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# The Use of Rabbit Model for the Efficacy of a Novel Epstein - Barr virus (EBV) Peptide Vaccine

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**Abstract**

The Epstein-Barr virus (Human herpes virus 4) encodes approximately 80 proteins, from which 15 possess at least 90 antigenic epitopes. A proportion of the immunogenic EBV proteins was found protective by immunization, when great many stimulate the T cell receptors (TCR), but less interact with the B cell receptors (BCR). Activation of B cells and subsequent antibody production has been related to at least3 envelope glycoprotein's (mainly to gp350/220), to the latency associated membrane proteins (LMPs) and to EBNA3/C. In acute infectious mononucleosis (IM) patients a considerable proportion (about 40 %) of the HLA B8 restricted cytotoxic lymphocyte (CTL) reactivity is directed against a single peptide (RAKFKQLL) of the transactivator protein BZLF1/Zta. The interaction of EBV peptides with the CD8 plus T cells is restricted by HLA I molecules, predominantly of the HLA-A subclass. An EBV vaccine can be designed to prevent any kind of infection (including prophylaxis of EBV-associated malignancies) or may be destined for therapeutic purposes only. The preventive vaccines protecting against acute disease (such as IM) contain, as a rule, the gp350/220 polypeptide (s) encoded by the BLLF1 gene. In contrast, vaccines destined for tumor prevention rather consist of peptides derived from latency associated nuclear proteins (EBNA 2, 3 and 6) and/or from the oncogenic latent membrane proteins (LMP1/LMP2A). We report the construction of a peptide vaccine, in which the selected EBV peptides (oligopeptides) coming from structural (gp350/220, gp110, gp85) as well as latency associated proteins (LMP1, EBNA2, EBNA3C/EBNA6 and others) were bound to micro-beads. Due to the growing importance of possible adverse effects of vaccines caused by adjuvant and/or by the presence of cross-reacting oligopeptides, the peptide vaccine seems to represent an acceptable solution avoiding autoantibody formation.

## INTRODUCTION

### Animal models for EBV infection

Several authors aimed to develop a suitable animal model for EBV infection. It is well known that EBV is a gamma herpes virus (member of subfamily *Gammaherpesvirinae*) that belongs to the genus Lymphocryptovirus; officially it has been labeled Human Herpes virus 4, abbreviation HHV-4 [1]. In early experiments, the rabbits have been infected with EBV-related lymphocryptoviruses [2-6], which mainly resulted in the establishment of lympho proliferative disease (LPD) and/or formation of malignant lymphomas. Alternatively, persistent infection of out bred laboratory and/or Balb/c mice infected with the murid herpesvirus (MuHV-4) has been described [7]. MuHV-4 is a relatively new Rhadinovirus (belonging to subfamily *Gammaherpesvirinae*), which had been isolated from free living rodents. Even though MuHV-4 in mice provides an interesting model of infectious mononucleosis (IM) - like disease and lymphoid proliferation including tumors [8-14], this model may not be suitable for the evaluation of a human vaccine. The first rabbit models reviewed by Hayashi et al., [15] took advantage of the administration of EBV-like lymphocryptoviruses. At least two different models of lymphoproliferative disease (LPD) induced by simian EBV-like viruses were described. First is the *Cynomolgus* EBV-induced T-cell lymphoma developing in the course of 2 - 5 months in intravenously (77 - 90%) and/or orally inoculated animals (82 - 89%). In these, the viral DNA could be detected in peripheral blood mononuclear cells (PBMC) by PCR from day 2 after oral inoculation, while the anti-VCA (viral capsid antibody) has appeared by 3 weeks later. Rabbit lymphomas and

their cell lines contained EBV-like DNA and expressed the EBV-encoded RNA-1 (EBER-1). The cell lines in question revealed typical chromosomal abnormalities and were tumorigenic when transferred into nude mice. Another (second) basic disorder is the EBV-like virus associated hemophagocytic syndrome (VAHS) appearing in rabbits infected with *Herpesviruspapio* (HVP). Rabbits inoculated intravenously with HVP producer cells showed increased titers of IgG class antibodies reacting with the specific capsid antigen (VCA). The majority of these rabbits (up to 85%) subsequently died of fatal LPD accidentally combined with VAHS; the latter syndrome encompassed bleeding and hepatosplenomegaly occurring within 22-105 days p.i. Cell-free HVP given by oral spraying has induced viral infection with sero conversion in a proportion of rabbits, out of which some died of LPD accompanied with VAHS. Atypical T lymphocytes containing HVP DNA and expressing EBER-1 were seen in many organs of these animals. Hemophagocytic histiocytosis was observed in lymph nodes, spleen and bone marrow which allowed to study the biology and pathogenesis of EBV infection. This model was found suitable also for experimental therapy. A brief overview of early animal models is given in Table (l). It shows that rabbits infected with human EBV (by the natural, i.e. oral and nasal routes) rarely develop lymphoproliferative tumors, in contrast to those infected with EBV-like lymphocryptoviruses (isolated from marmoset monkeys), which, as a rule, become a malignant LPD.

