Extraction of Equine Infectious Anemia Immunodiffusion Antigen with the Aid of the Chaotropic Agent, Thiocyanate

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Immunodiffusion antigen from spleens of horses infected with equine infectious anemia virus was prepared by methods employing freeze-thaw cycles and thiocyanate treatment. Thiocyanate (0.5 M) permitted the recovery of the greatest amount of antigen. Furthermore, it was most effective for recovery of immunodiffusion antigen from spleens which yielded unsatisfactory concentrations of antigen by the conventional freeze-thaw or water-extraction methods. The reactivity of the antigen did not appear to be affected by this chemical treatment.

Spleen from horses with acute equine infectious anemia (EIA) was shown by Coggins and Norcross (1, 2) to be a suitable source of antigen when used in an immunodiffusion test (ID) for detection of EIA-specific antibody. In our laboratory and that of Henson et al. (5) many spleens excised from EIA-infected horses were inadequate for extraction of EIA antigen when processed by the freeze-thaw method described by Coggins and Norcross (1, 2). Henson et al. (5) attributed this difficulty to low antigen concentration. They developed a method for partial purification and concentration of antigen from these spleens. Coggins and Norcross (1) reported that it appeared important to select animals for antigen preparation that showed acute disease symptoms within the 1st week after infection and to harvest the spleens after 3 to 4 days of elevated body temperature. The latter authors reported that spleens taken later in the disease were a poor source of antigen, possibly because sufficient antibody was present to mask the antigen.

The difficulty in extracting antigen from spleens of some horses led us to investigate the use of a chaotropic agent in the preparation of ID antigen for EIA. Hatefi and Haustein (4) have shown that chaotropic ions provide a simple, effective, and controllable means for increasing the solubility of organic molecules in aqueous solvents. We report the use of one of these ions, thiocyanate, as an aid in the preparation of EIA antigen from spleens considered to be satisfactory and unsatisfactory as a source of antigen when extracted by the freeze-thaw procedure (1, 2).

MATERIALS AND METHODS

Serum from horses infected with the Wyoming strain of EIA virus was stored at −70°C and served as the inoculum. Horses were inoculated intravenously with 10 ml of serum and sacrificed after 10 days. Portions of six spleens obtained from infected horses were employed in this study. Each spleen was extracted by the freeze-thaw method and evaluated as an antigen source by the ID assay. Four spleens were inadequate, one spleen was of marginal value, and one spleen was adequate as a source of EIA antigen.

We define an inadequate spleen as one from which suitable antigen cannot be obtained. A spleen designated of marginal value is one from which suitable antigen is obtained after concentration to one-fourth the original volume of the supernatant fluid obtained after the fifth sedimentation in the freeze-thaw process described below. An adequate spleen is one which yields an antigen solution which may require concentration but to not less than 60% of the original volume of supernatant fluid. A spleen from a normal pony was extracted as a control.

Extraction of antigen. Spleenic pulp was separated from stroma by scraping with a spatula. Pulp from each spleen was divided into three equal portions by weight for comparison of the amount of antigen extracted by the freeze-thaw method, with and without thiocyanate. Pulp from one spleen (B-13) was divided into 7 equal portions to determine the optimum molarity of NaSCN to employ.

Freeze-thaw extraction. A modification of the procedure described by Coggins et al. (2) was fol-
ollowed. Spleenic pulp was suspended in 0.08% NaN₃ (0.5 ml/g pulp), dispensed into 50-ml screw-cap centrifuge tubes, and frozen at −20 C. Frozen pulp was allowed to thaw at 25 C and sedimented at 34,000 × g for 30 min. The pellet was suspended in the supernatant fluid by carefully mixing the contents of each tube with an impeller attached to a Sorvall Omni-Mixer. Care was taken to prevent the introduction of air into the suspension. The freeze-thaw cycle was repeated five times. After the fifth centrifugation, the supernatant fluid was decanted and assayed for EIA antigen by the ID test (1). The antigen preparation was either concentrated by per-vaporation or diluted with 0.08% NaN₃ so that the intensity of ID precipitin lines was equivalent to diagnostic antigen obtained from E. E. Roth, of our department.

