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EVALUATION OF AN INACTIVATED BOVINE LEUKEMIA VIRUS PREPARATION AS AN IMMUNOGEN IN CATTLE

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Résumé

ESTIMATION DE LA VALEUR IMMUNOGENE CHEZ LES BOVINS D'UNE PREPARATION INACTIVEE DE VIRUS DE LA LEUCOSE BOVINE. — L'infection par le virus de la leucose bovine (BLV) est endémique dans de nombreux troupeaux des Etats-Unis, et sa limitation ou son éradication par un programme type « testage et élimination » serait très coûteuse. La vaccination pourrait être un moyen plus économique, et ce rapport résume nos travaux préliminaires dans le but de mettre au point un BLV inactivé et immunogène pour les bovins.

La source de BLV a été le liquide des cultures d'une lignée cellulaire de foetus et de rein d'agneau, et la quantité d'antigène glycoprotéique présent a été déterminée par immunodiffusion radiale. Le liquide de culture était incubé pendant 24 h à 37 °C, avec soit 0,05 % de formaline, 0,05 % de N-acetyléthylèneimine (AEI), ou 0,02 % d'éthylèneimine binaire (BEI). L'antigénicité de la glycoprotéine du BLV était notablement réduite par le traitement à la formaline, mais il n'y avait pas de perte détectable d'antigénicité après contact avec l'AEI ou le BEI. L'efficacité de l'inactivation par ces deux derniers produits a été contrôlée par un test d'induction d'un syncytium en culture cellulaire. Les deux composésLavaient détruit le BLV de façon satisfaisante après 8h à 37 °C.

Pour tester l'immunogénicité, quatre veaux reçurent une injection d'une préparation traitée par l'AEI, qui contenait 0,34 mg de glycoprotéine avec 10 % d'hydroxyde d'aluminium comme adjuvant. Il a fallu au moins deux injections pour obtenir un taux d'anticorps détectable par immunodiffusion en gel d'agar (AGID test). Le titre de neutralisation du virus de ces antisérums était de 1:32 ou 1:64. Les veaux vaccinés ont été éprouvés avec des lymphocytes d'animaux infectés, et un test ultérieur d'induction d'un syncytium montra que 3 veaux résistèrent à l'infection. L'année suivante, les tests sériques de neutralisation du virus, AGID et radioimmunologique décelèrent une baisse progressive du titre en anticorps, qui tombèrent parfois en dessous du seuil de détection. Par ailleurs les lymphocytes sanguins des veaux, inoculés au mouton, ne provoquèrent pas l'infection. Ces résultats permettent de conclure qu'il n'y a pas eu infection persistante par le BLV.

Introduction

Serologic surveys in the United States have shown a high incidence of bovine leukemia virus (BLV) infection in cattle (Baumgartener *et al.*, 1975 ; House *et al.*, 1977). This high infection rate would apparently preclude the use of a control program that requires slaughter or quarantine of affected animals, because of the severe economic consequences. Another approach to control, vaccination, would require the availability of a BLV immunogen that would not be infectious for cattle. The danger of an attenuated live virus vaccine would be two-fold : (1) BLV is a retrovirus and typically results in a persistent infection, presumably due to insertion of viral genetic information into cellular DNA ; (2) the transformation event that leads to neoplasia is believed to occur at the time of infection, so even if an attenuated virus did not result in persistent infection, lymphosarcoma could still occur.

The purpose of this report is to describe the preparation of an inactivated BLV immunogen, and to present results of preliminary experiments that suggest the « vaccine » might be useful for prevention of BLV infection.

Materials and methods

Cell culture

BLV and viral antigens were obtained from persistently infected fetal lamb kidney (FLK) cells (Van Der Maaten and Miller, 1976). Cultures were grown in 2-liter roller bottles that contained 200 ml Eagle's minimum essential medium with 5 % fetal calf serum and gentamicin sulfate, 50 mg per liter. The cells were subcultured at a ratio of 1:2 every 10 or 11 days. For efficient production of glycoprotein antigen, media was harvested on day 7 and again on the final day after subculture. To obtain BLV for infectivity assays and neutralization tests, we changed the media on a roller bottle and harvested culture fluid 24 hours later. The media for virus production contained polybrene (2 μg/ml) and 10 %, rather than 5 %, fetal calf serum. Virus preparations were clarified by passage through a 0.45 μ filter before use.

