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Review

Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine

Hans G. Bahnemann

Viral antigens for human and veterinary vaccines are still inactivated with formaldehyde. This is not an ideal inactivant and the problems of formaldehyde inactivation of vaccines are discussed. Vaccines inactivated with aziridines are superior in safety and antigenicity. Aziridines inactivate viruses in a first-order reaction and the inactivation rate and endpoint can be determined. The preparation and application of the aziridine compound binary ethylenimine (BEI) and the necessary conditions for and controls of the inactivation process are described and discussed. A computer program has been written for assistance in the use of BEI for controlled inactivation of viral antigens.

Keywords: Inactivation; binary ethylenimine; viral antigens

Introduction

A perusal of recent and current literature on the preparation of viral vaccines with inactivated antigen and, in particular, of experimental vaccines of this type shows that very often the inactivation is still obtained with formaldehyde and without the necessary controls. In the preparation of an inactivated viral vaccine the inactivation process is a very important step. The innocuity of the vaccine must be assured before the question of potency can be addressed.

It seems worthwhile to discuss the inactivation of viral antigens with reference to a few older (and perhaps forgotten) publications as well as some more recent studies. The procedures and process controls which must be applied in order to assure a safely inactivated vaccine will be described. The example of foot-and-mouth disease (FMD) vaccine preparation will be used as this vaccine is by volume the largest viral vaccine produced at present.

Inactivation with formaldehyde

For many years most of the viral vaccines with inactivated antigen were prepared with formaldehyde as inactivating agent. The work of Sven Gard and his collaborators with poliovirus during the period of 1956 to 1958 demonstrated that the inactivation of this virus with formaldehyde was not a linear or first-order reaction. Similar results were obtained for the formaldehyde inactivation of FMD virus by Wessln and Dinter in 1957 and by Graves in 1963.

A recent publication stating the linearity of formaldehyde inactivation of FMD virus comes to this erroneous conclusion because the infectivity titration for the inactivation slope was based on final readings of plaque forming units (p.f.u.) at 2 days. This is far too short a time for a reading with formaldehyde treated virus which is unique in having a markedly extended incubation period for the first replication cycle in cell cultures, as was shown by Schultz et al. in 1957 and Böttiger et al. in 1958. For points on the lower part of the inactivation slope Böttiger et al. needed 12 days to obtain a final p.f.u. reading.

The extended incubation period for formaldehyde treated virus also means that an innocuity test in animals is inappropriate for detecting small amounts of residual infectious virus. When this virus begins to replicate with a delay of between several days and 2 weeks, the animal is already beginning to produce antibodies. The new virus will then be neutralized by antibody and the animal has an abortive or subclinical infection. This subclinical infection can be detected by testing the animal for the virus infection associated antigen (VIAA), the viral RNA polymerase.

In 1975 Alonso et al. reported a study of cattle exposed to FMD and found that at 21 days postvaccination, of 18 animals vaccinated with formaldehyde inactivated vaccine five animals were positive for VIAA. Of 16 animals vaccinated with N-acetylthelylenimine (AEI) inactivated vaccine no animal was positive for VIAA. Pinto and Garland later found VIAA positive cases also in animals revaccinated with AEI inactivated vaccine, but emphasized that the response in these animals was much weaker and only transient. Alonso et al. were able to confirm this transient and weak response and also found, with binary ethylenimine (BEI) inactivated Al(OH)3 and oil adjuvant vaccines, a response to VIAA only after revaccination. The weak response to VIAA is caused by the presence of this antigen in the inactivated virus suspension used for vaccine preparation. However VIAA antibodies induced by safely inactivated vaccines are only detected after revaccination and the presence of such antibodies after primovaccination still indicates recent virus replication in the animal.

Lucam analysed in 1958 the FMD vaccination
Inactivation of viral antigens: H. G. Bahnemann

campaigns in France and suspected residual infectivity in some formaldehyde-inactivated FMD vaccines, which had passed official innocuity tests.

Recently Beck and Strohmaier studied viruses from field outbreaks of FMD in Europe by determination of their nucleotide sequences. They found that most of these isolates were related to virus strains in (formaldehyde-inactivated) vaccines. This led Strohmaier to the opinion that most of the FMD outbreaks in Europe in the last 20 years were 'homemade', i.e. were caused by vaccination. He made the recommendation that inactivation of FMD vaccine antigens should be changed from formaldehyde to first-order inactivants or that vaccination should be stopped altogether.