Unlike to the models described above, rabbits inoculated with human EBV either intravenously [23,24] and/or by intranasal/oral routes [25], did not develop overt disease. Just a proportion of them (4 out 10) showed the presence of EBV DNA in PBMCs and splenocytes and revealed the expression of corresponding RNA molecules such us EBERs. Lymphocytes expressing EBER-1 and latent EBV gene related proteins in their lymphoid tissues were found in 1 animal for longer than 200 days. The rest of three infected rabbits harbored the EBV DNA transiently; their PBMC extracts also expressed the mRNAs of non-structural or latency associated genes such as EBNA1, EBNA2 and BZLF1/Zta. The level of IgG class EA-D antibody reacting with an early antigen referred to as diffuse (as seen by IF in the nuclei of infected cells) has increased. EA-D antibodies were constantly maintained in all infected rabbits, whereas the levels of VCA-IgM and VCA-IgG (against a viral capsid antigen) increased just transiently and later on declined. Interesting enough, no EBNA1-IgG antibody was found. The infected rabbits revealed some scattered lymphocytes expressing EBER-1, LMP1, and EBNA2 in the spleen and/or lymph nodes. This suggests that EBV can infect rabbits by the intranasal or oral routes providing a useful model mimicking the natural and primary EBV infection in humans. Alternative follow up data came from the six out of seven rabbits inoculated intravenously with EBV [23,24]. In the latter, two out of six EBV animals showed EBV infection testified by the presence of EBV DNA in PBMC throughout the rest of their life. In addition, the high antibody titers against EA-D were maintained over 1000 days. A focal mass lesion was transiently observed by ultrasonography in the spleen of one rabbit. The two lifelong EBV DNA positive rabbits died on day 1522 or 1400; in them, autopsy revealed proliferation of lymphocytes expressing EBER-1 and/or LMP1 antigen accompanied with mild hemophagocytosis in the spleen or lymph nodes. Probably some EBV infected rabbits could not eliminate the virus and showed somewhat similar features to persistent EBV infection and/or mild sub-lethal hemophagocytosis. The lifelong infected rabbits might be regarded for a new useful animal model for persistent EBV infection occurring in almost all adult patients who recovered from IM.

## Characterization of our rabbit model

Recently, we have reported of our experience with oral/nasal infection of New Zealand white rabbits using human EBV prepared from B95-8 (producer) cells [26]. Blood was taken for serum preparation and for the isolation of PBMC purified by centrifugation on Ficoll-Paque PLUS-t gradient. The samples were obtained before infection and then on days 8, 28 and 98 post-infection (p.i.). Administration of either $3 \times 10^8$ (group A, 11 rabbits) or $1 \times 10^9$ (group B, 10 rabbits) EBV DNA copies per animal elicited subacute and/or persistent infection. In a large portion of animals the IgG class antibodies in plasma were detected by EA-D as well as VCA ELISA and by immunoblot (IB). The precipitation bands in the IB strips proved the presence of antibodies reacting mainly with the BZLF1/Zta transactivation polypeptide (69.2%), with the p54 early protein (53.4%) and with the p23 capsid protein (35.8%). No anti-EBNA1 antibody was detected throughout. Viral DNA could be found by PCR in PBMCs and/or in the spleen of 7 out of 21 infected rabbits (30%), even though 60-80% of infected animals developed a positive serological response as detected by at least 2 different techniques.

**Table l:** Brief overview of animal models of EBV infection and of related diseases*.

| Experimental animal | Virus administered | Typical outcome | References |
|---|---|---|---|
| Monkeys: cotton-top marmoset (*Sanguinus oedipus oedipus*), or common marmoset (*Callithrix jacchus*) | HHV-4/EBV | persistent infection, lymphoproliferation, LPD, malignant lymphoma | [16-20] |
| Rabbit | EBV-like (Maccacus)<br>EBV-like (Cynomolgus)<br>EBV-like (*H. Macacca nemestrina*)<br>EBV-like (*H. papio*) | LPD, lymphoma<br>IM - like disease<br>Hemocytophagic syndrome | [2-6] |
| Mice with SCID (severe combined immunodefficiency) | EBV DNA carrying cell lines | Multiple tumor formation | [21] |
| Mice outbred or inbred (Balb/c) | Murine herpesvirus (MuHV-4) | Persistent infection<br>lymphoproliferation | [7,8] |
| Rabbit | HHV-4/EBV | Persistent infection** | [25,26] |

*modified according to Rickinson and Kieff [22] and from Hayashi et al. [15], ** lymphoproliferative disease (LPD) occurs rarely

Summing up, in our EBV infected rabbits, viral DNA (vDNA) was present in B lymphocytes along with simultaneous expression of the LMP1 antigen in a relatively smaller proportion of animals and in the absence of EBNA1 antibody, a situation which might reflect the absence of corresponding EBNA1 polypeptide in the EBV DNA carrier B cells. The major pool of EBV latently-infected B cells are resting, peripheral blood class switched memory cells [27,28]. The transiently present EBV DNA (either in the PBMC and/or in splenocytes) can be accompanied by LMP1 antigen expression (Table 2). But if no viral proteins are expressed in the carrier B cells except of during their division, they are poorly recognized by the T helper cell response. We concluded our rabbits did not reveal a typical IM-like syndrome, in blood smears they just showed a mild increase of atypical mononuclear cells along with a higher proportion of lymphocytes. In addition we found that a proportion of rabbits has developed persistent EBV infection in the absence of EBNA1 antibodies.