Inactivation

Distilled water was used to make 5 % solutions of formalin and N-acetylethyleneimine (AEI). Binary ethyleneimine (BEI) was prepared by cyclization of 2 % 2-bromoethylamine hydrobromide in 0.02 N sodium hydroxide (Bahnemann, 1975). One ml of each inactivant was added to 99 ml of FLK culture fluid and the mixtures were incubated at 37 °C for 24 hours. Samples were then concentrated 50-fold in a non-agitated concentrator with a 15,000 MW selective membrane (Amicon Corp., Lexington, MA), and tested for glycoprotein antigen content by radial immunodiffusion (RID).

For the RID assay, we used gamma globulin from a BLV-infected cow at a dilution of 1:18 in 0.85 % agarose. The globulin had precipitating antibody to BLV glycoprotein antigen but not to p24 antigen (Phillips, Miller and Van Der Maaten, 1978). Buffer for the agarose was 0.05 M Tris-HCl, pH 7.2, with 8.5 % sodium chloride. Assays were conducted in a calibration plate (Miles Lab., Elkhart, IN) that contained 8.5 ml of the agarose-antibody mixture. Wells, 5 mm in diameter, were filled with 30μml of test material and plates were stored in a humidified chamber at room temperature for 5 days. Antigen quantity was determined by comparison to dilutions of isolated BLV glycoprotein (Phillips *et al.*, 1978). Protein content of the standard was determined by the procedure of Lowry *et al.*, 1951.

The inactivation of BLV infectivity by AEI and BEI was examined *in vitro* at various time intervals during the incubation period. The inactivants were neutralized by a 1:10 addition of 20 % sodium thiosulfate and then samples were tested for BLV with a syncytium induction assay (Benton *et al.*, 1978). For these tests, F-81 cat cells were seeded in 24-well tissue culture plates (Becton Dickinson, Oxnard, CA) at 50,000 cells per well. Twenty-four hours later, the cells were exposed to 100 μl of test material and incubated at 37 °C for 1 hour. One ml of media was then added and incubation was continued for 7 days with a media change every 48 hours. Throughout the assay we used Eagle's minimum essential medium with 0.25 % lactalbumin hydrolysate, 10 % fetal calf serum, polybrene (2 μg/ml) and 50 mg/liter gentamicin sulfate. At the end of incubation, plates were fixed in methanol, stained (Hemal-Stain, Danbury, CN), and ex__-mined for syncytia. Five or more syncytia in a well were recorded as positive for BLV.

Preparation of antigen

FLK culture fluids were inactivated with 0.05 % AEI at 37 °C for 24 hours. Proteins

were then concentrated 10-fold by ammonium sulfate precipitation as previously described (Miller and Van Der Maaten, 1977). This protein concentrate was dialyzed for 16 hours against 8 volumes of distilled water that contained 0.1 % sodium azide and the volume was then reduced to approximately 2 % of the original volume of culture fluid. A hollow fiber dialyzer/concentrator with a 10,000 MW cartridge (Amicon Corp., Lexington, MA) was used for dialysis and final concentration. The antigen preparation was clarified by centrifugation at 50,000 g for 1 hour and tested by RID to quantitate the amount of BLV glycoprotein antigen present. The presentation was then adjusted, by dilution or further concentration, to contain 75 μg antigen per ml.

Vaccination trials

Aluminium hydroxide gel was added to the BLV antigen to a final concentration of 10 %. Holstein calves, 6-12 months old, were injected intramuscularly with 5 ml of the antigen-adjuvant mixture at 2-week intervals. The calculated glycoprotein antigen content of each injection was 0.34 mg.