The FMD vaccine production regulations in several South American countries have for several years now permitted only the use of first-order inactivants. At least one European country has recently also adopted this position. Most of the FMD vaccine production laboratories in these countries apply the aziridine compound EI in the form of BEI. One group of laboratories uses diluted EI.

Inactivation with aziridines

The first report of a (bacterial) virus inactivation by ethylenimine, the basic aziridine substance, was published in 1935 by Raettig and Uecker. Hurst in 1957 was of the opinion that vaccines prepared with AEI as inactivant were antigenically superior to vaccines inactivated with formaldehyde and would guarantee inactivation of the virus. The antigenic superiority was later confirmed also for vaccines inactivated with BEI. ICI patented the use of AEI for inactivation of microorganisms in 1959. The first report on the inactivation of FMD virus by AEI was published by Brown and Crick also in 1959. This compound was subsequently used by a leading FMD vaccine production laboratory for many years in the preparation of inactivated antigens. However it did not come into general use because of its patent protection.

In 1961 Uecker reported the linearity of inactivation of bacterial viruses by ethylenimine derivatives and Graves and Arlinghaus described in 1967 the linearity of AEI inactivation of foot-and-mouth disease virus.

At ambient temperatures AEI is not stable and it therefore has to be kept at 4°C or preferably at −20°C. Fellowes remarked in 1965, that AEI has a low boiling point and very little is left in a biologic preparation at reaction temperatures of 20°C or above. This observation probably made him use an inactivation temperature of 25°C instead of the usual 37°C.

The problem of the stability of AEI was perhaps the reason for introducing the double dosing regimen for inactivation at 37°C, i.e. the application of two doses for 24 h each as described by Pay et al. This procedure is still being used with BEI by the same laboratory although EI is much more stable. An extended incubation of the antigen at 37°C damages the antigen, as was shown in comparative inactivations of FMD virus at 26°C and at 37°C. This damage is not due to the inactivant but is probably caused by the action of proteolytic enzymes present in the virus suspension.

Other laboratories continued to work on FMD virus inactivation by aziridines and in the early 1970s reports on ethylenimine, EEI, ethylenimine, EI and binary ethylenimine, BEI were published. Both EEI and EI are difficult to obtain in quantity. For this reason, as well as the ease of preparation and handling, BEI is now the preferred inactivating agent for FMD and other veterinary vaccines.

The viruses which have been reported as inactivated with BEI are given in Table 1. They belong to a variety of families of viruses with either RNA or DNA, which makes it very likely that most known viruses would be inactivated by an aziridine.

Inactivation with BEI

General considerations

Inactivation in vaccine preparation transforms an infectious antigen into a non-infectious one. This transformation step should therefore be done in a well identified intermediate area between the virus-containing and the virus-free area. Access to the intermediate area should be limited, and only be possible from the virus-containing side.

The antigen must be held in the intermediate area until completion of the necessary control tests (inactivation endpoint and innocuity). The facilities for holding of the inactivated antigen in this area, cold room or cooled storage tanks, should be able to accommodate a volume of at least 2–3 weeks production of virus in order to allow termination and if necessary a repeat of the control tests. Only after confirmation of the innocuity can the antigen be transferred to the virus-free area and be used for vaccine preparation.

The inactivation process should be done under slow agitation in two different vessels, with perhaps one quarter or a third of the time in the first vessel, and transfer of the virus suspension under inactivation in a closed system to the second vessel for the remainder of the time. This procedure is indicated in order to avoid pockets of the virus suspension into which the inactivant did not enter or reinfection from non-inactivated virus on the tank wall above the liquid level at the end of the inactivation period and after hydrolyzation of the inactivant.

The virus suspension should be checked to determine that it is at the desired temperature and is at a pH of ≈7.4 before the inactivant is added. It is also advisable to control the osmolarity of the virus suspension, which for cell culture produced FMD virus is ≈320–340 mOsM.

