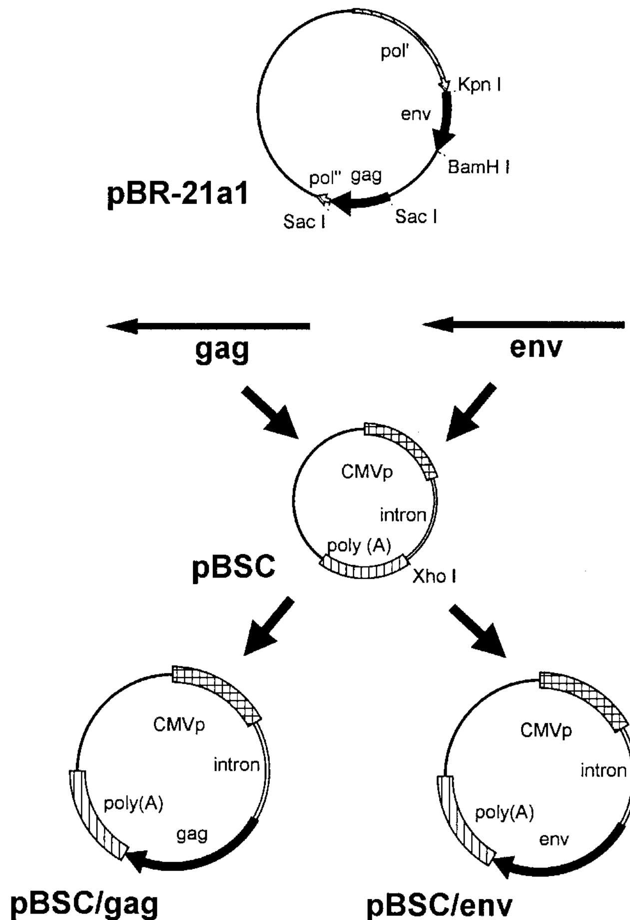

Fig. 1. Construction of vaccination plasmids

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.

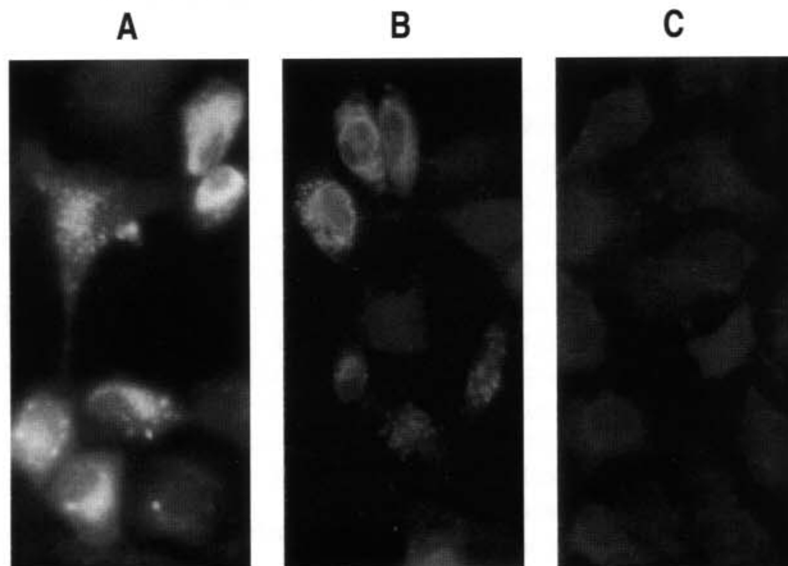

Fig. 2. Immunofluorescence tests with 293T cells transfected with pBSC/env (A), pBSC/gag (B) and pBSC (C). Cells were transfected and 24 h later stained with 1 : 20 diluted pool of sera from Balb/c mice that had been infected with the FV-P virus (see Material and Methods).