments in mass spectrometry facilitated high sensitive measurements of peptides and allowed to obtain peptide masses and partial sequences for identification of the protein spots by PSD-MALDI-MS or nanospray-ESI-techniques [5, 6]. On the other hand, some disadvantages of these techniques are:

a) the time consuming interpretation of the spectra
b) misleading data that may be obtained due to pro tein impurities
c) rather short partial sequences
d) high instrument costs of the mass spectrometer.

While identification and confirmation of known proteins are mostly straightforward by these techniques, the interpretation of the data for unknown or modified proteins is much more difficult. Hence, alternative approaches, e.g. by direct amino acid sequencing employing the Edman chemistry, serve as an additional method that allows more extended sequence results and, therefore, an unambiguous assignment of the investigated proteins. A high sensitive Edman type protein sequencer has to cover at least three innovations:

- the liquid handling part for minute sample and reagent amounts
- modifications in chemistry
- a highly sensitive detection system for the released amino acid derivatives and its on-line connection to the sequencer.

Liquid handling

In present day's sequencers, the consumption of reagents and solvents is relatively high; typically microliter volumes in the range of 20 to 200 are delivered due to the general construction of the machines and of the reactor, converter and the valves and their dimensions. The amounts of solvents are high, mainly in order to clean up and wash the delivery lines. The reagents adhere to the inner surface of the dosing system and represent liquid and dried remainders which may disturb the chemistry of the degradation and reduce the yields appropriately. We present an integrated approach to overcome these drawbacks. In the micro reaction and analysis system, all liquid handling devices as valves and reaction chambers necessary to perform the Edman degradation are fully integrated. The dimension of the valves and reactor and converter are considerably reduced. Therefore, the switching time of a valve (the response time) in the microreaction system is much faster than in common valves or valve systems. The smallest amounts of a reagent in sub micro liter amounts can be delivered. Due to the complete integration, no external connecting lines between the parts are necessary. The inner surface is much decreased, no hidden edges due to ferrules or fittings occur. Reagent delivery, washings and dryings of the lines can be substantially reduced [7, 8].

Modifications in chemistry and the detection system

Commercial sequencers are equipped with 1-IPLC and UV-detection. This limits the sensitivity to the upper femtomolar range. These systems cannot overcome the Lambert-Beer's Law. To gain higher sensitivity in protein sequencing, changes to the detection system are necessary, but require also changes in the chemistry. Generally two options are given:

a) a modification of the coupling reagent [9]
b) a post degradation fluorescent labelling, or an additional post-cleavage reaction which couples a high sensitive marker to the released amino acid derivative [10]. These methods allow to connect a capillary electrophoresis with laser induced fluorescence detection (CE-LIF) or a mass spectrometric (MS) detection system directly to the Edman sequencer. These innovations are under development.

1. Edman P, Begg G. A protein sequenator. Eur J Biochem 1967;1:80–97.

2. Klose J, Kobalz U. Two-dimensional electrophoresis of proteins. An updated protocol and implications for a functional analysis of the genome. Electrophoresis 1995;16:1034–59.

3. Brockstedt E, Peters-Kottig M, Badock V, Hegele-Hartung C, Lessl M. Identification of a new LI-1/CG-regulated protein and the corresponding gene in the mouse ovary: Mouse vas deferens protein (manuscripts submitted).

4. Brockstedt E, Rickers A, Kostka S, Laubersheimer A, Dörken B, Wittmann-Liebold B, *et al*. Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line, cleavage of hnRNP AI by caspase 3. J Biol Chem 1998;273: 28057–64.

5. Spengler B, Kirsch D, Kaufmann R. Metastable decay of peptides and protein in matrix-assisted laser-desorption mass spectrometry. Rapid Commun Mass Spectrom 1991;5:198–202.

6. Wilm M, Shevchenko A, Houtheave T, Breit S, Schweigerer L, Fotsis T, *et al*. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 1996;379(6564): 466–9.

7. Wurzel C, Wittmann-Liebold B. A new design of a wafer based micro Reaction System. In: Microreaction technology. W. Ehrfeld (editor) Springer;1998.p.219–24.

8. Wurzel C, Wittmann-Liebold B. New Approaches for Innovations in Sensitive Edman Sequencing. The Design of a Wafer Based Chip Sequencer. In P Jolles, H Jörnvall (editors.). Protein Sequences. Novel strategies and proteome analysis. Birkhäuser Verlag, Basel, in press.

9. Hess D, Nika H, Chow DT, Bures EJ, Morrison HD, Aebersold R. Liquid chromatography-electrospray ionization mass spectrometry of 4-(3-pyridinylmethylaminocarboxypropyl) phenylthiohydantoins. Anal Biochem 1995;224:373–81.

10. Tsugita A, Kamo M, Shikama N. Sensitization of Edman amino acid derivatives using the fluorescent reagent 4-aminofluorescein. J Biochem 1989;106:60–5.

Immunological Evaluation of Melanoma Patients Immunized with an Anti-idiotype Vaccine Mimicking NeuGe-containing Gangliosides

Mauro Alfonso,[1] Ana María Hernández,[1] Edmundo Rodríguez,[2] Noel Cordiés,[2] Rolando Pérez,[1] Ana María Vázquez[1]

[1]Center of Molecular Immunology. Havana, Cuba. E-mail: mauro@ictcim.sld.cu [2]National Institute of Oncology and Radiobiology, Havana, Cuba.

*I*ntroduction

Tumor associated gangliosides are very poorly immunogenic carbohydrate self-antigens. One strategy for inducing antibodies against gangliosides involves the use of anti-idiotype monoclonal antibodies (Ab2 MAbs) as antigen surrogates, a strategy which exploits the possibility that a ganglioside epitope of carbohydrate nature may be presented by a protein epitope on an antibody molecule, making it more immunogenic. In fact, Ab2 MAbs that mimic gangliosides highly expressed on tumor cells, such as GM3, GD3 and GD2, have been obtained. All of them had the property to induce circulating antibodies specific to the corresponding ganglioside when they were injected into syngeneic or xenogeneic animals. Promising results have been obtained in clinical trials where some of these Ab2 MAbs have been used together with BCG or QS21 adjuvants to treat cancer patients [1, 2].

We have generated and characterized an Ab2 murine MAb to a murine Ab1 MAb named P3, which recognizes specifically N-glycolyl sialic acid on several monosialo- and disialogangliosides. The IgG1 Ab2 MAb obtained was designated as 1E10 [3].

Here we describe the immune response induced in the first ten melanoma patients vaccinated with aluminum hydroxide precipitated-1E10 MAb.

*M*aterials and Methods

Patients with advanced malignant melanoma were treated with six doses of 2 mg of aluminum hydroxide-precipitated 1E10 MAb, injected intradermically into multiple sites at two -week intervals. Serum was obtained pre-treatment and fourteen days after each immunization.

