Articles

DNA Vaccine against Friend Erythroleukaemia Virus

(DNA vaccine / Friend leukaemia virus)

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Abstract. Plasmids carrying DNA copies of the *gag* and *env* genes of FV, which causes erythroleukaemia in susceptible mouse strains, were prepared. Expression of the cloned genes was confirmed by indirect immunofluoroscence in cells transfected *in vitro*. Immunization experiments were performed in DBA/2 mice. Animals were injected with three doses of plasmid either intramuscularly (100 μg DNA per dose) or intradermally (1 μg DNA per dose); in the latter case, a gene gun was used. The FV type A or P was used as a challenge. The immunization with *gag*- and *env*-derived vaccines resulted in protective immunity in a high proportion of mice.

DNA vaccines represent a new tool for the induction of both humoral and cellular immunity (Lewis and Babiuk, 1999). The antiviral efficacy of this type of vaccine was first demonstrated with the influenza virus (Ulmer et al., 1993). In the recent decade the immunizing effect of DNA vaccines has been demonstrated in a number of other systems comprising viruses, bacteria and parasites (Donnelly et al., 1997). Moreover, the DNA-based vaccine technology represents a potentially powerful entry into the field of immunological control of some non-infectious diseases such as cancer.

Among the retroviruses, human immunodeficiency virus 1 (HIV-1) has become the most frequent DNA-vaccine target, and HIV DNA vaccines are now in their first clinical trials (Formsgaard, 1999; Frey, 1999). DNA vaccines based on different HIV genes may prove to have the potential of efficient agents in AIDS. They can induce neutralizing antibodies and CTLs (cytotoxic T lymphocytes) against conserved viral proteins such as Gag (Qiu et al., 1999). However, the lack of realiable correlates of protective immunity makes a HIV vaccine design a difficult undertaking.

Preclinical studies aimed at claryfying different aspects of antiretroviral immunity induced by DNA vaccines require animal models. In the past, such studies included the simian immunodeficiency virus (Mossman et al., 1999), bovine leukaemia virus (Kučerová et al., 1999) and also the more distantly related murine leukaemia and sarcoma viruses. A number of interesting observations have been made in the course of these studies.

In the present series of experiments we used the Friend murine leukemia virus (FV) *gag* and *env* genes in the form of DNA vaccines. This virus differs from HIV in many important respects; however, some of its variants induce an immunosuppresion closely resembling AIDS, though mediated through different mechanisms. Friend virus, which induces erythroleukaemia in mice (Friend, 1957), is a complex of replication-defective spleen focus-forming virus (SFFV) and replication-competent Friend murine leukaemia virus (F-MuLV) (Teich et al., 1984; Kabat, 1989). In the receptive animals this complex induces polyclonal proliferation of immature erythroblasts, which leads to acute splenomegaly within a few days. The SFFV Env protein differs from the F-MuLV Env protein in size, by the presence of N-terminal sequences derived from the endogenous mouse retrovirus, the loss of a cleavage site and a few N-linked glycosylation sites, the composition of the transmembrane region, and lack of a cytoplasmic tail. Furthermore, it does not function as a viral structural protein. In spite of these differences, both the SFFV and MuLV Env proteins share some of the most important epitopes (for review see Ruscetti, 1995). The SFFV Env protein produced after the random integration of this virus DNA into the cell genome is responsible for the early stage of FV-induced disease. It binds, if properly processed and transported, to the erythropoietin (E) receptor, acting as a proliferative signal to erythroid precursor cells (Ruscetti, 1995; Lin et al., 1998). There are two types of SFFV *env*-gene products. One confers complete E-independence of infected cells (SFFV type P), while the other does not

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Abbreviations: FV – Friend murine leukaemia virus, SFFV – spleen focus-forming virus.