| Virus | Nucleic acid | Ref. |
|-------|-------------|-----|
| 1 African swine fever | DNA | 31 |
| 2 Bluetongue | RNA | 32 |
| 3 Bovine leukaemia | RNA | 33 |
| 4 Bovine rhinotracheitis | DNA | 34 |
| 5 Bovine rhinovirus | RNA | 35 |
| 6 Bovine viral diarrhoea | RNA | 36 |
| 7 Eastern equine encephalomyelitis | RNA | Unpublished |
| 8 Foot-and-mouth disease | RNA | 30 |
| 9 Newcastle disease | RNA | 10 |
| 10 Porcine parvovirus | DNA | 37 |
| 11 Pseudorabies | DNA | 38 |
| 12 Rabies virus | RNA | 39 |
| 13 Vesicular stomatitis | RNA | 34 |
The pH and osmolarity of the virus suspension affect the velocity of inactivation. Figures 1 and 2 show the results of a study some years ago of the effect of pH and osmolarity on the inactivation rate of EI. The experimental conditions were the same as given previously. It can be seen from Figure 1 that an increasing alkalinity reduces the inactivation rate. This is in contrast to formalin inactivation, where the velocity of inactivation was found to increase with increasing alkalinity. Figure 2 shows that increasing osmolarity also slows down the inactivation rate. The pH and osmolarity effects on the inactivation rate are perhaps due to conformational changes in the viral capsid proteins which affect the permeability for the inactivant.

Preparation and application of inactivant

The inactivant was called binary ethylenimine or BEI because it is prepared from two substances, 2-bromo-ethylamine HBr (BEA) and NaOH, and also to distinguish this preparation from pure EI. BEA converts in an alkaline solution to ‘binary’ ethylenimine. The active substance is the ethylenimine ring as in all aziridine compounds.

The BEA solution is 0.1 M or 20.5 g l⁻¹ of a 0.175 N NaOH solution. The conversion to BEI is completed in 30–60 min at 37°C and is accompanied by a pH drop from ≈12.5 to ≈8.5. The formation of BEI is indirectly controlled by visualizing this change with the pH indicator β-naphthol violet (BNV). From a 1% aqueous stock solution of BNV, 0.5 ml is added per litre of NaOH solution. The colour of the BEA solution changes at 37°C in ≈15 min from violet to orange upon formation of BEI. The BEI preparation should not be used for inactivation if the colour has not changed to orange.

The 0.1 M BEI preparation contains only 0.5% EI and therefore is much easier to manage than concentrated EI or AEI. However it should still be handled with care and prepared in a closed vessel which allows transfer of the BEI in a closed system to the inactivation vessel.

Higher concentrations of BEI, such as 1 or 2 M, can be prepared. But the preparation and handling of such BEI solutions requires much more attention and precautions as the EI concentration is much higher.

It is recommended to use the 0.1 M BEI preparation with a final BEI concentration in the virus suspension of between 1 and 3 mM. For an inactivation at 37°C the 0.1 M BEI is added at 1.5% for a final BEI concentration of 1.5 mM or at 26°C at 3% for 3 mM BEI. At these temperatures and BEI concentrations and for a pH of ≈7.5–7.6 and at 320–340 mOsm of the virus suspension, the inactivation rate for FMD virus is around one log of virus per hour.

The inactivation rates (log virus h⁻¹) published for some other viruses with 1 mM BEI and at 37°C are as follows: vesicular stomatitis virus 1.2⁴⁴, bovine rhinotracheitis virus 1.1⁴⁴, equine encephalomyelitis virus 1.2 (unpublished), pseudorabies virus 1.2⁴⁸, rabies virus 4.1⁹⁹ and Newcastle disease virus 0.5¹⁸.

Na-thiosulphate hydrolyses BEI. The BEI inactivation is stopped by the addition of a 1 M sterile Na-thiosulphate solution at 10% of the volume of the BEI solution used. It should also be used for hydrolysis of BEI in any spills or for cleaning of the vessel in which the BEI solution was prepared.

In-process controls

The inactivation process must be accompanied by appropriate in-process controls. This begins with the BEI preparation. As described above, the formation of the ethylenimine ring is monitored in a simplified manner. If necessary, a colorimetric determination of the produced EI can be performed using the method described by Epstein et al.¹⁴¹, and has been used by Czelleng et al. to determine EI. However it should still be handled with care and prepared in a closed vessel which allows transfer of the BEI in a closed system to the inactivation vessel.

The inactivation of viruses with BEI is a first order or linear reaction. For each inactivation, the rate has to be determined. This is done by taking samples for infectivity titration during the early part of the inactivation process, at 0, 1, 2, 3 and 4 h for example. The inactivation rate is obtained from the infectivity titres by calculation of the regression coefficient and is used to calculate the inactivation endpoint.