The presence of Ab3 in the sera of patients was determined in a solid-phase ELISA using 1E10 MAb and irrelevant isotype-matched mouse MAbs. To ascertain whether Ab3 present in patients' sera shared idiotopes with P3 MAb (Ab1), their sera were assessed for their ability to inhibit the binding of biotinilated 1E10 to P3 (Ab1) in an ELISA assay.

Binding of patients' Ab3 to purified gangliosides was determined using an indirect ELISA assay described previously and the specificity was assessed by high -performance thin-layer chromatography (HPTLC) and enzyme immunostaining [4].

Immunoglobulin isotypes and subclasses were determined by the anti-1E10 and anti-ganglioside ELISA using antihuman class and subclass specific reagents (Pharmingen).

*R*esults and Discussion

We have generated the 1E10 g-type anti-idiotype monoclonal antibody (Ab2 MAb) [3] that induced anti-anti-idiotypic antibodies (Ab3) in syngeneic and xenogeneic animal models, characterized by bearing P3 MAb idiotopes but it failed to elicit Ab3 antibibodies with the same antigen specificity than P3 MAb (Ab3, Id+Ag-). The 1E10 MAb was able to inhibit pulmonary metastases in murine models (un - published data).

A clinical trial is been carried out for patients with advanced malignant melanoma treated with six intradermal injections of aluminum hydroxide-precipitated 1E10 anti-idiotype MAb. Toxicity has included local reaction at the site of injection with induration and erythema, sometimes associated with mild pain that resolved in a few days (24-48 h). Fever (Grade I-II, WHO) and chills occurred in only a few patients (toxicity as grade I). The median survival time was 47 (95% confidence interval, 35-69) weeks with a 50% one-year survival at the moment of evaluation.

The analyses of the humoral immunity induced by immunization with 1E10-aluminum hydroxide was performed by testing sera obtained from patients before vaccination and two weeks after each immunization. Hyperimmune sera from 9 of the 10 patients included in the study developed anti-anti-idiotypic-Ab3 antibody response capable to inhibit Ab2 binding to Ab1 (Ab3 1d). An anti-ganglioside antibody response was induced in the patients against N-glycolyl-containing gangliosides (Ab3 Ag+) and the specificity was confirmed by HPTLC immunostaining. The isotype of the antibody response induced against 1E10 MAb was predominantly IgG (IgG4 and IgG2 subclasses) showing antibody titers ranging from 1:10,000 to 1:100,000, while the isotype of the antibody response against gangliosides was IgG (mainly IgG2) and IgM, showing antibody titers ranging from 1:100 to 1:3,200.

In conclusion, our vaccinal preparation has demonstrated to be immunogenic and non-toxic in most of the 10 melanoma patients included in the study.

1. McCaffery M, Yao TJ, Williams L, Livingston PO, Houghton AN, Chapmann PB. Immunization of melanoma patients with BEC2 anti-idiotypic monoclonal antibody that mimics GD3 ganglioside: enhanced immunogenicity when combined with adjuvant. Clin Cancer Res 1996;2: 679–86.

2. Foon KA, Sen G, Hutchins L, Kasbala OL, Baral R, Banerjee M, *et al*. Antibody responses in melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. Clin Cancer Res 1998;4:1117–24.

3. Vázquez AM, Pérez A, Hemández AM, Macías A, Alfonso M, Bombino G, *et al*. Syngeneic anti-idiotypic monoclonal antibodies to an anti-NeuGc-containing ganglioside monoclonal antibody. Hibridoma 1998;17:527.

4. Alfonso M, Lanne B, Ifverssen P, Vázquez AM, Pérez R, Portoukalian J, *et al*. Antigen-specific primary immune response of human B lymphocytes after *in vitro* immunization with GM3 ganglioside. Hum Antibod Hybridomas 1995;6(3):102–12.

Goals and Feasible Techniques of Proteome Analysis in the Year 2000

Brigitte Wittmann-Liebold,[1] Christian Scheler[2]

[1]Max-Delbrueck-Centrum for Molecular Medicine, Robert-Roessle-Str. 10 D-13125 Berlin-Buch. E-mail: liebold@mdc-berlin.de [2]Wita GmbH. Warthestr. 21. 14513 Teltow, Germany. E-mail: scheler@wita.de; http://www.wita.de

Proteome analysis offers several new approaches in bioscience research. Compared to genome analysis which defines a static situation of the cell, the proteome describes dynamic states of protein expression during cell development, differentiation or in cell disorders. Therefore characterization of protein extracts of different cell states becomes increasingly important for identification of disease-associated proteins, of development of early diagnosis markers, and for drug screening. Many proteins show variants and modifications which contribute to their functions like phosphorylation or glycosylation. In opposite, gene analysis does not allow to identify or predict these modifications and therefore only reflects a static situation of the inherited information. Furthermore, there is no direct correlation between mRNA and protein concentration, due to different half life times of mRNA and proteins and protein processing and translocation [1]. Nonetheless, the knowledge of genetic sequences stored in databases is essential for identification of proteins by mass spectrometric techniques used in proteome analysis.

A prerequisite for proteome analysis is a method for a high resolution separation of protein extracts of entire cells. At present two-dimensional electrophoresis (2DE) is the only available method of choice with a resolution of more than 5000 proteins. The best separation technique was developed by Klose and Kobalz [2], which enables these high resolutions in combination with unambiguous identification of the resulting protein spots. We used a combination of in-gel digestion [3] of the protein spots followed either by matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) peptide mass fingerprinting or nano-electrospray ionization mass spectrometry (ESI-MS) for protein identification. We built up 2D-databases for heart muscle proteins, and for B-cell proteins identified at different stages in IgM-induced apoptosis.

Future techniques will implement more and more automation of the different analysis steps leading to high throughput characterization and identification of many proteins of the cell extracts. These possibilities open a wide range of new research fields, e.g. in antibiotic screening [4], identification of proteins associated with apoptosis [5], drug effects [6], infectious diseases [7] and cancer [8].

Such new techniques for proteome analysis, for instance, imply the development of automates of the 2DE method and reaction devices on wafer basis for the analysis and derivatization of minute sample amounts as developed in our lab. Further steps involve the combination and integration of the different partially automated wet laboratory techniques starting from two-dimensional separation, gel staining, spot cutting, in-gel digestion to mass spectrometry. Dry lab techniques like automatic spot detection, matching of 2DE gels, database searches and storage of information in suitable databases have also to be automated and combined with the wet lab methods to achieve a high throughput analysis of proteomics in the future.

1. Haynes PA, Gygi SP, Figeys D, Aebersold R. Proteome analysis: biological assay or data archive? Electrophoresis 1998;19: 1862–71.

2. Klose J, Kobalz U. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. Electrophoresis 1995;1:1034-59.

3. Otto A. Thiede B, Müller EC, Scheler C, Jungblut, P. Identification of myocardial proteins by 2-D gel electrophoresis using an efficient sample preparation for mass spectrometry. Electrophoresis l996;17:1643–50.