Three serologic tests were used to evaluate antibody response to the BLV antigen. Virus neutralization (VN) assays were done with the procedure of Diglio and Ferrer (1976), except that the quantities of virus and serum were 100 μl rather than 1 ml. The agar gel immunodiffusion (AGID) test for antibody to BLV glycoprotein antigen and the radioimmunoassay (RIA) for antibody to p24 antigen have been described elsewhere (Miller and Van Der Maaten), 1977 ; Devare *et al.*, 1977).

Vaccinated calves were challenged with 2.5-5 × 10^3 peripheral blood lymphocytes from a persistently infected steer. The lymphocytes were isolated by Ficoll-hypaque flotation (Muscoplat *et al.*, 1974), suspended in saline, and 0.25 ml was injected intracutaneously. Three to 5 weeks after challenge, vaccinates and controls were tested for BLV infection with the syncytium induction assay (SIA) (Ferrer and Diglio, 1976).

Results

Evaluation of inactivants

Three different collections of FLK culture fluids were tested for BLV glycoprotein antigen after exposure to AEI, BEI, or formalin. The results of each trial were similar and are illustrated in Table 1. There was no loss of glycoprotein antigen in the fluids treated with AEI or BEI, whereas treatment with formalin destroyed one-third to one-half of the antigen's precipitating activity. Because AEI and BEI were superior to formalin with respect to preservation of BLV glycoprotein, we tested only the former 2 compounds for inactivation of infectious BLV. The results are presented in Table 2. The titer of BLV infectivity

Table 1. — Effect of inactivants on antigenicity of BLV glycoprotein.

Inactivant	Antigen μg/ml
None	85
AEI	85
BEI	80
Formalin	40
Control (1)	80

(1) held at 4 °C.

Table 2. — Inactivation of BLV.

Inactivant	Time (hours)					
	0	1	2	4	8	24
None	256 (1)	128	32	32	16	0
AEI	—	64	32	8	0	0
BEI	—	128	16	16	0	0
Control (2)	—	128	32	32	32	16

(1) Reciprocal of virus dilution positive for BLV.
(2) Held at 4 °C. All others at 37 °C.

declined progressively in the untreated as well as in treated samples, but at 8 hours, only the AEI and BEI-treated materials were negative for BLV. By 24 hours, infectious virus was detected only in untreated fluid that had been held at 4 °C.

Immunogenicity in cattle

Four calves were given the BLV antigen preparation. Ten days after each injection, sera were collected and tested by AGID. None of the calves produced BLV antibody in response to the first injection. Following the second exposure, 2 calves had positive AGID reactions and they were challenged with 5 × 10^3 lymphocytes (experiment 1). The other 2 calves were given a third injection and then they also were serologically positive by AGID. They were challenged with 2.5 × 10^3 lymphocytes (experiment 2). In each challenge experiment, 1 control calf was also inoculated.

Results of VN and RIA tests on sera from calves in experiment 1 are presented in figs. 1 and 2 respectively. At 6 and 24 weeks after challenge, SIA results indicated that the control calf and 1 vaccinate (no. 19) had become infected with BLV. The persistence of relatively high VN antibody titers in these 2 animals supported results of the virus isolations. The RIA titers of both vaccinates declined progressively and at 6 months after challenge their titers were very low, although still considered positive (1:20 and 1:30). The BLV-negative vaccinate (no. 49) was AGID negative 9 months after challenge, RIA negative at 10 months, and by the 17th month, no VN antibody was detected.

Serologic test results of calves in experi-

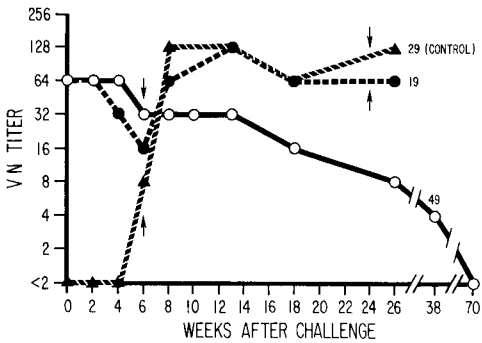

Fig. 1. — Virus neutralization titers, challenge experiment 1. Arrows indicate a positive virus isolation.