When comparing the rabbit models aiming to mimic human EBV infection, namely the results described by Okuno et al. [25], with our published results [26], it can be seen that the antibody levels to different EBV antigens in the Okuno`s experiment revealed significant increases only in the four EBV DNA positive rabbits, but showed no elevation in the rest 6 out of 10 infected animals which remained negative for EBV DNA. In contrast, as confirmed by two different tests against the same antigen (namely antibodies to the p54 non-structural polypeptide as detected by EA-D ELISA and by immunoblot), we found a positive serologic response in relatively more rabbits, i.e. also in some of those in which the EBV genome could not be detected. Furthermore, we did not observe the rare pattern of continuous vDNA detection, which had been described by Okuno et al. [25], in one of their ten EBV infected rabbits. In our hands, the vDNA presence as detected by PCR in 7 out 21 animals (30 %) was rather transient. This finding, however, does not contradict to intermittent transcription, since Okuno et al. [25], found EBNA1, EBNA2, and BZLF1 mRNA molecules in PBMCs of some infected animals. For example in rabbit no. 4, such transcripts were intermittently detected from day 7 to day 249 p.i., while in rabbit number 5, the EBNA2 transcript was found on day 14 only or the BZLF1 mRNA was expressed on days 14 and 21. Finally, in Okuno`s experiments the BZLF1/Zta mRNA was detected in two rabbits (numbers OK8 and OK10) on day 14 only. In our hands [26], the occurrence of EBV DNA in PBMCs and spleen was less frequent than the serological response. In 6 occasionally EBV DNA positive carrier rabbits, also the LMP1 protein could be detected by IF at matching intervals. Since the purpose of our experimental model was not to prove a lifelong EBV persistence, we did not search for EBV DNA at the extremely late intervals such as on day 1400 and 1522. Takashima et al. [23], described the persistence of EBV DNA in the spleen of a proportion of rabbits in result of intravenous inoculation by a very high virus load, when confirming the presence of LMP1, EBNA2 and Zta mRNAs in the EBER-positive lymphocytes. It seems that both, the route of virus administration as well as the source of the inoculated virus, might be essential for further virus spread and for the efficiency of elimination of the latent virus genome [29,30]. Since our experiment encountered a relatively representative number of animals (together 21) which had been infected with two slightly different EBV doses, we set forward the idea to define the most reliable and simple techniques suitable for the efficacy of protective EBV polypeptides in the non-lethal rabbit model. The epitopes in question have been selected according to the data from literature [31] and also regarding to an independent computer analysis [32,33].

**Table 2:** Proportion of rabbits exhibiting lymphocytosis* and atypical mononuclear cells** in peripheral blood smears as compared to the results of DNA and LMP1 testing.

| Location/Test | Day 8 | Day 28 | Day 98 |
|---|---|---|---|
| PBMC/DNA (PCR) | 5% | 10% | 22 % |
| PBMC /LMP1 (IF) | 5 % | 10 % | 22% |
| Atypical Mo*/ smears | 35.7 % | 29.4% | 23.5 % |
| Lymphocytes**/ smears | 57.1 % | 66.6 % | 35.3 % |
| serum/EA-D ELISA | 52.0 % | 65.0 % | 50.0 % |

*lymphocytes up to 60%; **atypical mononuclear cells in the range of 10-20%

## The immunogenic EBV proteins and their protective epitopes

As mentioned above, the EBV (HHV-4) genome encodes approximately 80 proteins, from which 15 (or 16) possess one, but as a rule, several antigenic epitopes (together at least 90, Table (3)). Great many of them stimulate the T cell receptors (TCR), but a few interact with the B cell receptors (BCR). Activation of B-cells and subsequent antibody production has been attributed to the envelope glycoprotein gp220/350, to the LMP1 and EBNA1 polypeptides. The rest of the immunogenic proteins stimulate the TCRs located either on the cytotoxic or on the helper T cells. The interactions mediated by the cytotoxic (CD8) T cells (CTL) are restricted by the HLA I molecules, predominantly of the HLA-A subclass. In patients with acute IM a considerable proportion (of about 40 %) of the HLA B8 restricted CTL reactivity is directed against a single peptide (RAKFKQLL) of the transactivator protein BZLF1/Zta.

## The EBV peptide vaccine: a novel approach for vaccine design

The EBV vaccines designed so far fall into two categories: those preventing any kind of infection (can be used for prophylaxis of EBV-associated malignancies) and those designed for therapeutic purposes (to be used in subjects already infected). Preventive vaccines protecting against acute disease (such as IM) contain, as a rule, some of the epitopes of gp350/220 polypeptide(s) encoded by the BLLF1 gene. Vaccines destined for tumor prevention consist of peptides derived from latency associated nuclear proteins (EBNA2, 3 and 6) and/or from latent membrane proteins (LMP1/LMP2A). In addition, besides of the recently described recombinant immunogenic EBV polypeptides and/or their mixes, new perspectives were opened by construction of random overlapping strongly immunogenic scrambled polypeptide(s). Our novel approach is based on careful selection of antigenic peptides (oligopeptides) coming from both structural as well as non-structural and/or latency associated

**Table 3:** The immunogenic EBV coded proteins (15), the corresponding oligopeptides (90) and their target cell receptors.

| Protein | Gene | Epitope number | Target cells (for stimulation) | Reference |
|---|---|---|---|---|
| gp350 (gp340) | BLLF1 | 3<br>2<br>1 | B cells Helper CD4⁺/T cells<br>Cytotoxic CD8⁺/T cells (CTL) | [34-36] |
| gp85 | BXLF2 | 3 | | [37] |
| Zta/ZEBRA | BZLF1 | 2 | Cytotoxic T cells | [38] |
| Rta | BRLF1 | 7 | Cytotoxic T cells | [39,40] |
| regulator protein* | BMLF1 | 3 | Cytotoxic T cells | [40] |
| DNA poly cofactor** | BMRF1 | 2 | Cytotoxic T cells | [41] |
| Bcl-2 | BHRF1<br>BALF1 | 2 | Helper CD4⁺/T cells (CD3⁺/CD4⁺/CD8⁺) T cells | [42] |
| EBNA1 | BKHF1 | 3 | B cells | [43] |
| EBNA2 | BYRF1 | 2 | Cytotoxic T cells | [40]<br>[44] |
| EBNA3a (EBNA3) | BLRF3 BERF1 | 11 | Cytotoxic T cells | [36], [40], [44-47] |
| EBNA3c (EBNA6) | BERF3 BEFR4 | 10 | Cytotoxic T cells | [36], [40], [44-48] |
| EBNA4 (EBNA3b) | BERF2a BEFR2b | 9 | Cytotoxic T cells | [49] |
| EBNA 5 (EBNA-LP) | BamH1 W | 1 | | [40] |
| LMP1 | BNLF1a | 7 + 5 | (CD8+ as well as CD4+ T lymphocytes)***<br>B lymphocytes | [37], [40], [49], [50] |
| LMP2A,B | BNLF1b,c | 15 + 2 | (CD8+ as well as CD4+ T lymphocytes)*** | [40]<br>[51] |
| Total 15 | | 90 | | |