(SFFV type A), possibly owing to the amino acid substitutions in its transmembrane domain. Still, it is likely that the type A Env protein also alters the growth of erythroid cells by reason of its interactions with the E receptor resulting in a strong decrease of their requirement for E (Li et al., 1991). The subsequent pathogenic steps involve a site-specific integration of SFFV DNA. This integration results in a rearrangement and activation of the PU.1 transcription factor (Moreau-Gachelin et al., 1989; Paul et al., 1991). It is strongly suspected that high levels of the PU.1 protein and the simultaneous loss of expresion of the p53 tumour-suppressor gene (Mowat et al., 1985; Munroe et al., 1990) are responsible for transformation and generation of transplantable erythroleukaemia cells of unlimited growth potential.

Only some mouse strains are susceptible to FV. The mouse genome contains several genes that are extremely important for the outcome of infection with this virus (Chesebro et al., 1990). Among these are the so-called *Fv1-Fv6* genes whose products give rise to FV susceptibility via non-immunological mechanisms. Four major histocompatibility complex (MHC) genes, and one non-MHC gene denoted *RVF-3*, also play an important role in the development of, and recovery from, FV-induced erythroleukaemia, most probably through regulation of the host immune responses (Hasenkrug and Chesebro, 1997).

A large body of information on the specific immune reactions to FV has been accumulated in the last two decades. The complexity of these reactions in FV-induced disease and the current problems and paradoxes which need further analysis have been described and thoroughly discussed (Hasenkrug and Chesebro, 1997).

In the past, a number of experimental vaccines, e.g. purified Env protein (Kleiser et al., 1986), Env-derived peptides (Bayer and Hunsmann, 1987), killed virus (Ishizara et al., 1991), recombinant vaccinia virus expressing F-MuLV *env* and F-MuLV *gag* (Earl et al., 1986; Miyazawa et al., 1992) and gene-modified (interferon α-expressing) FV-transformed cells (Ferrantini et al., 1993) have been used in successful immunization against FV. It has been the aim of the present study to examine the possibility to confer protection against FV-induced disease by DNA vaccines containing either the *env* or the *gag* gene of F-MuLV. To the best of our knowledge, DNA vaccination against FV has not yet been reported.

Material and Methods

Viruses, animals and cells

Friend virus types A and P were obtained, through the courtesy of M. J. Koury of Vanderbilt University, Nashville, USA, in the form of frozen sera from infected mice. These materials contained 10^4/ml infectious units of FV type A and 10^5/ml infectious units of FV type P, as shown by *in vivo* titration in DBA/2 mice. To prepare virus stocks, 7 x 10^1 or 7 x 10^2 infectious units of FV type A or P, respectively, were injected into 4–5-week-old Balb/c mice (Charles River, AnLab, Prague) and after 10 days the animals were bled out. Their plasma was collected and stored at -70°C. The virus titres were expressed as erythroleukaemia-inducing doses 50 (ELID50). Three to four-week-old DBA/2 mice (Charles Rives, AnLab, Prague) were used in all experiments, five mice per each ten-fold dilution.

293T cells were obtained from J. Kleinschmidt, DKFZ, Heidelberg, Germany. They were maintained in Dulbecco's modified Eagle's medium (D-MEM) (BRL, Karlsruhe, Germany) supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Friend leukaemia cell lines FLC 3cl8 and FLC IFN-α cl11 were kindly provided by M. Ferrantini, Istituto Superiore di Sanita, Rome, Italy, and were maintained in RPMI (Sigma-Aldrich, Irvine, UK) supplemented with 10% foetal calf serum (ZVOS, Hustopeče, Czech Republic), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.3 mg/ml G418. The last three passages before using them for immunization (see below) were made in non-selecting media. Clone FLC 3cl8 was isolated from the FLC 3 cell line, originally derived from a DBA/2 mouse in which erythroleukaemia had been induced by F-MuLV. These cells are highly oncogenic for syngeneic mice after intraperitoneal inoculation. They induce ascitic tumours with metastatic foci in the liver and spleen. Clone FLC IFN-α cl11 was derived from FLC 3cl8 after transfection with a plasmid derived from a retroviral vector carrying murine IFN-α1 cDNA. These cells were reported to be non-oncogenic for mice (Ferrantini et al., 1993).