4. Marzocchi B, Magi B, Bini L, Cellesi C, Rossolini A, Massidda 0, *et al.* Two-dimensional gel electrophoresis and immunoblotting of human serum albumin modified by reaction with penicillins. Electrophoresis 1995;16:851–3.

5. Brockstedt E, Rickers A, Bommert K, Dörken B, Wittmann-Liebold B, Otto A. Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line. J Biol Chem 1998;273:28057–64

6. Anderson NL, Anderson NG. Proteome and proteomics: new technologies, new concepts. and new words. Electrophoresis 1998;19:1853–61

7. McAtee CP, Fry KE, Berg DE. Identification of potential diagnostic and vaccine candidates of *Helicobacter pylori* by "proteome" technologies. Helicobacter 1998;3:163–9.

8. Zeindl-Eberhart E, Jungblut PR, Otto A, Rabes HM. Identification of tumor-associated protein variants during rat hepatocarcinogenesis. Aldosereductase. J Biol Chem 1994;269:14589–94.

"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"
"ISPMB Québec l'an 2000"

6th International Congress of Plant Molecular Biology

June 24-28, 2000. Québec, Canada

Organizer:
The International Society for Plant Molecular Biology and Université Laval

Contact:
Congress Secretary, ISPMB
c/o Agora Communication
2600, boulevard Laurier (suite 2680)
Sainte-Foy, QC, Canada G1V 4M6
Tel.: 418.658.6755
Fax: 418.658.8850
E-mail: dhagora@microtec.net
URL: http://www.uga.edu/ispmb

Intranasal Immunization Using HBsAg-Acemannan Formulations: Kinetics and Duration of Serum IgG Responses

Aguilar JC, Pichardo D, Leal MJ, Crombet L, Pentón E, Muzio V, Guillén G

División de Vacunas. Centro de Ingeniería Genética y Biotecnología.
AP 6162, CP 10600, Ciudad de La Habana, Cuba.

*I*ntroduction

The potency of an adjuvant and the duration of the response generated are two important parameters in the evaluation of any new vaccine formulation. Although the ability of CT to act as a mucosal adjuvant has been confirmed by a large number of researchers [1], CT and some other related proteins do not fulfill the classical definition of an adjuvant. CT stimulates an immune response against itself, and its adjuvant activity depends on its immunogenicity [2].

The purposes of this work are: a) to investigate the duration of the antibody (Ab) response obtained using acemannan as a nasal adjuvant for hepatitis B surface antigen (HBsAg), and b) to show the kinetic of antibody appearance in serum using CT and alum as controls and as nasal and systemic adjuvants, respectively.

*M*aterials and Methods

The schedule was carried out in 8-week-old Balb/c female mice with four inoculations: days 0, 14, 28, and 56, and the extractions were performed by retroorbital punction: days 26, 42 and 70. Titers were determined using the conventional ELISA for the detection of specific mouse IgG antibodies. All groups except group 4 were immunized nasally using volumes of 50 μL; group 4 was immunized through the subcutaneous route using volumes of 100 μL. Vaginal washes were performed with 100 μL of sterile PBS on day 70.

The concentration of acemannan was determined by the Antrona colorimetric method and was referred as mg of total hexoses/mL.

The statistical analysis was performed using the Student's *t* test and the F test to determine variance homogeneity ($p < 0.05$ was considered a significant difference).

*R*esults and Discussion

An statistical analysis after a second dose did not evidence significant differences between the alum control group and the nasal group with the same quantity of antigen. The group of mice immunized with CT as adjuvant by the nasopharyngeal route generated an antibody response higher than that of the group with the lower quantity of antigen and was not significantly different from that of the group with equal quantity of HBsAg.

After three immunizations, there was no statistical significant difference between the group of mice immunized intranasally and systemically with 2 μg. In the same way, there was no significant difference between the group of mice immunized with CT as adjuvant and the equivalent formulation under assay, both with l0 μg of HBsAg.

Considering the strong increase in titers from the second to the third dose, we worked on determining if this increase followed the same slope for the different groups assayed, after a fourth dose applied one month after the third inoculation.

Using acemannan as an adjuvant it is possible to obtain a response after a fourth dose. This response is higher than that obtained with CT in serum, as evidenced by comparing G2 and G5.

Then, through the nasopharyngeal inoculation of HBsAg mixed with polysaccharides it is possible to attain responses that are able to exceed in quantity and quality the response generated by systemic inoculations using alumina. Additionally, considering the fact that a strong mucosal antibody response was induced only by nasopharingeal route, the efficient and potentially innocuous human inoculation of HBsAg becomes a real possibility.

*C*onclusions

1. Nasal inoculations of HBsAg-acemannan formulations resulted in a long-term response.
2. The slope of the IgG response curve is higher for groups immunized with acemannan than for groups immuniced with alum and CT which generated stronger responses after four inoculations.

1. McGhee JR, *et al*. Vaccine 1992;10(2): 75–88.

2. Elson CO. Fed Proc 1987;46:1778.

Immune Response against Hepatitis C Virus Core Induced by DNA Administration Is Modulated When Additives Are Combined with the Core Encoding Vector

Liz Álvarez-Lajonchere, Santiago Dueñas-Carrera, Lázaro J Lorenzo, Dagmara Pichardo, Juan Morales

Centro de Ingeniería Genética y Biotecnología. PO Box 6162, Ciudad de La Habana, Cuba. E-mail: juan.morales@cigb.edu.cu

Introduction

DNA vaccines represent a novel means of expressing antigens *in vivo* for the generation of both humoral and cellular immune responses. Attempts to enhance immune responses against DNA-encoded antigens have included the combination of DNA constructs with liposomes, proteins and plasmids encoding cytokines or co-stimulatory molecules [1]. In this work, we evaluate the humoral immune response against the HCV core when different additives are combined with the DNA-encoding plasmid. We also compare protein and DNA immunization approaches in relation to the magnitude of the induced humoral immune response and the IgG2a/lgG1 antibodies ratio in the serum of immunized Balb/c mice.

Materials and Methods

Plasmids

The pAEC-K6 is an expression plasmid that contains the human cytomegalovirus immediate early promoter, the SV40 terminator, and the polyadenilation sequences and the bacterial replication origin from pUC. The pIDKCo is a pAEC-K6-derived expression vector containing the sequence encoding the first 176 aa of the HCV core protein.

Co. 120 protein

The Co. 120 is an *Escherichia coli* -derived protein containing the first 120 aa of HCV viral polyprotein. This HCV core protein has been estimated to be 95% pure by Coomassie staining and immunoblot analysis.

Plasmid DNA immunization

Pathogen-free Balb/c female mice of 6 to 8 weeks of age were purchased from CENPALAB (Ciudad de La Habana, Cuba) and used for all *in vivo* studies. Mice were injected at 0 and 3 weeks in the quadriceps muscle, with 100€µg of plasmid DNA in a 100 µL final volume of 0.9% NaCI solution. Co.120 protein was administered (10 µg per dose) in similar conditions. Serum samples were taken 5 and 14 weeks after primary immunization.