**Extraction with thiocyanate.** The spleenic pulp was mixed with an equal weight of 0.08% NaN₃ and allowed to stand at 4 C for 1 hr and subsequently stirred. The final concentration of NaSCN was obtained by adding an equal volume of NaSCN solution of twice the molarity desired. The suspension was allowed to stand for 10 min at 25 C and dialyzed at 4 C overnight against 0.02 m phosphate buffer (pH 7.1). A 240-ml amount of dialysis buffer was used per g of spleenic pulp. After dialysis, the suspension was mixed and sedimented by centrifugation at 27,000 × g for 20 min. The supernatant fluid was collected by decanting, and the pellet was resuspended in an equivalent volume of phosphate buffer (pH 7.1) and sedimented as above. The supernatant fluid was again collected by decanting, and the pellet was washed once more as described above. The supernatant fluids were pooled and an equal volume of saturated (NH₄)₂SO₄ solution was slowly added with stirring in an ice bath. Prior to use, the pH of the saturated (NH₄)₂SO₄ solution was adjusted to 7.1 with NH₄OH. Protein was allowed to precipitate for 1 hr at 4 C after the addition of the final increment of (NH₄)₂SO₄ solution, and the precipitate was sedimented by centrifugation at 27,000 × g for 15 min. The supernatant fluid was discarded, and the pellet was dissolved in a minimal volume of 0.02 m phosphate buffer, pH 7.1, and dialyzed overnight against the same buffer. After dialysis, undissolved material was sedimented by centrifugation at 27,000 × g for 15 min and discarded. The supernatant fluid was concentrated by per-vaporation at 4 C or diluted with distilled water until the antigen formed precipitin lines in the ID tests which were equivalent to that obtained with diagnostic antigen.

**Extraction without thiocyanate.** This procedure was included as a control for the extraction with thiocyanate. The method described above for extraction with thiocyanate was followed except distilled water was employed in the place of the thiocyanate solution.

**Assay of antigen.** The ID test described by Coggins and Norcross (1) was employed to assay for antigen. Five milliliters of 2% Noble agar in 0.15 M borate buffer, pH 8.7, was poured into a 100-mm plastic petri dish, was allowed to stand 3 to 4 hr, and was overlaid with 15 ml of 1.0% Noble agar in 0.15 M borate buffer, pH 8.7. After standing at 25 C for at least 1 hr, a template with one central and six peripheral punches was employed to make wells in the agar. The wells were 7 mm in diameter and 3 mm apart. Antigen, positive and negative control sera, and test sera were placed in appropriate wells. The petri dishes were incubated at 24 C and examined for precipitin lines at 24, 48, and 72 hr.

**Evaluation of antigen.** Antigenicity was determined by ID against positive and negative sera. The amount of antigen present was evaluated by comparing the intensity of precipitin lines obtained with experimental antigen was those obtained with diagnostic EIA antigen. Diagnostic antigen was prepared by the freeze-thaw extraction method. Experimental antigen preparations were diluted with 0.02 M phosphate buffer, pH 7.1, or concentrated by per-vaporation until precipitin lines in ID tests were comparable to those obtained with diagnostic antigen. The volume of each antigen preparation was recorded. Antigen concentration is expressed as the number of horse diagnostic tests which were extracted from 1 g of spleenic pulp.

**RESULTS**

The results of an experiment to ascertain the concentration of NaSCN which extracted the greatest quantity of EIA antigen from spleenic pulp is presented in Table 1. The greatest quantity of antigen was extracted from equivalent portions of spleen by both 0.5 and 0.7 M NaSCN. The amount of antigen extracted by 0.3 M NaSCN was only slightly greater than that extracted without NaSCN. A concentration of 0.1 M NaSCN had little or no effect on release of EIA spleenic antigen. When the NaSCN was 1.0 M, EIA spleenic antigen recovered was less than the control. No EIA antigen was detected when the spleenic pulp suspension was 2.0 M NaSCN.

Figure 1 is a photograph of an ID test. Diagnostic EIA antigen and EIA antigen extracted from spleenic pulp suspension made 0.5 M with respect to NaSCN were in alternate peripheral wells. A known EIA-positive serum was in the center well. Deviation and complete

| Table 1. Effect of thiocyanate on the extraction of EIA spleenic antigen |
|-------------------|------------------|
| NaSCN molarity    | Diagnostic tests per gram of spleen |
| 0.0               | 4.0              |
| 0.1               | 4.0              |
| 0.3               | 6.2              |
| 0.5               | 12.0             |
| 0.7               | 12.0             |
| 1.0               | 2.5              |
| 2.0               | 0.0              |

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fusion of precipitin lines confirms that the EIA antigen preparations have a common antigenic determinant.