*post-transcription regulator, **DNA polymerase processivity factor ***additional data according to Haigh et al., 2008 [52].

proteins bound to micro-carrier (Figure 1). Any construct based on the epitopes of latency proteins might be useful for immuno prophylactic therapy following bone marrow and/or heart transplantations as well as for prevention of EBV-related tumors such as lymphomas and nasopharyngeal carcinoma. Due to the growing importance of the immunogenic epitopes selected as future vaccine components, at least the half of them has been patented not only for their natural amino acid (aa) sequence, but also for different possible chemical variations [31]. Due to the large number of epitopes, which were declared immunogenic but not always protective in animal experiments, computer design was used to elucidate their three dimensional structure. When considering the 3 envelope glycoproteins and/or the additional 9 predominantly non-structural polypeptides, they were found to possess at least 52 immunogenic oligopeptides; from them a final number of 15 epitopes has been selected for further testing (Table 4).

The selected epitopes were grouped into 19 combinations (at least 1 but occasionally 2 structural oligopeptides was/were combined with either 1 or 2 non-structural peptides). Each epitope combination was coupled to the same carrier bead together with at least 3 PRR (pattern recognition receptor) agonist molecules, which were expected to function as adjuvant. These adjuvants (PRR agonists) such as LPS (lipolysaccharide), IC (poly I/poly C), CpG (cytosin- phosphate-guanosin) and/or R848 (resiquimod) were first tested separately, to achieve the best synergistic effect as determined in human DCs. This was important, since any of particle bound phagocytosed adjuvants and immunogenic peptides might interfere with some of the immune stimulation pathways. The combination of epitope binding with the correct PRR agonist was expected to induce different pathways of antigen processing as compared to the soluble epitope/PRR mixes. The choice of a proper combination of immunogenic peptides along with corresponding PRR agonist createda final carrier system referred to the pathogen-mimicking Stealth Microparticles™ (PMM). The biocompatible micro particles for PMM preparation were manufactured by GalenBio (California, USA) according to their experience in manufacturing micro particles (particles larger than 100 + µm) at a commercial scale (1000 + L lots). The procedure in question was performed under cGMP (good manufacture practise)/ISO (international standard organization/9001) control to achieve non-toxic coupling chemistry compliant for chromatographic separations of FDA/EMEA licensed biologics (Figure 1). Proprietary coupling chemistries assured the attachment of precise amounts of the chosen combination of epitopes and PRR agonists forming the micro particles. The coupled ligands were stable for years at room temperature. The selected adjuvant combinations together with the given antigenic epitopes were repeatedly monitored in DCs. The effect of PMM in mature as well as immature DCs (G106 cells) and in macrophage cultures. The activation of alternative pathways was expected to function also in antigen presenting cells (APC). The results of *in vitro* stimulation experiments are summarized in Table (5).

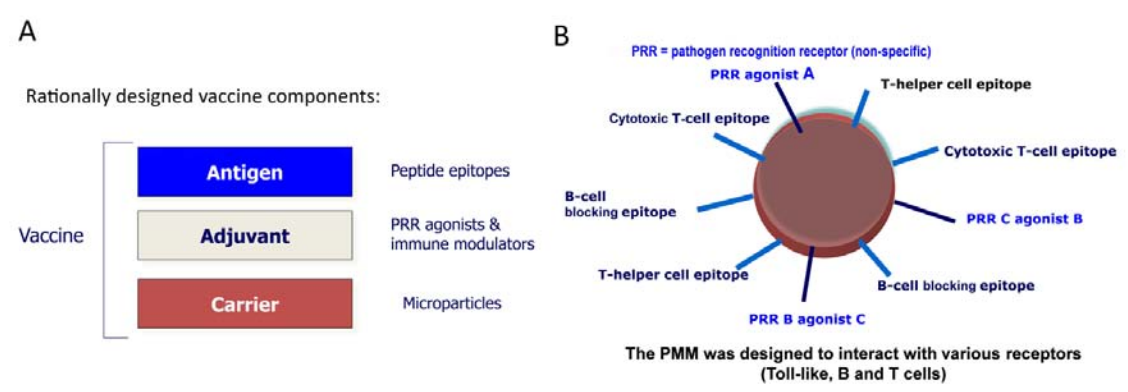


**Figure 1 Design of the novel peptide vaccine.** A. (in the left) shows that the vaccine design consisting of a carrier nanoparticle to which the PRR agonists as well as the EBV oligopeptides bind. As shown on scheme B (in the right), the pathogen mimicking particle (100+ μm in size) binds 2 or 3 different peptides, from which at least one should come from a structural glycoprotein. The peptides must stimulate both, the B lymphocytes as well as T cells. As described below, the selected EBV oligopeptides and the corresponding PRR agonists were used in 19 combinations.