Preparation of plasmids

For DNA immunization, plasmid pBSC, into which genes coding for viral proteins were cloned, was constructed (Šmahel et al., 2001). Plasmid pBR-21A1, carrying the whole genome of F-MuLV (clone 57), was kindly provided by W. Ostertag, Hamburg, Germany (Oliff et al., 1980). This plasmid was digested with *Sac*I or *Kpn*I and *Bam*HI to get fragments with the *gag* or *env* gene, respectively. These fragments were blunt ended and inserted into vector pBSC, which was digested with *Xho*I and blunt ended. The resulting plasmids were denoted pBSC/env and pBSC/gag (see Fig. 1). Plasmid pNLS-lacZ, kindly provided by J. Kleinschmidt, DKFZ, was used for monitoring the effectiveness of transfection. Its expression was detected by staining with X-gal (Fermentas, Vilnius, Lithuania).

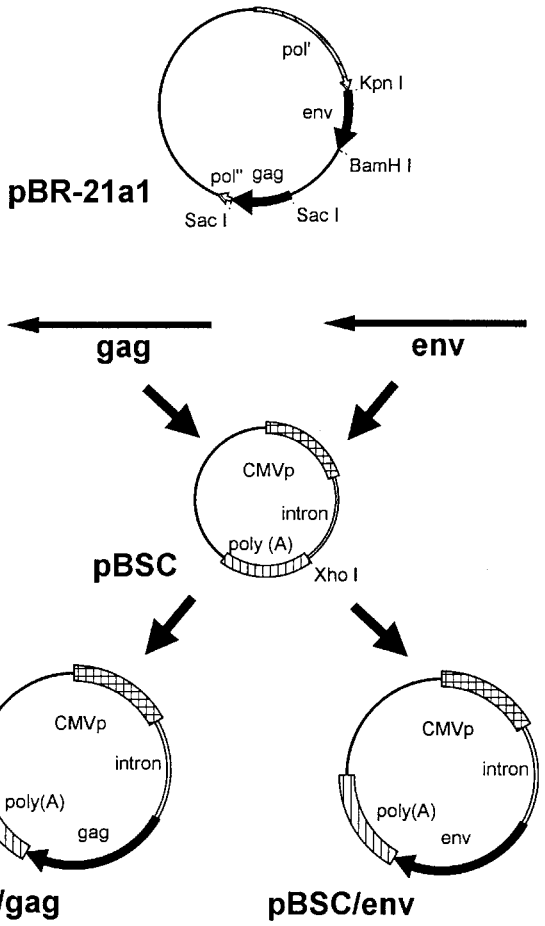

Fig. 1. Construction of vaccination plasmids

Transfection and immunofluorescence test

To demonstrate the expression of the genes cloned, pBSC/env and pBSC/gag, and the control plasmids pBSC and NLS-lacZ were transfected by calcium-phosphate precipitation (Chen and Okayama, 1987) into 293T cells at transfection doses of 20 μg of pBSC/env, pBSC/gag and pBSC per 4 x 10^5 cells and 1 μg of pNLS-lacZ per 2 x 10^3 cells. After 24 h the cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS for 10 min, permeabilized with 1% Triton X-100 (Serva, Heidelberg, Germany) in PBS for 5 min, and incubated with a 1 : 20-diluted serum pool from Balb/c mice that had been infected with FV-P. As a secondary antibody, swine anti-mouse IgG antibody labelled with FITC (Sevac, Prague, Czech Republic) was used. The immunofluorescence test (IF) was also used to determine the antibody presence in the sera from immunized mice, which were used diluted 1 : 100. This dilution was found to be fitting the purpose; however, because of the very small amounts of sera available from individual animals, no systematic quantitative investigations could be performed. For detecting Gag and Env antibody, 293T cells transfected with the respective plasmids were fixed with paraformaldehyde and incubated for 2 h with diluted serum samples. The treated cells were washed five times with PBS and exposed to 1 : 100 diluted FITC-labelled anti-mouse IgG antibody (Sevac, Prague, Czech Republic). Sera giving clear fluorescence (usually in more than 25% of cells) were considered antibody-positive. Control positive and negative sera were included into each test. The specifity of the test was confirmed by using sera from mice immunized with either only the *gag* or *env* vaccine (see below).