Figure 2 is a photograph of an ID test which shows that EIA antigen extracted in the presence of NaSCN is specific. EIA antigen is in the center well. A known negative EIA serum is in well 1; known positive EIA sera are in wells 2, 4, and 6; a known positive EIA field test serum is in well 3; and a known weak positive serum is in well 5. No precipitin line was formed with the negative EIA serum in well 1. Precipitin lines formed with EIA-positive control sera in wells 1 and 6 extend into well 1 with no deviation. Precipitin lines of identity were formed with the positive field test serum (well 3) and positive control sera (wells 2 and 4). Precipitin lines formed with EIA-positive control sera in wells 4 and 6 deviate with the standard weak EIA-positive serum in well 5. No nonspecific lines were detected.

Data presented in Table 2 compare efficacy of the freeze-thaw and NaSCN methods in the extraction of EIA antigen. In all spleens from diseased horses, 0.5 M NaSCN effected the most efficient extraction of antigen. No antigen was detected when a normal spleen (H-38) was extracted. Antigen extracted by the freeze-thaw method and without NaSCN required pervaporation to a pulpy consistency to obtain an antigen which would give precipitin lines in ID tests equivalent to diagnostic EIA antigen. Due to viscosities, antigen prepared by these procedures could not be pipetted into agar wells for ID tests. No precipitin lines formed against known EIA-positive sera when these preparations were not concentrated to a pulp consistency. The freeze-thaw method was a more effective procedure for extracting the antigen than the procedure employed without NaSCN.

EIA antigen extracted from marginal or inadequate spleens in the presence of 0.5 M NaSCN yielded an antigen which could be concentrated to a point where precipitin lines

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**Table 2.** Comparison of efficacy of freeze-thaw, with thiocyanate-, and without thiocyanate-extraction methods to release EIA spleenic antigen

| Horse designation | Spleen evaluation a | Diagnostic test/gram of spleenic pulp |
|-------------------|--------------------|-------------------------------------|
|                   |                    | Freeze-thaw | 0.5 M NaSCN | No NaSCN |
| Jake              | Inadequate         | 0.0         | 4.5         | 0.0      |
| H-106             | Inadequate         | 3.0         | 5.9         | 0.0      |
| H-107             | Inadequate         | 4.5         | 8.6         | 0.0      |
| H-28-2            | Inadequate         | 2.0         | 4.5         | 2.0      |
| B13               | Marginal           | 4.2         | 12.0        | 4.0      |
| H94-1             | Adequate           | 13.1        | 14.5        | 11.5     |
| H-83              | Normal             | 0.0         | 0.0         | 0.0      |

a A portion of each spleen was evaluated by the freeze-thaw method.
equivalent to diagnostic antigen were formed with known EIA-positive sera. Antigen prepared in this manner was in a form which was easily pipetted into agar wells. Antigen preparations more concentrated than the diagnostic antigen were easily diluted to the optimum concentration for ID tests. A greater final volume of adequate antigen preparation per gram of splenic pulp was obtained from each spleen extracted with 0.5 M NaSCN than was obtained by freeze-thaw or without NaSCN methods. No detectable loss of antigen activity occurred when EIA antigen extracted with 0.5 M NaSCN was stored at 4°C for 2 months. No change in antigenic activity was detected when a volume of diagnostic antigen was exposed to 0.5 M NaSCN, dialyzed, and concentrated to its original volume. Similar treatment of a volume of diagnostic EIA antigen with 2.0 M NaSCN resulted in loss of all detectable antigen reactivity.

DISCUSSION

The ID test developed by Coggins and Norcross (1) is of great value for the diagnosis of EIA. One of the major problems associated with this test is an adequate method for extracting and preparing antigen. The source of antigen is spleens from horses experimentally infected with EIA. The course of the disease is monitored, and the spleen is taken after 3 to 5 days of elevated body temperature. This reaction usually occurs within 9 to 11 days postinoculation. In our experience many spleens taken from horses inoculated for the purpose of antigen production are not suitable as a source of antigen. Henson et al. (5) reported a similar problem. The method presented here for extracting EIA antigen with thiocyanate alleviates this problem.

For this study, we selected EIA-infected spleens which were inadequate, of marginal value, and adequate as an EIA antigen source when extracted by the freeze-thaw method (1).