Results and Discussion

In this work, 10 groups of 5 animals were studied to analyze the effect of different additives over the anti-core specific humoral response generated after intramuscular DNA vaccination. Group 1 (control) was immunized with the pAEC-K6 plasmid. Mice were also inoculated with the pIDKCo plasmid alone (group 4) or combined with 100 mM $CaCl_2$ (group 5), 1% PEG 6000 (group 6), Freund's adjuvant (group 7), and 100 µg of sonicated calf thymus DNA. The truncated Co.120 protein was either evaluated alone (group 3) or in conjunction with the pIDKCo plasmid either simultaneously (group 2) or alternatively (primary immunization for group 9 and booster for group 10). Determination of anti-core specific antibodies in immunized mice was performed at 5 and 14 weeks after primary vaccination by an ELISA assay against the Co.120 protein. Although $CaCI_2$ and PEG have been widely used to facilitate DNA incorporation in eukaryotic cells [2], it was observed a very little and transient effect on the stimulation of anti-core immune responses when combined with plasmid DNA. Something similar accounts for the combination of pIDKCo with sonicated calf thymus DNA or Freund's adjuvant, and no statistical differences in the anti-core immune response were detected in comparison to the vector alone. However, Co.120 protein administration induced a definite stronger antibody response alone or co-injected with the pIDKCo plasmid (data not shown).

lnterestingly, the IgG2a/lgG1 ratio of anti-core antibodies in immunized mice was remarkably different. The separate immunization with plasmid DNA and protein, or their combination, rendered a mixed pattern of IgG2a/lgG1 anti-core antibodies. However, anti-core antibodies generated in all the other mice showed a clear bias towards the IgG2a subclass (Figure). In contrast, anti-core antibodies in naturally infected individuals are predominantly IgG1 [3]. Our results indicate that it is possible to manipulate the immune responses generated by DNA vaccination.

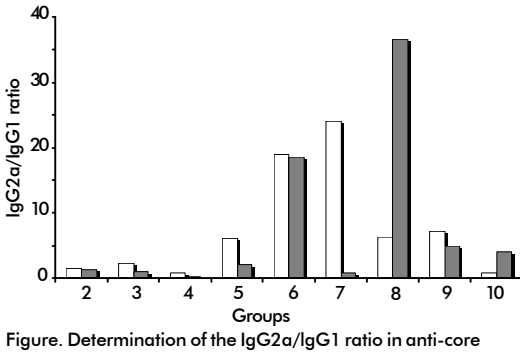

Figure. Determination of the IgG2a/lgG1 ratio in anti-core positive mouse sera. Serum samples (1:50 diluted) were pooled and evaluated for antibody subclass determination, at 5 (open bars) and 14 (stripped bars) weeks after primary immunization, in an ELISA assay against the Co. 120 protein.

1. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. Annu Rev Inmunol 1997;15:617–48.

2. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Press, New York, 1989.

3. Chen M, Sallberg M, Sonnerborg A, Weiland O, Mattsson L, Jin L, *et el.* Gastroenterology 1999;116(1):135–43.

Modelling of Hepatitis C Proteins

Asutosh Yagnik, Armin Lahm, Anna Tramontano

IRBM P. Angeletti Via Pontina Km 30.600 - I00040 Pomezia (Rome).
URL: http.//www.irbm.it; E-mail: Tramontano@irbm.it

*I*ntroduction

Hepatitis C virus (HCV) has been identified as the causal agent of most transfusion-associated non-A non-B hepatitis infections. It afflicts millions of people world wide and its infection has a chronicity rate of about 70% [1]. During the last 10 years, tremendous progress has been achieved in our understanding of the biology of hepatitis C virus, but neither a vaccine nor an effective antiviral therapeutical agent have been yet developed.

HCV has a single -stranded RNA genome of about 9.6 kb in length which encodes a precursor polyprotein composed of 3010-3030 residues. The precursor polyprotein comprises the structural proteins (C, E1, E2) and the nonstructural proteins NS2-NS5B, and is cleaved into individual proteins. The proteolytic cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, NS5A/NS5B sites is mediated by a chymotrypsin-like viral serine proteinase encoded within the NS3 protein. NS5A has been shown to be the RNA -dependent RNA polymerase. Several experimental evidences suggest that HCV E2 is a fundamental candidate antigen for a vaccine against hepatitis C virus. NS3 protease and NS5B are important targets for the development of antivirals.

Models of all these three proteins were built using different techniques and have been very useful in suggesting critical experiments. In two cases (NS3 protease and NS5B), their general features have been confirmed by X-ray crystallography.

*M*aterials and Methods

CLUSTALW was used for multiple sequence alignments, PHD for secondary structure prediction, TOPITS, THRADER2 and ProCyon for fold recognition. Insight II was used for model building. The stereochemical quality of the models was examined using PROCHECK. The description and references for these software packages can be found at the URL: http://www.irbm.it/irbm-course97.

*R*esults and Discussion

Since the E2 protein sequence does not share significant sequence homology with any known protein, we applied several protein secondary structure prediction and fold recognition techniques. This led to the construction of a model based on the E protein of tick-borne encephalitis virus (1SVI3). Mapping all available experimental data onto this structure allowed the binding interactions between E2 and its proposed cellular receptor CD81 to be localised, as well as a rough model for the tertiary and quaternary structure of the envelope glycoproteins E1 and E2 to be proposed [Yagnik A, Lahm, A T, unpublished results].

The NS3 serine proteinase domain shows only limited homology to cellular serine proteinases. Despite this low sequence conservation, we built a homology model of this domain that has helped us in directing experimental work, even in the absence of detailed structural information [2]. The three-dimensional structure of the HCV NS3 proteinase domain has now been solved by X-ray crystallography [3] and revealed that we correctly predicted the general topology, as well as several unique structural features of the enzyme, including the positions of the residues that determine the shape of the S1 substrate binding pocket and the presence of a tetrahedral metal binding site.

An inital partial model of the NS5B RNA-dependent polymerase was based on the coordinates of the poliovirus 3Dpol X-ray structure. For the sequence alignment we took advantage of the presence of conserved sequence motifs in RNA polymerases and of the secondary structure prediction for HCV NS5B. Superposition of the crystal structures of T7 DNA polymerase and of HIV reverse transcriptase, both in complex with a primer/template substrate, allowed us to identify NS5B residues that might be involved in substrate recognition and/or catalysis. Based on the now available NS5B crystal structure, we know that most of the palm domain and the fingers domain topology was correctly predicted. The model also correctly predicted the elements forming the proposed active site (A Lahm, unpublished results).

In conclusion, our results demonstrate that even models based on very low sequence homology, and therefore necessarily approximate, can be extremely useful in the drug discovery process.

1. Clarke B. Molecular virology of hepatitis C virus. J Gen Vir 1997; 78:2397–410.

2. Pizzi E, Tramontano A, *et al*. Molecular model of the specificity pocket of the hepatitis C virus protease: implications for substrate recognition. Proc Natl Acad Sci USA 1994;91: 888–92.