**Table 4:** The immunogenic epitopes selected for the EBV peptide candidate vaccine[#].

| Protein/gene | Epitopes | | Polypeptide position[***] | | Epitope target | |
|---|---|---|---|---|---|---|
| | considered[*] | selected[**] | start | stop | T cell | B cell |
| gp350/220/BLLF1 | 14 | 4 | 541 | 556 | | + |
| | | 11 | 859 | 873 | + | |
| gp42/BZLF2 | 5 | 1 | 45 | 62 | | + |
| gp110/BALF4 | 4 | 1 | 774 | 778 | | + |
| dUTPase/BLLF3 | 8 | 1 | 223 | 242 | | + |
| Rta/BRLF1 | 2 | 1 | 101 | 117 | + | |
| ?/BMRF2 | 1 | 1 | 191 | 202 | | + |
| vIL-10/BCRF1 | 2 | 1 | 85 | 90 | | + |
| p138/BALF2 | 3 | 1 | 502 | 510 | + | |
| LMP1/BNLF1 | 5 | 2 | 43 | 52 | | + |
| | | 3 | 97 | 106 | | + |
| | | 5 | 159 | 167 | + | |
| EBNA1/BKRF1 | 3 | 1 | 556 | 574 | + | |
| EBNA2/BYRF1 | 2 | 1 | 276 | 295 | + | |
| EBNA3A | 3 | 1 | 803 | 815 | + | |
| Total | 52 | 15 | | | 7 | 8 |

[*]the total number of epitopes considered [**]epitopes chosen for immunization (numbered according to their position in the polypeptide chain) [***]the amino acid (aa) numbers within each polypeptide chain [#]according to Söllner et al. [32,33].

**Table 5:** Cytokine production in DCs/ stimulated by PMM.

| Interleukin | Natural production | Production in DCs | | Function(s) |
|---|---|---|---|---|
| | | Immature cells | Mature cells | |
| IL-1β | DCs, macrophages | Yes | Yes | T cell differentiation, IL-2 receptorexpression |
| IL-2 | T cells | Yes | Yes | Proliferation of T/CD3 lymphocytes as well of B lymphocytes |
| IL-6 | Macrophages, T cells | No | Yes | B cell proliferation and differentiation, IgG production |
| IL-8 | macrophages | Yes | Yes | Capillary adhesion and diapedesis, promotion of inflammation |
| IL-10 | T cells | No | Yes | Anti-inflammatory lymphokine |
| IL-12 | DCs, macrophages | No | Yes | NK cell activation, IFNγ induction, promotion of CTL response |
| TNFα | Macrophages, T cells | No | Yes | B cell proliferation, cytotoxic T cell differentiation, apoptosis indection |
| IFNγ | T cells, NK cells | No | Yes | MHC I expression, macrophage activation |

## Efficacy of the selected EBV epitopes in rabbit model

Together 60 rabbits were immunized with 19 different epitope combination formulas (labeled epitopes 1-19) consisting of 15 peptides coming from 12 EBV (or EBV coded) proteins. A group of 3 rabbits was left for infection (along with the virus challenge) in the absence of immunization (control I). In addition, the 21 not immunized rabbits which were infected with EBV as already described, served as further control (control II) [26]. After drawing blood for hematological and serological examinations (at interval F0A, i.e. before immunization and at interval F0B, 30 days post-immunization), the rabbits were challenged with a high dose ($10^7$ cells/0.1 ml) of EBV prepared from the producerB95-8 cells ($3x10^8$ DNA copies). Then at intervals F1 (day 8 post-infection, p.i.), F2 (day 28 p.i.) as well as at autopsy (day 108 p.i.), the blood samples were drawn for hematological, serological, biochemical and morphological examinations. The native blood smears were taken directly at autopsy, air dried and stained by immersion into "DIA-Fix Panoptic" stain solution for 10-20 sec. Additional blood smears were fixed either in para formaldehyde and/or in acetone (for IF staining). The blood drawn into isotonic EDTA/PBS to prevent clotting was layered (under sterile conditions) over the Ficoll-Paque PLUS-t solution and centrifuged to separate the blood serum from erythrocytes and PBMCs. The separated PBMC were used for preparation of smears as well as for DNA and/or RNA extractions. Serological tests at each given interval (F0A, F0B, F1 and F2) were made by ELISA and by immunoblot. At last autopsy interval, i.e. on day 108 p.i., blood was drawn and handled as described above [26]. In addition, spleen, thymus and occasionally also the liver and lung tissue were quickly frozen for DNA extraction or blocks were frozen in liquid propan/butan (-76°C) for cryostat sectioning. The results were summarized in Table (6) show the positive rate of each technique (EA-D ELISA, immunoblot, vDNA detection by PCR and IF staining of LMP1 antigen) considered for reliable from the point of view of false positive reading.

The value of individual tests assessing the replication of EBV in infected animals was determined as follows: 1. Serum antibody response to the non-structural early antigen (originally designated as diffuse antigen EA-D/p54) examined by ELISA. 2. Serum antibody response detected by immunoblot (IB) allowing to distinguish the presence of antibodies to latent, capsid ordinates shows serum dilutions at which the ISR value was determined. The abscissa shows time intervals post-challenge (day 0 means the virus administration day, day 108 the autopsy day). In addition to the group number, the individual animal numbers are also shown in each group. Some of the immunized and challenged animals showed an EA-D response similar to mock-immunized controls (example is group 2, classified into category III). The EA-D response in control rabbits was the highest by day 108 and early proteins (antigens available on the commercial strip: EBNA1, p18, p23, BZLF1, p54 and p138). 3. Detection of the EBV DNA in PBMC and spleen by polymerase chain reaction (classical and/or nested PCR). 4. Visualization of LMP1 protein in PBMC smears by indirect IF. The PBMC counts in native blood smears did not confirm the development of the IM-like syndrome (compare Table (2)). Nevertheless, the elevated