Definition and detection of disease

Spleens, livers and lungs were excised four weeks after challenge for macroscopic inspection and histological examination. Enlargement of the spleen associated with erythroblastoid infiltration of spleen, liver and lung and necrotic foci in the spleen were considered as evidence of disease. The normal weight of DBA/2 mouse spleen and liver is 0.05–0.15 g and 0.2–0.9 g, respectively. In diseased mice, spleens were enlarged 5 to 50 times. Enlargement of other organs was not observed.

Immunization and challenge experiments

Four to five-week-old DBA/2 mice were immunized either with three intramuscular doses given at three-week intervals of 100 μg each of pBSC/gag, pBSC/env or control pBSC, or with 1 μg of each of the above mentioned

plasmids via intradermal inoculation with a gene gun (Bio-Rad, Hercules, CA). The intramuscular injections were performed into the quadriceps femoris muscle. For intradermal inoculation, gold particles coated with vaccination plasmids (according to the producer's instruction) were shot into the abdominal skin under the helium pressure of 400 psi. Into the second immunization experiment, lethally irradiated FLC 3cl8 and lethally irradiated and non-irradiated FLC IFN-α cl11 were also included. These cells were administered intraperitoneally in two doses (in all instances 10^6 cells/dose) at the terms of the second and third DNA immunizations; these animals were not given the plasmids. Two weeks after the last immunization dose the mice were intraperitoneally challenged with approximately 20 ELID50 of either FV-A or FV-P. Just prior to the challenge, a blood sample was taken from the tail of each mouse. Four weeks after challenge the mice were sacrificed and their spleens, livers and lungs were removed for weight determination and histological examination.

Neutralization test

Five serum pools were prepared from sera taken at challenge: (i) Env^+Gag^- from pBSC/env-immunized animals, (ii) Env^-Gag^- from pBSP/env-immunized animals, (iii) Env^-Gag^+ rom pBSC/gag-immunized animals, (iv) Env^-Gag^- from pBSC/gag-immunized animals and (v) Env^-Gag^- from animals injected with control pBSC. All sera were inactivated for 30 min at 56°C. Serum pools diluted 1 : 50 were mixed with equal volumes of FV-P virus diluted to contain 20 ELID50 per 0.1 ml. The mixtures were incubated for 30 min at room temperature and 0.2 ml of each mixture was inoculated intraperitoneally into a group of six mice. A control group of seven mice was inoculated with the same volume of a mixture in which PBS was substituted for serum. Similarly as in the immunofluorescence antibody assay, only one serum dilution was tested, because only very little amounts of sera were available. The animals were observed for four weeks, then humanly killed and inspected for the disease.

Statistical analysis

Data obtained were analysed in 2 x 2 contingency tables by 2-tailed Fisher's exact test. Any difference between groups was considered statistically significant if $P < 0.05$. Calculations were performed using the Prism software, version 3.0 (GraphPad Software, Inc., San Diego, CA).