3. Yan Y, Li Y, Munshi S, *et al*. Complex of NS3 protease and NS4A peptide of BK strain hepatitis C virus: a 2.2 Å resolution structure in a hexagonal crystal form. Protein Sci 1998;7:837–47.

Near Patient Testing. Technologies and Applications

Gary Thorpe

Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Birmingham, B15 2TH, England. E-mail: G.H.Thorpe@bham.ac.uk

A number of technological advances are allowing near patient testing (NPT) by inexperienced operators and increasing usage is occurring in clinical chemistry, haematology and microbiological applications. A range of systems is currently available, and more are in development. These include desk-top analysers, small dedicated instruments, and simple disposable non-instrumental systems in which results are interpreted visually [1].

Desk-top analysers are based on either dry chemistry reagent strips or cassettes containing all the liquid reagents required for a test, and several can use whole blood samples, or perform multiple tests simultaneously. Small dedicated inexpensive instruments such as those for blood glucose measurement also have sophisticated features and many of the operator-dependent steps associated with previous systems have been eliminated. Further developments in biosensors, microminiaturisation, the use of reagent arrays such as "DNA chips", and microfluidic integrated circuits offer the extended future application of small analysers, the first application of DNA chips in NPT probably being used for the identification of pathogens and their antimicrobial resistance potencial [2].

One of the key aims of NPT is the development of systems that are either non- or minimally invasive. Most research and development of non invasive and minimally invasive technologies has focused on the development of glucose analysis systems. Systems for glucose analysis are already extremely advanced in comparison to systems for other analytes. These include numerous technologies, ranging from semi-invasive biosensor and iontophoretic approaches to completely non-invasive systems using infrared spectroscopy and light polarisation, which are currently at a variety of development and validation stages for glucose measurement. The successful development of a clinically acceptable, non-invasive glucose meter is likely to lead the way for the development of similar NPT systems for other analytes.

Simple disposable non-instrumental systems have been widely used in NPT for many years. Urine dipsticks have been widely used and similar technology now allows several tests to be performed simultaneously. These forms of assays have been adapted for general and immunoassay chemistries to a range of formats, such as dipsticks and platforms, and can use a range of specimens including urine, whole blood serum/plasma, swab extracts, faeces and saliva [3]. The systems can he designed to incorporate integral procedural controls, which indicate that the operator has performed the test appropriately, and that the reagents are active.

Non-radioactive labels, monoclonal antibodies, and advances in reagent support membranes and reagent deposition have enabled the development of rapid and simple immunoassays where the presence of analyte can be indicated by a coloured response on the device. Alternatively, the intensity of the coloured change can be proportional to concentration, or the response used to produce positive or negative symbols to indicate the presence or absence of the analyte. Such systems have proved to be sensitive and rapid, and have been applied in diverse areas including assays of pregnancy hormone, luteinising hormone, drugs of abuse, occult blood, microalbuminuria, and in numerous microbiological assays including antigen and antibody markers of infectious diseases.

In conclusion, new technology is allowing opportunities to increase for NPT. Future developments will extend the type of systems available, the substances that can be rapidly detemined in smaller quantities of body fluids, and the introduction of minimally invasive systems.

1. Hobbs FDR, Delaney BC, *et al.* A review of near patient testing in primary care. Health Technology Assessment 1997;1(5): 1–229.

2. Borriello SP. Near patient microbiological tests. British Medical Journal 1999: 319;298–301.

3. Price CP, Thorpe GHG, *et al.* Disposable integrated immunoassay devices. In Principles and Practice of Immunoassay 1997:581-603. eds. Price CP and Newman DJ. Stockton Press NY USA.

Why Does the Early Immune Response Fail to Mediate Better Control of HIV-1 Replication?

P Borrow,[1] X Wei,[2] SE Gloster,[1] R Harrop,[1] NA Jones,[1] J Lewis,[1] MC Riffkin,[1] P Newton,[3] I Williams,[4] M Stevenson,[5] JA McKeating,[6] P Balfe,[3] GM Shaw[2]

[1]The Edward Jenner Institute for Vaccine Research, Compton, Newbury, Berkshire RG20 7NN, UK, E-mail: persephone.borrow@jenner.ac.uk [2]Department of Medicine, University of Alabama at Birmingham, 613 Lyons-Harrison Research Building, 701 19th St. South, Birmingham, AL 35294, USA. [3]Deptartment of Virology and [4] Department of STDs, Royal Free and University College London Medical School. [3]The Windeyer Building, 46 Cleveland Street, London WIP 6DB, UK. [4]The Mortimer Market Centre, Mortimer Market, off Capper Street, London WC1E 6AU, UK. [5]Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Centre, 373 Plantation Street, Worcester, MA 01605, USA. [6]School of Animal and Microbial Sciences, University of Reading, Whiteknights, Reading RG6 2AJ, UK.

*I*ntroduction

The events that take place in the first six months following HIV-1 infection are critical determinants of the subsequent disease course. During this period, a "setpoint" load of persisting virus is established, which is a good predictor of the length of time before AIDS develops [1]. We are studying virus-immune system interactions in a panel of patients during and shortly after HIV-1 seroconversion to identify immunological factors that influence the setpoint viral load established. These studies will facilitate the development of therapeutic and prophylactic strategies to modulate these early events so that subsequent progression to AIDS is delayed as far as possible.

*M*aterials and Methods

Peripheral blood samples are being collected at sequential timepoints over the early course of infection from patients undergoing HIV-1 seroconversion. The viral load, $CD4^+$ T cell count and clinical status of the patients are being monitored. Virus isolates are being derived from the patients and characterised *in vitro*, and sequence changes in the *in vivo* viral quasi-species analysed. The specificity and heterogeneity of the virus-specific $CD8^+$ T cell response are being mapped, and other aspects of the antiviral immune response also characterised.

*R*esults and Discussion

We (and others) have shown that virus-specific $CD8^+$ CTL responses are mounted very early after HIV-1 infection [2, 3] and play an important role in control of early virus replication [4].

Current work is defining how qualitative differences in the early CTL response affect the efficiency of containment of virus replication, and is investigating how the virus evades T cell control. We have found that viral variants bearing mutations that confer escape from recognition by epitope-specific CTLs can be selected during early HIV infection [4 and unpublished data], and are examining their impact on the efficiency with which virus replication is contained.

We are also analysing the contribution that other mechanisms make to the inability of the early immune response to control virus replication more completely, including resistance of HIV-infected cells to antiviral actions of T cells, exhaustion of high affinity clones of virus-specific $CD8^+$ T cells, and deficits in $CD4^+$ T cell functions. A broad virus-specific $CD8^+$ T cell response may be best equipped to counteract the viral immune evasion strategies and mediate optimal containment of early virus replication.

1. Mellors JW, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science 1996;272: 1167–70.

2. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MBA. Virus specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 1994;68:6103–10.

3. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, *et al*. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994;68:4650–5.

4. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, *et al*. Antiviral pressure exerted by HIV-1-specific cytotoxic T. lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nature Med 1997;3:205–11.

Contact:
IDICT, Dpto Comercial
y Marketing
Industria y San José
PO Box 2213, CP 10200.
Ciudad de La Habana, Cuba
Tel.: (537) 626531
Fax: (537) 338237; 626531.
E-mail: comercial@idict.cu

Collection: Directories of Science and Technology

Instituto de Información Científica y Tecnológica

❍ Directory "Biosciences in Cuba 1999/2000". 5th Edition. It is the most complete guide of Cuban and foreign organizations in this sector resided in Cuba. It contains 345 research institutions, manufacturing companies, university centers, information centers, consulting offices, trade firms and institutions that offer services in the sector of the biosciences.

❍ Biographical directory "Who is Who in Cuban Sciences" More than 555 personalities from more than 120 Cuban scientific institutions. Soon it will be available in CD ROM.

IDICT
INSTITUTO DE INFORMACION CIENTIFICA Y TECNOLOGICA

Mapping of Epitopes Recognized by Anti-pvIII Monoclonal Antibodies using Synthetic Peptides

Nelson S Vispo, Glay Chinea, Yanet García, Neysi Ibarra, Osvaldo Reyes

Pharmaceutical Division. Centro de Ingeniería Genética y Biotecnología, AP 6162, Ciudad de La Habana, CP 10600, Cuba. E-mail: farma2@cigb.edu.cu

*I*ntroduction

Display of peptides on the surface of filamentous phage by fusion to the *N* terminus of pIII and pVIII is a powerful tool for the identification and/or characterization of ligands for many different target molecules (antibodies, receptors or other proteins). Using a variety of display methods, vast libraries of short random-sequence peptides have also been constructed. Affinity selection of such libraries using monoclonal (MAb) and polyclonal antibodies has revealed novel ligands (mimotopes) which mimic the binding properties of the natural ligand [1].

Several different techniques for the identification and/or characterization of positive clones have been described. However, none of these procedures is suitable for rapid and sensitive analysis of large numbers of clones. Here we describe the characterization of a set of mouse MAbs that recognize phage coat proteins. These MAbs represent a useful tool for investigating phage assembly and structure.

*M*aterials and Methods

To identify the regions of pVIII involved in MAb-pVIII binding, the peptide spot synthesis approach as previously described by Frank [2] was used.

*R*esults and Discussion

Spot synthesis in combination with peptide library techniques serve as a useful tool to study protein-peptide interactions on continuous cellulose membranes. In order to determine the location of the epitopes recognized by the anti-pVIII MAbs, we used the method of spot synthesis in cellulose membranes to synthesize 12 overlapping peptides of 8 and 9 amino acids encompassing the first 20 residues of pVlll. Inspection of the crystallographic structure of the bacteriophage indicates that residues between Y_{21} and S_{50} have minimal or no accessibility to antibodies after the phage capsid is formed. Eight was chosen as minimal peptide length because it corresponds to two helical turns and such peptides would include the spatially close residues observed in alpha helical conformations.

The table shows the reactivity profile of the four MAbs tested against the overlapping peptides. MAbs 65/55/53 (I), 97/28/32 (II) and 60/55/80/2 (III) recognize only spot No. 12, which indicates that they share the same lineal epitope comprising residues D_{12}-E_{20}. T_{19} and/or E_{20} are essential residues in the interaction, but residues D_{12}, S_{13} and A_{16} could also be part of the contact surface. MAb 20/21/54 (IV) weakly recognizes spots No. 5 and 12. The corresponding peptides from pVIII monomers contiguous in the three-dimensional structure of the capsid form a continuous patch on the surface of the phage. lt suggests that this epitope is topographic, which is consistent with previous studies showing that it is destroyed by SDS-PAGE treatment of the phage (in press).

In the past few years there has been a surge of interest in the use of MAbs to study filamentous bacteriophages, the usefulness of the monoclonal antibodies on this technology not only serves as a tool for investigating phage assembly and structure, but also for improving selection and screening of positive clones from peptides and protein libraries displayed on phage as identification, to detect coat epitopes not inhibited by the foreign protein fused.

1. Castagnoli L, Vetriani C, Gonfloni S, Vispo NS, Cesareni G. Selection from a peptide library of the antigenic determinants of a protein. Generation of antibodies by cell and gene immortalization. Terhorst C, Malavasi F, Albertini A editors. Year Immunol, Basel, Karger 1993;7:41–9.

2. Frank R. Spot-Synthesis: An easy technique for the positional addressable, parallel chemical synthesis on a membrane support. Tetrahedron 1992;48:9217–32.

Table

Exposed aa are shown in bold			Anti-pVIII MAbs			
Spot	pVIII peptide	Sequence	I	II	III	IV
1	1–8	**AE**GD**DP**A**K**				
2	2–9	**E**GDD**P**A**KA**				
3	3–10	GD**DP**A**KA**A				
4	4–11	D**DP**A**KA**AF				
5	5–12	**DP**A**KA**AF**D**				Weak
6	6–13	**PAKA**AF**DS**				
7	7–14	A**KA**AF**DS**L				
8	8–15	**KA**AF**DS**LQ				
9	9–16	**A**AF**DS**LQ**A**				
10	10–17	AF**DS**LQ**A**S				
11	11–18	F**DS**LQ**A**SA				
12	12–20	**DS**LQASA**TE**	Strong	Strong	Strong	Weak

Strong recognition

Weak recognition

Efecto de la inmunoterapia sobre larvas infectantes (LI) de Trichinella spiralis *implantadas en músculo estriado en modelo experimental*

Gabriela Reveles,[1] Jesús Muñoz,[2] Sergio J Saldívar,[1] María A Moreno[1]

[1]Centro de Biología Experimental. [2]Instituto de Investigaciones Odontológicas. Universidad Autónoma de Zacatecas. Apartado Postal 12. Guadalupe, Zac. México. 98600

Introducción

La enfermedad causada por *Trichinella spiralis* es la trichinellosis. En el hombre, se ha reportado en casi todo el mundo, su prevalencia es alta en Europa y Asia, y se asocia con la ingesta de carne infectada mal cocida o cruda, principalmente de cerdo. En México, la investigación epidemiológica relacionada con trichinellosis indica que esta enfermedad tiene una frecuencia hasta de 8,1% de la población general, por lo que se considera un problema de salud pública. Zacatecas, Jalisco, Estado de México y Chihuahua, son los estados con mayor frecuencia de brotes de trichinellosis reportados oficialmente de 1938 a 1995 [1].