**Table 6:** Cumulative statistics of the four examination methods at detecting EBV infection in rabbit model.

| EA-D | | vDNA | | IB | | LMP1 | | Sum of | | | Group |
|---|---|---|---|---|---|---|---|---|---|---|---|
| P | T | P | T | P | T | P | T | positives | Total | Per cent | |
| Category I | | | | | | | | | | | |
| 1 | 8 | 0 | 8 | 1 | 6 | 0 | 8 | 2 | 30 | 6.66 | 1 |
| 0 | 6 | 0 | 6 | 0 | 4 | 0 | 5 | 0 | 21 | 0 | 6 |
| 0 | 8 | 0 | 10 | 0 | 6 | 0(1)* | 8 | 0 | 32 | 0 | 12 |
| 0 | 9 | 0 | 12 | 0 | 6 | 0 | 9 | 0 | 36 | 0 | 13 |
| 1 | 8 | 0 | 10 | 3 | 5 | 0 | 8 | 4 | 31 | 12.9 | 14** |
| 0 | 9 | 0 | 6 | 0 | 5 | 0(1)* | 8 | 0 | 28 | 0,00 | 15 |
| 0 | 9 | 0 | 8 | 0 | 5 | 0 | 8 | 0 | 30 | 0 | 18 |
| Category II | | | | | | | | | | | |
| 3 | 8 | 0 | 7 | 2 | 5 | 0 | 8 | 5 | 28 | 17.85 | 3 |
| 3 | 8 | 0 | 10 | 3 | 4 | 0 | 8 | 6 | 30 | 20 | 4 |
| 2 | 9 | 1 | 12 | 5 | 6 | 1 | 9 | 9 | 36 | 25 | 8 |
| 2 | 9 | 2 | 12 | 1 | 6 | 1 | 9 | 6 | 36 | 16.66 | 9 |
| 2 | 8 | 1 | 8 | 1 | 4 | 1 | 7 | 5 | 27 | 18.52 | 10 |
| 1 | 8 | 2 | 10 | 2 | 6 | 2 | 8 | 7 | 32 | 21.87 | 16 |
| 0 | 9 | 2 | 12 | 2 | 5 | 1 | 9 | 6 | 35 | 17.14 | 17 |
| 3 | 9 | 0 | 12 | 1 | 6 | 0 | 9 | 4 | 36 | 11.11 | 19 |
| Category III | | | | | | | | | | | |
| 6 | 9 | 1 | 8 | 4 | 4 | 1 | 7 | 12 | 28 | 42.85 | 2 |
| 2 | 6 | 0 | 6 | 3 | 3 | 0 | 5 | 5 | 20 | 25 | 5 |
| 2 | 9 | 2 | 12 | 6 | 6 | 1 | 9 | 11 | 36 | 30.55 | 7 |
| 5 | 6 | 3 | 8 | 2 | 4 | 2 | 6 | 12 | 22 | 54.55 | 11 |
| 7 | 9 | 3 | 12 | 5 | 5 | 2 | 8 | 17 | 34 | 50 | Control 1 |
| 32 | 58 | 10 | 75 | 31 | 36 | 9 | 60 | 82 | 231 | 35.5 | Control 2 |

*not specific IF in the absence of EBV DNA; **transferred to category II

value of lymphocytes (slightly over 60%) and the presence of atypical mononuclear cells (slightly over 10 %) could have been regarded for EBV-related. Taken together, in the immunized and challenged animals the four above mentioned methods could be used to estimate the degree of in vivo protection. The IgG antibodies measured by ELISA to a commercially available early EBV antigen (p54 protein, an EBV DNA polymerase cofactor) were quite specific, since no binding of pre-infection serum samples to micro plates was observed. The commercial EA-D test was also useful, because no peptide (epitope) of this kind had been used for immunization. For both reasons, we considered the positive antibody (ISR) values against this antigen for real indicator of EBV replication in the lymphatic tissues of challenged animals. According to the frequency (positive rates) of EA-D antibodies in result of the EBV challenge of immunized rabbits three categories were created to assess their protection status. Rabbits of category I encountered the immunization groups 6, 12, 13, 15 and 18 (Table 6), which developed no EA-D (early) antibodies at any of the post-inoculation intervals tested. Furthermore, a low level antibody response in just one post-infection interval (on day 8) in 1 of 3 rabbits developed after challenge in groups 1 and 16 (Figure 2) along with a potent EA-D antibody increase in a single rabbit at day 108 after challenge (not shown). While the rabbits from immunization group l were still regarded to belong into category I, those from groups 14 and 16 were re-classified into category II. The reason being that in the former the early antibody titer occurred at a very high level (ISR value over dilution 1: 160), while in the latter the IB showed more antibodies, along with additional tests which turned out positive. Figure (2) also shows that the frequency of EA-D antibodies within immunization groups belonging into category I, was considerably different from those found in the immunization groups 2 and 11 (comprising category III). The results in latter category were closely similar to mock-immunized controls, manifesting no protection. All the other immunization groups were collected into the largest category II (examples shown on Figure (3)). In these, the individual immunization groups revealed either two positive samples by two different immunized animals at the same interval (groups 7, 9 and 10), or two positive samples from the same animal at two different intervals (group 8) or, finally, three positive serum samples at two different intervals coming from two different immunized animals (groups 3, 4 and19). As judged from EA-D ELISA results, the rabbits of category I originally showed either full protection (groups 6, 12, 13, 15 and 18) or at least partial but still convincing protection (group 1). The protection in the latter immunization group was regarded for partial, the serum from rabbit number 4998, in addition to positive ELISA on day 8, precipitated the BZLF1/Zta early polypeptide on day 28 post-challenge indicating the onset of virus replication.