The endpoint is the most important parameter to be determined and is a function of the inactivation rate, the reaction time and the volume of the virus suspension. The minimum endpoint is defined as being one log(10) lower than the titre which gives one infectious unit in the total volume under inactivation. For example: One infectious unit in a volume of one litre has a titre of 10⁻¹. The titre is determined and is a function of the inactivation rate, the reaction time and the volume of the virus suspension. The minimum endpoint is defined as being one log(10) lower than the titre which gives one infectious unit in the total volume under inactivation. For example: One infectious unit in a volume of one litre has a titre of 10⁻¹.
log $10^{-3}$ (for the usually expressed titre in ml). The minimum endpoint for this volume therefore is $10^{-4}$. For a successful inactivation the calculated endpoint has to be lower than the minimum endpoint. The difference between the calculated and the minimum endpoint, or the DIM (Difference of Inactivation endpoint to Minimum) value has to be positive. It is important to determine the DIM value for each inactivation process for an assessment of a successful inactivation.

After termination of the inactivation each virus suspension has to be tested for innocuity on cell cultures. This can be done by inoculation of at least two roller bottles or similar cell culture vessels and two subsequent blind passages at 48 h intervals. The cell cultures for this test have to be prepared in the virus-free area and the test should be done in the intermediate area. Only after determination of the inactivation endpoint and completion of the innocuity test can the virus suspension be considered to be properly inactivated.

**Other applications of BEI**

The major application of BEI will be in the preparation of inactivated antigen for vaccines. But there are other areas in which BEI can be used. Since BEI does not react with proteins it can be used for inactivation of adventitious viruses in biological preparations from animal or human tissues or fluids.

The BEI treatment of bovine serum used for cell cultures has been reported in 1976. The usefulness of this method was later confirmed in 1984 by Heuschele, who applied it over a 6-year period for the inactivation of adventitious bovine viral diarrhoea virus in calf serum used in primary or secondary animal cell cultures.

BEI can also be used for inactivation of viruses in enzyme preparations of animal origin. A commercial trypsin preparation was treated with BEI without any loss of activity (unpublished results). It is very likely that other biologicals, like Factor VIII, could be treated with BEI for inactivation of adventitious viruses.

**Safety**

Pure aziridines are highly toxic and have to be handled with special precautions and extreme care. This high toxicity is the reason for the 0.1 M preparation of BEI. At this molarity the BEI preparation contains only 0.5% EI and the vapour pressure at this concentration is low enough that for temperatures under 50°C no EI will escape into the atmosphere (the boiling point for EI is 57°C).

On the basis of experiments in laboratory animals, aziridines are considered to be carcinogenic substances. According to Dermer and Ham, however, no cases of human cancer caused by EI have ever been reported. Fellowes cites Hurst who states that an injection of 0.5 mg of AEI into rats did not produce any tumours during an observation period of 515 days.

The total annual production of FMD vaccine worldwide is probably between 700 and 800 million doses, of which nearly 500 million doses are produced in South America alone. Furthermore between 70% and 80% of all FMD vaccines are inactivated with BEI, which means that about 500 million doses of FMD vaccine are inactivated with BEI and applied in cattle annually. The majority of these cattle are revaccinated many times over the years. No increase in the incidence of cancer in cattle after vaccination with BEI inactivated vaccines has ever been reported from any country. Neither is there any reason to expect any increase, as the residual BEI after inactivation is hydrolyzed with Na-thiosulphate.

**Computer program**

A computer program has been written to assist with the calculations needed for a controlled inactivation of viruses with BEI or any other first-order inactivant. The screen menu for the program is given in Figure 3. The program runs with DOS and is available in English (vine.exe) or in Spanish (vins.exe). Copies of either program can be obtained by sending a formatted double-density/double sided 5.25 in. diskette. Requests from South America should be sent to the Centro Panamericano de Febre Aftosa, Caixa Postal 589, 20001 Rio de Janeiro, Brasil. Requests from North America, Europe or other countries can be sent to the Pan American Health Organization, attention DIC/USA, 525-23rd Street, NW, Washington, DC 20037, USA.

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**Program for Inactivation of Viruses**

- **Preparation and use of BEI**
- **Components of the BEI Solution**
- **Inactivation Process Control**
- **Titer in Plaque Forming Units**
- **Titer in Infectious Dose 50 %**
- **Inactivation Rate and Endpoint**
- **Exit Program**

**Figure 3** Screen menu of the computer program for inactivation
Inactivation of viral antigens: H. G. Bahnemann

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Inactivation of viral antigens: H. G. Bahnemann