Results

Expression of env and gag after in vitro transfection

293T cells were transfected with plasmids pBSC/env, pBSC/env, pBSC or pNLS-lacZ. Up to 50% efficiency of transfection with control plasmid pNLS-lacZ was observed in repeated tests. Approximately the same percentages of cells were positive with sera from FV-infected or *gag*- or *env*-plasmid immunized mice (Fig. 2A and 2B). We did not detect any signal in control, non-transfected cells, in cells transfected with the parent control plasmid pBSC (Fig. 2C), or in transfected cells treated with control mouse sera.

Virus titration in mice

Stocks of both FV-P and FV-A prepared in Balb/c mice were titrated in parallel in DBA/2 mice. The results of the titration are shown in Table 1.

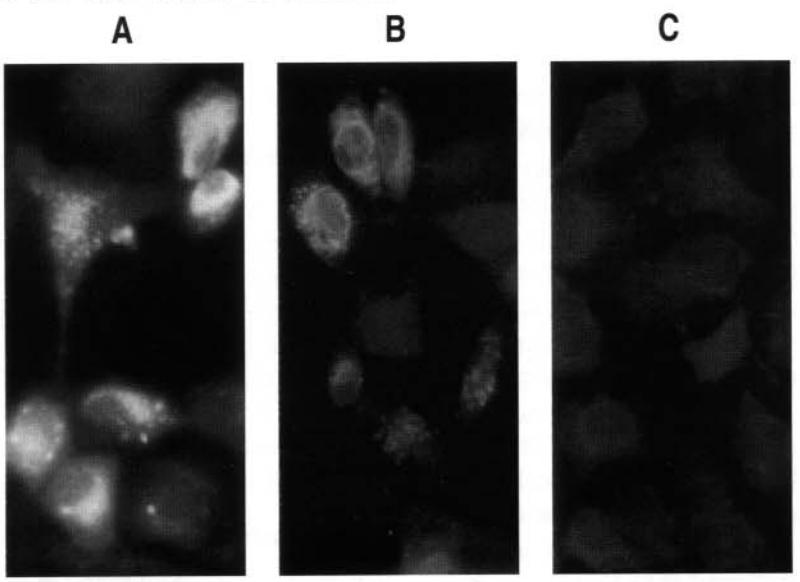

Fig. 2. Immunofluorescence tests with 293T cells transfected with pBSC/env (A), pBSC/gag (B) and pBSC (C). Cells were transfected and 24 h later stained with 1 : 20 diluted pool of sera from Balb/c mice that had been infected with the FV-P virus (see Material and Methods).

Table 1. Titration of Friend erythroleukaemia virus stocks

Virus dilution	Animals with erythroleukaemia [a]	
	FV-A	FV-P
10^{-1}	5/5	5/5
10^{-2}	5/5	5/5
10^{-3}	5/5	5/5
10^{-4}	5/5	5/5
10^{-5}	5/5	5/5
10^{-6}	2/5	5/5
10-7	0/5	0/5

[a]No. of mice with disease over No. of mice inoculated,

Table 2. Results of the second immunization experiment

Group	Animals with erythroleukaemia[a]	P
control	8/8	
pBSC	7/8	
pBSC/gag	2/10	P < 0.01
pBSC/env	2/10	P < 0.01
FLC 3cl8* (irradiated)	7/10	NS
FLC IFN-α cl11* (irradiated)	2/10	P < 0.01
FLC IFN-α cl11	0/7	P < 0.01

[a]see Table 1

First immunization experiment

In the first experiment plasmids pBSC/env, pBSC/gag or pBSC were administered either intramuscularly or intradermally. The mice were challenged with approximately 20 ELID50 of either A or P virus. As indicated in Fig. 3A and 3B, all control mice, whether untreated or injected with the control pBSC plasmid, developed the disease. A weak protection against virus-induced erythroleukaemia was observed both in mice immunized with pBSC/env and pBSC/gag. When taking into account mice challenged with both A and P viruses, *env* immunization protected 9 out of 19 mice, and *gag* immunization 2 out of 20 mice. Only the effect produced by pBSC/env was statistically significant ($P < 0.05$). While protection by pBSC/env against both the A and P type of virus was evident, immunization with pBSC/gag seemed to be slightly effective against FV-P only. Mice declared as protected were free of erythroblastoid infiltration in their spleen, liver and lungs, and the weight of all these organs was within normal limits.