Taken together, when calculating the sum of positive rates of both serological tests (IB and ELISA), the original conclusion must be modified by immunization group 14, so that this group was finally transferred into category II. If accepting the assumption that category I rabbits must not encompass animals showing latent infection (Table 7), the immunization group 16

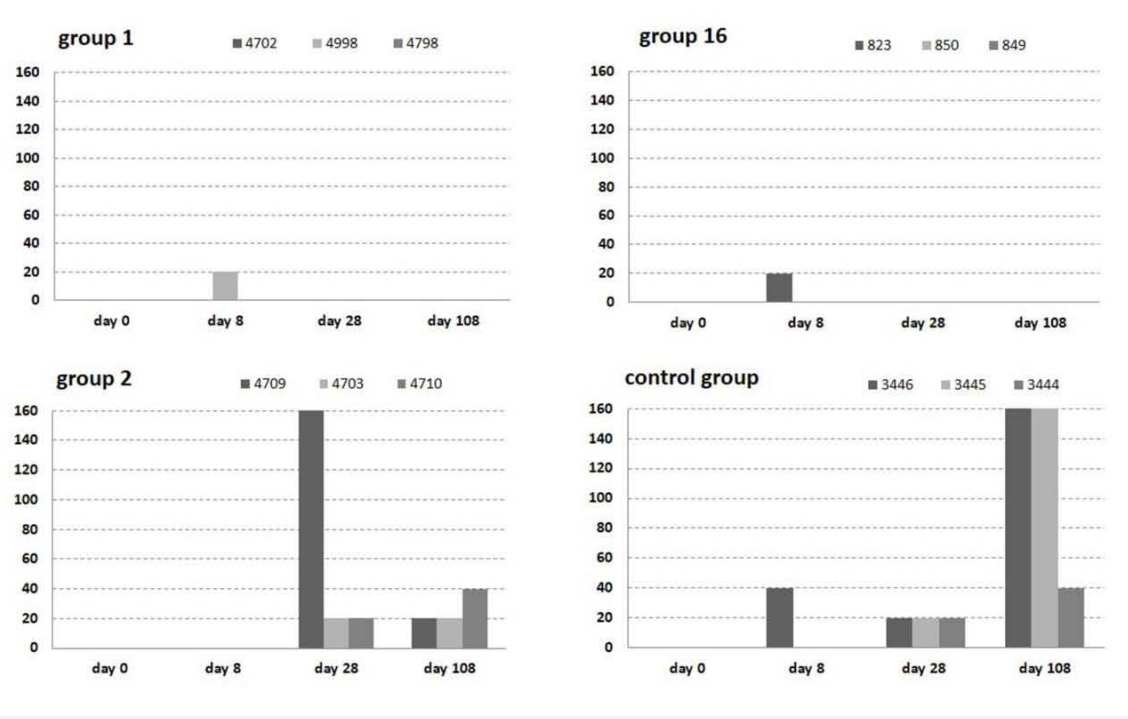


**Figure 2 Immunization groups of category I as compared with those of category III (group 2) and controls.** Immunization group 1 shows a minimal EA-D antibody response on day 8 post challenge. Alternatively, group 16 rabbits developed a minimal EA-D response as found by ELISA, but showed a more antibody response as detected by immunoblot as well the presence of latent vDNA. Therefore, we classified this group into category II. The rabbits of immunization groups 6, 12, 13, 15 and 18 developed no EA-D response at all (data not shown). One of 3 rabbits within group 14 showed a potent EA-D response by day 108 post challenge and therefore, it has been transferred into category II (see Table (6) for details).