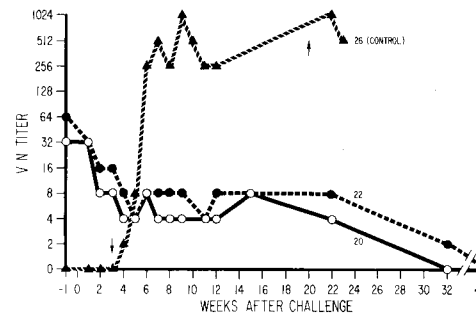

Fig. 3. — Virus neutralization titers, challenge experiment 2. Arrows indicate a positive virus isolation.

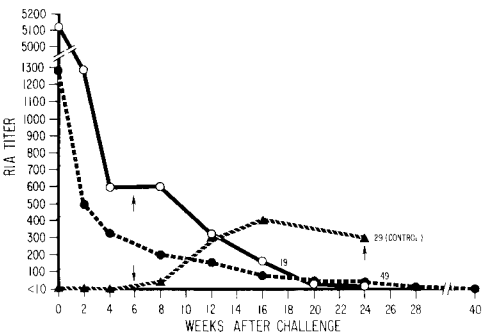

Fig. 2. — Radioimmunoassay titers, challenge experiment 1. Arrows indicate a positive virus isolation.

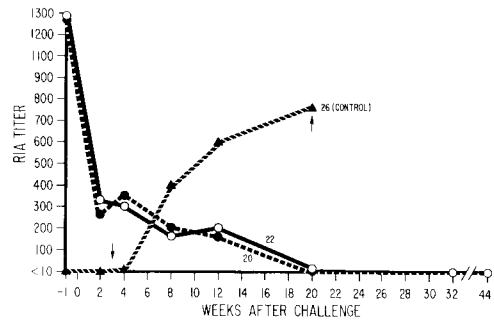

Fig. 4. — Radioimmunoassay titers, challenge experiment 2. Arrows indicate a positive virus isolation.

ment 2 are given in figs. 3 and 4. BLV infection was established only in the control animal, as determined by SIA results at 3 and 20 weeks after challenge. VN antibody titers in the vaccinates remained stable from 4 weeks after challenge through the 24th week, but RIA titers continued to decrease during this period. The vaccinates became AGID negative 4 months after challenge and at 8 months, all serologic tests were negative except that 1 calf had a 1:2 VN titer. This animal (no. 22) was VN negative 11 months after challenge.

The 3 vaccinated calves that remained negative for BLV after challenge were also tested for infection by inoculation of sheep. Blood leukocytes were isolated by the hypotonic shock method (Stock and Ferrer, 1972), washed, and 2 sheep were inoculated from each donor calf. The number of cells given ranged from 0.9-1.2 $\times$ 10^9 and all injections were by the intraperitoneal route. The sheep were subsequently examined for BLV infection by monthly AGID tests of their sera. All 6 sheep remained negative during a 6-month observation period.

Discussion and conclusion

Formalin is a commonly used inactivant for killed vaccines, but results of our RID assays indicate that it has a deleterious effect on the BLV glycoprotein antigen. We believe preservation of this antigen is extremely important for production of an effective vaccine because the virion glycoprotein is responsible for stimulation of neutralizing antibodies.