It is clear that the amount of recoverable EIA antigen increased when infected spleens were extracted with 0.5 M NaSCN (Table 2). In addition to more antigen obtained per gram of splenic pulp, the utility of the final product should be considered. The antigen obtained from all EIA-infected spleens except B-13 and H94-1 by freeze-thaw and without NaSCN methods necessitated concentration to a pulpy consistency to detect precipitin lines when employed in the ID test. We considered these antigen preparations as unsatisfactory. Antigen extracted from the same spleens with 0.5 M NaSCN was in the liquid state, and the hemoglobin content was greatly reduced. They could be pervaporated or diluted to the optimum antigen concentration and accurately pipetted into agar wells for the ID test. Antigen extracted from spleens B-13 and H94-1 by freeze-thaw and without NaSCN procedures was satisfactory for use in ID tests.

It is not clear why the amount of antigen extracted from infected spleens varies. Henson et al. (5) found that the infected spleens employed in his work all had comparable amounts of antigen by immunofluorescence but were not all of equal quality as antigen source for ID. It is apparent from our results that the amount of antigen recovered also varied from one spleen to another. Apparently, in spleens we term adequate (H94-1) antigen is easily extracted. Freeze-thaw treatment of splenic pulp from this spleen slightly improved the antigen yield over water extraction, and the amount of antigen extracted with the aid of NaSCN was not greatly increased. It is significant, however, that the presence of NaSCN did increase the amount of antigen recovered, thus indicating that the anion did aid in release of the antigen and had no detrimental effect on the antigen. Thiocyanate appears to have greatest value when splenic antigen is difficult to extract in adequate yields. When spleens which yielded unsatisfactory antigen by the freeze-thaw procedure were extracted in the presence of 0.5 M NaSCN, a satisfactory antigen was obtained in all cases. This is important from an economical standpoint since few, if any, spleens need be discarded because of low antigen yield. When the efficacy of extracted EIA antigen in the presence of NaSCN from inadequate, marginal, and adequate spleens is compared to the freeze-thaw method and the method without NaSCN, it is apparent that the thiocyanate extraction procedure is superior.

The concentration of NaSCN employed for antigen extraction must be controlled. A final thiocyanate molarity of 0.5 to 0.7 is optimum (Table 1). Apparently, at lower thiocyanate concentrations less antigen is released, and at higher molarities (1.0 and above) reactivity of the antigen is altered.

Loss of standard antigen reactivity in the presence of 2.0 M NaSCN is evidence that the antigen will not tolerate the extracting agent at concentrations of 1.0 M and above. That 0.5 M NaSCN is not detrimental to the antigen was proven by no detectable loss of antigen reactivity when a volume of diagnostic EIA antigen was made 0.5 M NaSCN.
Antigen extracted with NaSCN and standard EIA antigen have a common antigenic factor since in ID tests precipitin lines of identity are formed (Fig. 1). If 0.5 M NaSCN alters the antigen, it is in a manner that does not affect the antigenic determinant. At concentrations above 1.0 M NaSCN this antigenic determinant may be altered.

Hatefi and Haustein (4) applied the term chaotropic to those inorganic anions which favor the transfer of apolar groups to water. These authors discuss several effects which these anions have on proteins. They reported that several anions including thiocyanate can be used to increase the water solubility of a variety of organic compounds which are sparingly soluble in water. In 7.0 M thiocyanate, riboflavine is 660 times as soluble as in buffer alone. The salting-in effect of NaSCN was not due to alteration of the riboflavine structure. Another effect of chaotropic agents reported by Hatefi and Haustein (4) was solubilizing of membrane-bound protein. In the presence of 2.0 M NaSCN, 40% of the electron-transport particle protein, 24% of Bacillus subtilis membranes, and 27% of erythrocyte membranes were solubilized. Therefore, this anion provides an effective means for resolution of membranes and multicomponent enzymes. Dandliker and his associates (3) employed NaSCN to dissociate antigen-antibody complexes without destroying the immunospecific activity of the antibody. They did not present data on the effect of the anion on the antigen.

The mechanism by which NaSCN augments the extraction of EIA antigen from EIA-infected spleens is speculative at this time. Other chaotropic agents such as sulfate, fluoride, chloride, iodide, acetate, chlorate, and nitrate may be as effective as thiocyanate in releasing EIA antigen from spleenic pulp. However, according to Hatefi and Haustein (4), the most denaturing, depolymerizing, and solubilizing chaotropic anion is thiocyanate, and it may, therefore, be the chaotropic agent of choice.

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