Second immunization experiment

The experiment was repeated using the same plasmids, but only intradermal inoculation of DNA and the FV-P virus for challenge were used. For control purposes immunization with irradiated FLC 3cl8 and FLC IFN-α cl11 cells and non-irradiated FLC IFN-α cl11 cells were performed. Ten mice were included in each group. Three mice inoculated with non-irradiated FLC IFN-α cl11 cells developed intraabdominal ascitic tumours after the first inoculation; this might have been due to the appearance of rare revertants because the last three passages of these cells prior to immunization were performed in the absence of G418 (see Material and Methods). These three animals were eliminated from the experiment. Again, all mice were challenged with approximately 20 ELID50. The results are shown in Table 2. The disease developed in all untreated mice and in all but one animal treated with the control plasmid. The immunization effect was even more marked than in the first experiment. Eighty per cent of mice immunized with either pBSC/env or pBSC/gag were protected.

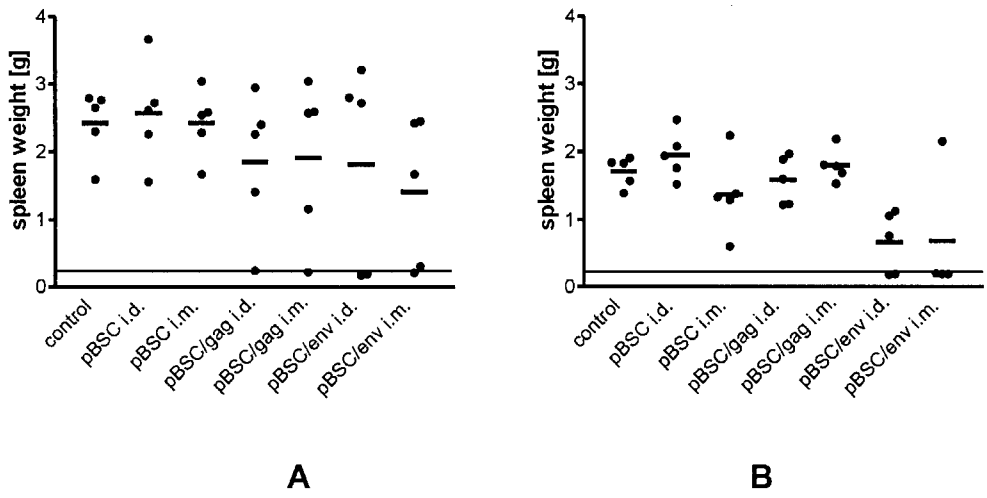

Fig. 3. The first immunization experiment – weight of spleens of mice challenged with the P type virus (A) and the A type virus (B). Mice were injected with three doses of plasmids given either intramuscularly (100 μg/dose) or intradermally (1 μg/dose). The thinline marks the maximum weight of normal spleen in DBA/2 mice. Short thick bars indicate the average weight of spleens in each group.

Serological response to vaccination and its relationship to protection to challenge

A proportion of immunized mice possessed the anti-Env and/or anti-Gag antibodies at the time of challenge. It was of interest to find out whether there was a relationship between the antibody presence and the protection to challenge. The results of this analysis are shown in Tables 3 and 4. Although the protection was seen somewhat more frequently in Env- and Gag-antibody-positive animals, the difference was quite small. In this respect it is noteworthy that in the groups immunized by the plasmids all those who were protected were free of detectable Env or Gag antibody at the challenge; on the other hand, the only two antibody-positive animals were not protected. These findings demonstrate a lack of association between the antibody presence and the protective immunity. It may also be of interest that after challenge, at the end of the observation period, no striking differences were detected in the occurrence of antibodies in the successfully immunized mice and those who developed the malignancy (results not shown).