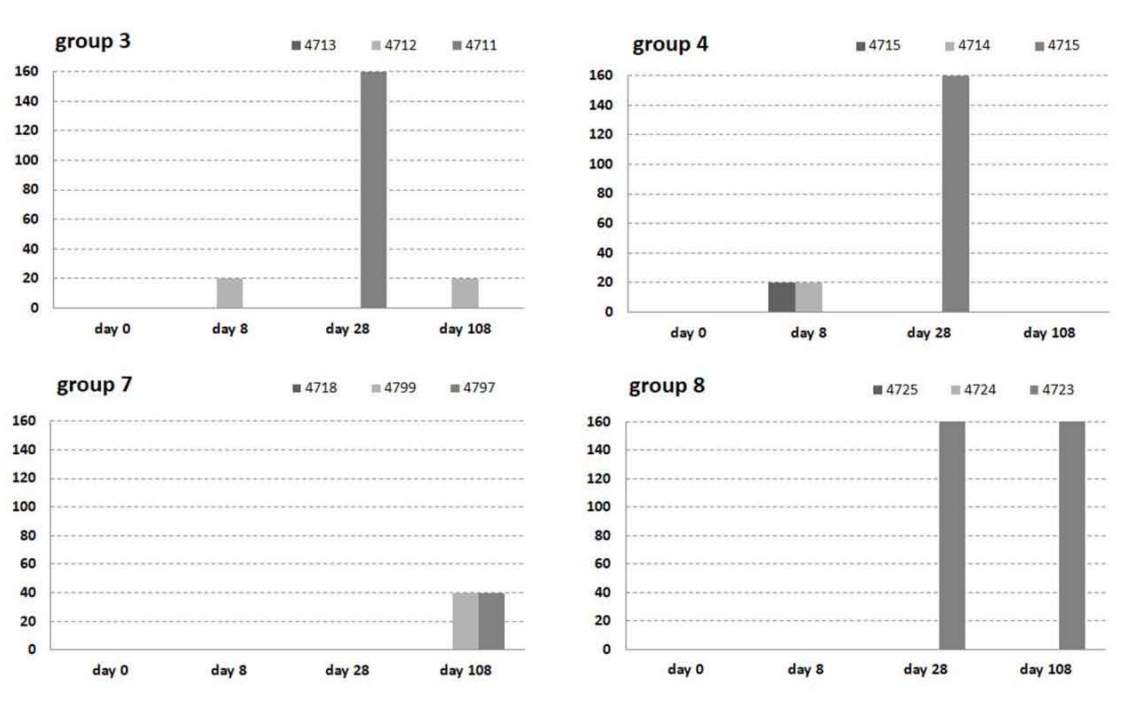


**Figure 3 The serology in rabbits showing no sufficient protection after immunization (category II).** The majority of immunization groups which were challenged with high infectious doses of EBV showed a moderate EA-D response. The following antibody reaction patterns were observed within this category: A. Antibodies from 2 different rabbits at 3 different intervals (group 3) or at 2 different intervals (group 4, similar groups 5 and19 not shown); B. Antibodies from 2 different rabbits in a single interval (group 7, other similar groups not shown); C. Antibodies from the same rabbit at 2 different intervals (group 8). Immunization group 7 was finally transferred to category III (compare Table (6)).

**Table 7:** EBV DNA and LMP1 antigen detection in rabbits of the immunization categories II and III.

| F1/PBMC | | F2/PBMC | | Aut/PBMC** | | Aut/Spl | Group | Rabbit number |
|---|---|---|---|---|---|---|---|---|
| LMP1 | DNA | LMP1 | DNA | LMP1 | DNA | DNA | | |
| Category II* | | | | | | | | |
| negat | negat | negat | positive | negat | negat | negat | 7 | 4718 |
| negat | negat | positive | positive | negat | negat | negat | | 4799 |
| negat | negat | negat | negat | positive** | negat | negat | | 4797 |
| | | | | | | | | |
| negat | negat | negat | negat | negat | negat | negat | 8 | 4725 |
| negat | negat | negat | negat | negat | negat | negat | | 4724 |
| negat | negat | positive | positive | negat | negat | negat | | 4723 |
| | | | | | | | | |
| negat | negat | negat | negat | negat | negat | negat | 9 | 1755 |
| negat | negat | negat | positive | negat | negat | negat | | 4999 |
| negat | negat | positive | positive | negat | negat | negat | | 4997 |
| | | | | | | | | |
| negat | negat | negat | negat | negat | negat | negat | 10 | 320 |
| negat | negat | positive | positive | ND | ND | ND | | 995 |
| negat | negat | negat | negat | ND | ND | ND | | 4899 |
| | | | | | | | | |
| negat | negat | negat | negat | negat | negat | negat | 14 | 817 |
| posit | negat | positive | positive | positive | positive | negat | | 818 |
| negat | negat | negat | negat | ND | ND | ND | | 819 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Category III | | | | | | | | |
| negat | negat | negat | negat | ND | ND | ND | 2 | 4709 |
| negat | negat | negat | negat | ND | ND | ND | | 4703 |
| negat | negat | negat | negat | negat | negat | positive | | 4710 |
| negat | negat | posit | negat | ND | ND | ND | 11 | 550 |
| negat | negat | unclear | positive | negat | posit | positive | | 530 |
| negat | negat | posit | negat | ND | ND | ND | | 540 |
| *immunization group 16 not included **probably artifact | | | | | | | | |

was regarded to belong into category II from the very beginning regardless to minimal EA-D antibody response. Thus, the category II of rabbits was grouped according to positive rates of both, IB and ELISA results and in addition, taking into account the presence of vDNA. In fact, the animals of category II fell into two subgroups: 1. animals which received epitope mixes exerting a minimal (or slight) protection despite of the antibody response and 2. animals obtaining epitope combinations not exerting a sufficient protection. Briefly, the category II consisted of immunization groups that neither fit into category I (containing the protective immunization schedules) nor into category III (the fully inefficient immunization schedules).

In a larger group of parallel control (infected but not immunized) rabbits, which had been examined independently, the positive rate of EBV DNA and/or LMP1 antigen detections was lower as compared to the rate of EA-D/p54 antibody response. The latter control cohort also showed that the incidence of LMP1 antigen in the PBMC smears in the majority of cases coincided with EBV vDNA detection in corresponding PBMC extracts at the same given intervals. However, in rabbit nr.4797 the LMP1 antigen seemed positive in the absence of the DNA (Table 7). Such possibly false positive IF reading of the LMP1 antigen as noted in the absence of was considered for an artifact but not vice versa (rabbit nr. 530 on day 108 post-challenge).

In the category III immunization groups at least one rabbit from each group revealed positive DNA findings. In the rabbit number 4710 (group 2) the EBV DNA was present in spleen at autopsy, while in rabbit number 530 (group 11), it was found in two PBMC extracts (on days 28 and 108) as well a in the spleen extract (at autopsy day). In this immunization group, 5 animals out of 9 revealed the EBV DNA in the PBMC as well assplenocyte extracts and nearly always expressed the LMP1 antigen.

## CONCLUSIONS

This paper relates to experimental immunization of rabbits with 19 different micro particle formulations using 15 epitope mixes selected from 12 structural as well as non-structural and/or latency associated immunogenic EBV proteins. The peptides in question were chemically linked to micro particle carriers in various combinations. The carefully selected virus-coded carrier bound epitope combinations along with the PRR agonists created the candidate peptide vaccine useful for immunotherapeutic and/or prophylactic use. Six out 19 combinations of the selected immobilized oligopeptides coming from seven immunogenic proteins (including two glycoproteins) bound together with three PRR agonist molecules to the same micro particle, elicited an efficient stimulation of APCs *in vitro* and provided satisfactory protection in our rabbit model *in vivo*. The advantage of the peptide vaccine presented here is the low probability of the induction of autoimmune responses, such as the ASIA syndrome [53], which may occur in a proportion of immunized patients.

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