Ciclo vital de *T. spiralis*

Cuando un huésped consume larvas infectantes (LI) de un músculo estriado, éstas son liberadas por acción de las enzimas digestivas y son transportadas al intestino delgado donde sufren 4 mudas, madurando a adultos. Éstos están capacitados para la fecundación, dando origen a larvas recién nacidas (LRN), migrando a la lámina propia y después a vasos linfáticos y mesentéricos o directamente al torrente sanguíneo vía porta, de ahí a músculo estriado, donde se lleva a cabo el implante y el proceso de LRN a LI. En alrededor de un mes se forma una cápsula y el quiste se forma después de 6 a 9 semanas [2]. Después de 6 meses, comienza a calcificarse, concluyendo ésta, aproximadamente a los 18 meses. A pesar de la calcificación, las LI siguen viables por años. La infección por *T. spiralis* induce en el huésped una fuerte respuesta inmune local a nivel intestinal y posteriormente se da la respuesta sistémica. Sin embargo, su actividad es insuficiente para evitar el implante de LI, por la cual, si se utilizan antígenos que sean potentes inmunógenos capaces de estimular el sistema inmunitario de manera activa, dirigirán el ataque inmunológico [3] contra el implante de las LI. Teniendo como base este conocimiento, se propone la alternativa de la inmunoterapia para despertar una respuesta inmune amplificada contra las LI de *T. spiralis* implantadas en músculo estriado.

Objetivo

Aplicación de inmunoterapia con antígeno soluble total para modificar el implante de las LI de T. *spiralis* en músculo estriado.

Material y Métodos

Se utilizaron 10 ratas Long Evans machos de 2 meses y medio de edad que se infectaron vía oral con aproximadamente 500 LI y se dejaron un año en observación con la finalidad de que, en músculo estriado, las LI estuvieran calcificadas. Previa a la infección, las ratas se sangraron para evaluar la respuesta inmune por técnicas inmunológicas, microinmunodifusión (MID) e inmunoelectrotransferencia (IET), se tomó biopsia para observar directamente al microscopio y otra fracción se fijó en formol al 10% para ser procesada y teñida con hematoxilina-eosina. Se hicieron 2 grupos de 5 animales, el primero fue de control y el segundo recibió inmunoterapia aplicada por 5 días consecutivos en dosis ascendente de 100 a 500 µm. Al día 10, se dio un refuerzo con 500 µm. A los 15 días posteriores, ambos grupos se sangraron para realizar técnicas inmunológicas, se tomó biopsia para observar directamente al microscopio y una fracción se fijó en formol al 10% para ser procesada y teñida con hematoxilina-eosina Al día 30 posterior a la inmunoterapia, los animales se sacrificaron, tomando muestras de sangre para técnicas inmunológicas y biopsia de diafragma, masetero, lengua y pierna, las cuales se observaron por compresión directamente al microscopio de luz y se fijaron para tinción de hematoxilina-eosina. Así mismo, se llevó a cabo digestión del tejido muscular para cuantificar el número de las LI y obtención del antígeno soluble total, al cual se le realizó electroforesis en geles de poliacrilamida.

Resultados

Los animales que recibieron inmunoterapia subieron de peso 10 g como promedio, su pelo se hizo brillante y menos erizo, la MID inicial fue de 2 a 3 bandas y la final de 1 banda. En la IET inicial se detectaron 5 péptidos de 33, 43, 45, 48 y 67 kD. Posterior a la inmunoterapia, se detectaron únicamente 3 péptidos de 43, 45 y 48 kD. En la compresión inicial, al observarla al microscopio de luz, se encontraron LI de *T. spiralis* calcificadas. Posterior a la inmunoterapia, hubo un aumento de vascularización, aumento de acúmulos de grasa, aumento de pofimorfonucleares y disminución del número de LI calcificadas.

Discusión

Una vez que ha llegado, la LRN evoluciona a LI en músculo y se enquista, evadiendo la respuesta inmune del huésped y no existe ningún tratamiento alternativo para remover las LI. En este estudio, se estableció la alternativa de la inmunoterapia con el antígeno soluble total, el cual tuvo un efecto sobre la respuesta inmune en contra de las LI de *T. spiralis* y mejoró el estado físico del animal, estableciendo un campo de estudio en el tratamiento de las parasitosis.

1. Correa D, De la Rosa JL, Ortega-Pierres MG. Trichinellosis: human epidemiological data in Mexico from 1939 to 1995. ICT9. Trichinellosis 1996;519–23.

2. Reveles RG, Villalobos R, Saldívar S, Moreno A. Histologic experimental implant of *T. spiralis*. ICT9. Trichinellosis 1996;79–85.

3. Moreno GA, Muñoz EJ, Rumayor RA. The 45 kDa antigen of *Trichinella spiratis* induce protection in experimental infections. ICT9. Trichinellosis 1996;261–7.

Inmunización de ADN en ratones con variante truncada de la gB del virus herpes simple 2

Ana B Pérez,[1] Juan Fló,[1] Mauro Saturnega,[2] Francisco Baralle[2]

[1]Instituto "Pedro Kourí" Autopista Novia del Mediodía, km 6½, La Lisa, Marianao 13, Ciudad de La Habana, Cuba. E-mail: anab@ipk.sld.cu
[2]International Center of Genetic Engineering and Biotechnology Padriciano 99, Trieste 34 1 00, Italia.

*I*ntroducción

La infección por virus herpes simple (VHS) humano constituye un problema de morbilidad en humanos a nivel mundial. Numerosos estudios han demostrado el papel de la inmunidad mediada por células (IMC) en el control de esta infección [1, 2]. La recurrencia de la infección latente ha sido relacionada a una disminución de la IMC antiviral [3]. La inmunización en modelos animales de algunas glicoproteínas o construcciones de ADN que expresan los componentes virales, proveen protección parcial o total contra el reto viral [4, 5]. La glicoproteína (gp) B forma parte de la envoltura viral y contribuye a la penetración del VHS a la célula hospedera. En nuestro estudio, usamos un modelo de vacuna de ADN para investigar la capacidad de desencadenar una respuesta inmune en animales inmunizados e inducir protección frente al reto viral de una forma truncada de la gB del VHS_2.

*M*ateriales y Métodos

El gen codificante de la gB truncado en su extremo 5' (sin 400 pb) fue amplificado por PCR a partir del genoma viral obtenido de células Vero infectadas. Dicha secuencia fue clonada en un vector $pcDNA_3$ digerido por *Bam*HI, para ser expresado bajo el control del promotor de CMV humano. La construcción fue chequeada por digestión con enzimas de restricción y secuenciación, y se verificó su expresión usando el sistema de transcripción/traducción TNT en lisado de reticulocitos de conejo (Promega). Tras la purificación del ADN con kits de mega prep Qiagen, 100 µg del plásmido recombinante fueron inyectados en ratones C57BI por vía intramuscular en un esquema de tres dosis con intervalos semanales. Se usó como control un grupo de animales inmunizados con el plásmido y como control positivo, animales inmunizados con 10^6 PFU del virus también por vía intramuscular.

Una parte de los animales fueron retados intravaginalmente con el virus. En el resto de los animales se evaluó la reacción de hipersensibilidad retardada (DTH) por inoculación del virus o control celular en la oreja de aquellos y medición del grosor del pabellón auricular a las 24 h.