To further examine the nature of the antibodies developed, the neutralization test with the sera either possessing or not possessing antibodies at the time of challenge was performed. No neutralization activity was observed. All animals developed the disease, and its course and pathological findings did not differ among the different groups of animals (results not shown).

Discussion

The FV model has repeatedly been used to elucidate the mechanisms involved in protective immunity against retrovirus-induced diseases. Several types of vaccine have been successfully employed to prevent FV-induced erythroleukaemia in mice. Correlation between protection and antibody development, CTL response and lymphocyte proliferation has been reported (for review see Hasenkrug and Chesebro, 1997), suggesting that both humoral and cell-mediated immune reactions are involved. Immunoprotective determinants have been identified in both the Env and Gag proteins and some evidence has been obtained that these determinants induce immune responses that interfere with different steps of the pathogenetic process (Ruan and Lilly, 1991; Hasenkrug et al., 1997; Uenishi et al., 1998). This has suggested that reactivity against a multiplicity of epitopes is needed to induce a full protection. It is noteworthy that immunization with the Gag protein has been reported in mouse strains in which immunization with the Env protein failed (Ishizara et al., 1991), which in its turn underlines the importance of genetic factors in the development of FV-induced disease and in protection from it.

It was not the purpose of the present experiments to analyse the different parameters of immunity against FV, but simply to explore whether efficient immunization by DNA vaccines against FV-induced leukaemia was possible. The results indicated that protective immunity in DBA/2 mice was indeed achieved by their immunization with DNA copies of viral genes. Both *env*- and *gag*-based vaccines were effective and the protective effect attained was comparable to that observed after immunization with continuous FV-transformed cell lines.

Although originally not planned for the present series of experiments, we could not resist the temptation

Table 3. Presence of anti-Env antibody and protection to challenge with FV

Group	No. of animals	No. of animals with *env* antibody before challenge	Protection in	
			env + antibody animals	*env* – antibody animals
pBSC/env	10	1/10	0/1	8/9
FLC 3cl8* (irradiated)	10	4/10	2/4	1/6
FLC IFN-α cl11* (irradiated)	10	1/10	1/1	7/9
FLC IFN-α cl11	7	7/7	7/7	-
All	37	13/37 (35.1%)	10/13 (76.9%)	16/24 (66.7%)

Table 4. Presence of anti-Gag antibody and protection to challenge with FV

Group	No. of animals	No. of animals with *gag* antibody before challenge	Protection in	
			gag + antibody animals	*gag* – antibody animals
pBSC/gag	10	1/10	0/1	8/9
FLC 3cl8* (irradiated)	10	7/10	3/7	0/3
FLC IFN-α cl11* (irradiated)	10	4/10	4/4	4/6
FLC IFN-α cl11	7	7/7	7/7	-
All	37	19/37 (51.4%)	14/19 (73.3%)	12/18 (66.6%)

to test sera collected at the time of challenge for the presence of both Env and Gag IgG antibodies. The protection was apparently independent of the Env and Gag IgG antibodies. The absence of protective immunity in several animals with anti-Env antibody may have indicated that these antibodies were not directed against the virus-neutralizing epitopes. The absence of the Env antibody in most of the protected mice may have been due to a late development of this antibody, since it has been shown that even in successfully immunized animals, neutralizing antibodies develop rather after than before the challenge with FV (Earl et al, 1986). These data demonstrated that in DNA-immunized animals the presence or absence of either Env or Gag IgG antibodies – as determined in the present study – was not a reliable indicator whether resistance to challenge had developed. These findings might be interpreted as a proof that cell-mediated immunity played a decisive role in the protection induced. It will be the purpose of future experiments to determine the mechanism of protection induced by FV DNA vaccines in different phases of the immunization process.

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