We thought the altered antigenicity produced by formalin probably was a result of protein precipitation, so we decided to look for an inactivant that would act in a different way. Graves and Arlinghaus (1967) have shown that the inactivation of foot-and-mouth disease virus by AEI occurs through interaction with viral RNA, and there is no evidence of significant effect on protein components in the virus. This report prompted us to investigate AEI, and a similar compound, BEI, as potential inactivants for a BLV vaccine. Both chemicals were found to be superior to formalin with respect to their preservation of BLV glycoprotein antigen. BEI has the advantage of being less dangerous for laboratory personnel because it can be prepared as a dilute solution from a non-toxic compound, whereas AEI is extremely toxic and flammable (Bahnemann, 1975). Both AEI and BEI were effective inactivants of BLV infectivity, although the rate of inactivation was slower than expected when compared to results obtained with foot-and-mouth disease virus (Brown and Crick, 1959 ; Bahnemann, 1975). Perhaps the presence of an envelope on BLV particles increases the time required for the chemicals to interact with viral nucleic acid.

As expected, multiple injections of BLV antigen were required to induce serologic responses in calves and the VN titers produced were relatively low, 1:32 or 1:64. In later experiments we have found that antigenicity of the vaccine is enhanced if the concentration of aluminum hydroxide adjuvant is increased from 10 % to 50 %.

Following challenge with BLV-infected lymphocytes, 3 of 4 vaccinated calves appeared to have been protected. The primary evidence for this conclusion was the absence of viremia, as indicated by the negative SIA results. Supporting evidence was the eventual disappearance of BLV antibodies and demonstration that blood leukocytes from the calves were not infectious for sheep.

In calf n° 49, the vaccinate that became infected after challenge, there was a marked disparity in the serologic response pattern observed with the RIA and VN or AGID tests. Even though virus infection was confirmed 6 weeks after challenge, the RIA titer continued to decrease, just as it did in non-infected calves. The VN titer, however, stayed at prechallenge levels, and a strong AGID response was maintained. These results reflect an independent serologic response of animal no. 49 to the 2 major BLV antigens. The internal virion polypeptide, p24, is used for RIA, whereas the AGID test, and presumably the VN test, measure antibody to envelope glycoprotein antigens. We have previously reported that many cattle with naturally acquired BLV infections have AGID antibody to the glycoprotein antigen but not to p24 (Miller and Van Der Maaten, 1976 ; Miller *et al.*, 1977).

We recognize that the 2 vaccine challenge experiments reported here represent an extremely limited experience and that similar tests must be done on a much larger scale. The results were certainly encouraging, however, and expanded studies for further evaluation of the BLV immunogen have been initiated.

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Summary

Bovine leukemia virus (BLV) infection is endemic in many cattle herds in the United States, and control or eradication by standard « test and removal » programs would be very costly. Vaccination might be a more economically acceptable approach, and this report summarizes our preliminary efforts to develop an inactivated BLV immunogen for cattle.

Culture fluids from the fetal lamb kidney cell line were the source of BLV and a radial immunodiffusion assay was used to determine the quantity of glycoprotein antigen present. Culture fluid was incubated for 24 hours at 37 °C with either 0.05 % formalin, 0.05 % N-acetylethyneimine (AEI), or 0.02 % binary ethyleneimine (BEI). Antigenicity of BLV glycoprotein was substantially reduced by formalin treatment, but there was no detectable loss of antigenicity after exposure to either AEI or BEI. The effectiveness of the latter 2 chemicals for inactivation of BLV was tested with a syncytium induction assay in cell culture. Both compounds had satisfactorily destroyed BLV after 8 hours at 37 °C.

To test immunogenicity, 4 calves were injected with an AEI-treated preparation that contained 0.34 mg glycoprotein with 10 % aluminum hydroxide as adjuvant. At least 2 injections were required to produce detectable antibody in the agar gel immunodiffusion (AGID) test. Virus neutralizing titers of these antisera were 1:32 or 1:64. Vaccinated calves were challenged with BLV-infected lymphocytes and subsequent syncytium induction assays indicated that 3 calves resisted infection. In the next year, virus neutralization, AGID, and radioimmunoassay tests on sera showed a progressive decrease of antibody titers, and eventually they fell below detectable levels. Furthermore, blood lymphocytes from the calves were not infectious upon inoculation into sheep. These findings supported the conclusion that persistent BLV infections had not been established.

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