Se colectaron sueros de los animales para medir la respuesta de anticuerpos mediante un ELISA indirecto, y se evaluó la respuesta de células T *in vitro* frente al virus, mediante el análisis por citometría de flujo de la expresión de CD25 en células CD4+ incubadas en presencia de antígenos virales.

*R*esultados y Discusión

Los animales inmunizados con el plásmido recombinante, al ser retados con el virus, llegaron a 50% de supervivencia (dato preliminar), mostrando un ligero nivel de protección, al ser comparados con aquellos que recibieron el plásmido solo, los cuales murieron en su totalidad. Por su parte, los animales inmunizados con el virus se protegieron, no presentando signos de infección genital, ni se recuperó virus en los lavados vaginales después de la inoculación viral. Se comprobó una respuesta de células T *in vivo* frente al virus a través de la medición de la DTH, la cual fue significativa al compararse con el control celular ($p < 0,05$). No se detectaron títulos de anticuerpos en los animales inmunizados con ADN, en cambio sí altos títulos en aquellos inoculados con el virus. Al ser enfrentados los esplenocitos de animales inmunizados con el plásmido recombinante a antígenos virales, y analizados por citometría de flujo, obtuvimos un incremento evidente en la expresión de CD25 en células T $CD4^+$ que corrobora el reconocimiento y activación de éstas por estos antígenos.

La ausencia de anticuerpos detectables se explica por la localización intracelular de la proteína expresada, un hecho que limita la accesibilidad del antígeno para el reconocimiento y estimulación de las células B [6]. Por otra parte, la ubicación en el citoplasma de la proteína traducida debe garantizar su accesibilidad al complejo LMP y, por tanto, la presentación eficiente a las células T citotóxicas, la cual es fundamental en la respuesta antiviral. Aunque estudios recientes demuestran que células musculares transfectadas pueden inducir respuestas de linfocitos T citotóxicos [7], parece ser importante la liberación del antígeno al espacio extracelular para ser endocitado por células presentadoras de antígenos inductoras de inmunidad. Existen evidencias de la dependencia de la presentación de antígenos asociados a MHC II para desencadenar respuestas CTL a vacunas de ADN [8]. El bajo nivel de protección en los animales retados pudiera estar relacionado con esto.

1. Nash AA, *et al*. J Gen Virol 1980;48: 351–57.

2. Carr JA, *et al.* J Virol 1997;71:7799–803.

3. Iwasaka T, *et al.* Infect Immun 1983;42: 955–64.

4. Cehimi J, *et al.* Eur J Immunol 1993;23: 1826–30.

5. Bourne N, *et al*. J Infect Dis 1996;173: 800–7.

6. Roitt I. Immunology 1996;4–7.

7. Sigal L. J, *et al.* Nature 1999;398:77–80.

8. Whitton JL, *et al*. Vaccine 1999;17: 1612–9.

PCR Fingerprinting of Multidrug-resistant Nosocomial Isolates of Acinetobater baumannii

Alejandro M Viale,[1] Adriana S Limansky,[1] M Cristina Guardati,[2] Maria I Zamboni[2]

[1]Instituto de Biología Molecular y Celular (IBR, CONICET), Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario Suipacha 531.
[2]Hospital Municipal de Emergencias Clemente Álvarez (HECA), 2000 Rosario, Argentina.
E-mail: amviale@infovia.com.ar

Introduction

Acinetobacter baumannii is frequently associated to nosocomial infections. This Gram-negative microorganism seems also widely distributed in hospital environments, and has been isolated from skin of both staff personnel and patients as well as from many inanimated materials. The appearence of strains of this pathogen possessing resistance to multiple antibacterial agents constitutes an emerging problem in the medical practice. Therefore, the accurate identification of clonal relationships of isolates present in nosocomial environments constitutes a pre-requisite to evaluate reservoirs, sources, modes of transmission as well as clonal disseminations of this pathogen. We evaluated in this work several methodologies (including phenotypic as well as genotypic procedures) for the characterization of *A. baumannii* isolates obtained in a hospital (HECA) of Rosario City. We found that RAPD analysis employing degenerate primers constitute the most discriminative procedure for the epidemiologic analysis of clinical isolates of *A. baumannii*.

Materials and Methods

A total of 43 clinical isolates of *A. baumannii* (including 37 multiresistance strains) obtained in HECA between November 1994 and June 1998 were analized. Bacterial isolates were characterized phenotypically by biotyping (API 20E system) and antimicrobial susceptibility was determined by disk diffusion tests. Genotypic characterization was done by random DNA amplification (RAPD) using a published set of degenerate primers [1], as well as by REP-PCR [2]. Total envelope proteins were obtained and analized by SDS-PAGE.

Results and Discussion

Genotypic analysis using REP-PCR indicated the presence of 8 different amplification profiles among the 43 different *A. baumannii* isolates. In turn, RAPD analyses using the 19-mer primer 19 and the 14-mer primer 5314 [1] indicated the existence of three and two additional amplification patterns, respectively. Interestingly, RAPD analysis could discern five distinct clones among the multidrug-resistant isolates, whereas REP-PCR revealed only two among the same strains. lt is worth noting that among the above strains, imipenem-resistant and sensitive isolates could be distinguished by RAPD but were indistinguishable by the other genotypic or phenotypic procedures.

Phenotypic analyses indicated the presence of 8 different antibiotypes, which showed little correlation to those found by genotyping. A similar lack of correlation and lower discrimination was observed by envelope protein analysis, which showed the presence of 6 different profiles. Thus, these methodologies were not judged of utility for the characterization of *A. baumannii* strains.

The epidemiological analysis of the multidrug-resistant *A. baumannii* strains by RAPD indicated that imipenem-sensitive clone A (20 isolates) was predominant from November 1994 to November 1996, and was subsequently replaced by imipenem-resistant clone B (11 isolates). The replacement was correlated with the introduction of imipenem as the predominant antibiotic to treat these infections in HECA. Moreover, this methodology allowed a clear distinction between epidemic and sporadic clones of *A. baumannii*.

Conclusion

RAPD using degenerate primers provided the largest discriminative procedure to distinguish *A. baumannii* strains isolated in our medium, differentiating even clonely-related imipenem-resistant from imipenem sensitive isolates. This provides us with a fast, reliable and relatively non-expensive methodology for epidemiological studies of pathogenic bacteria.

1 . Limansky AS, Sutich EG, Guardati MC, Toresani IE, A Viale. Genomic Diversity among *Streptococcus agalactiae* Isolates Detected by a Degenerate Oligonucleotide-primed Amplification Assay. J Inf Dis 1998;177:1308–13.

2. Reboli AC, Houston ED, Monteforte JS, Wood CA, R Hamill. Discrimination of Epidemic and Sporadic Isolates of *Acinetobacter baumannii* by Repetitive Element PCR-mediated DNA Fingerprinting. J Clin Microbiol 1994;32:2635–40.