Acta vet. scand. 1975, *16*, 251—257.

From the Department of Forensic Medicine, Veterinary College of Norway, Oslo.

SPECIES SPECIFICITY IN THERMOSTABLE AND ETHANOL INSOLUBLE TISSUE ANTIGENS

I. IMMUNIZATION OF RABBITS AND GOATS WITH BOVINE ANTIGEN

By

Arne E. Andersen

ANDERSEN, ARNE E.: *Species specificity in thermostable and ethanol insoluble tissue antigens. I. Immunization of rabbits and goats with bovine antigen.* Acta vet. scand. 1975, *16*, 251—257. — Kidney and spleen antigens from cow, sheep, goat, European moose, reindeer, deer and roe deer were prepared by boiling and ethanol precipitation and tested against rabbit and caprine anti-bovine kidney sera. Two different levels of antigen concentration were used for immunizing animals in the various groups. In the group using high antigen concentrations, the precipitation reaction obtained initially disappeared after further inoculations, probably due to immunological tolerance. Sera tested from animals inoculated with low level antigen concentration showed a variation in the immunological response. The caprine anti-bovine sera showed distinct species specific reaction, while rabbit antisera showed either species specific or organ specific reaction, with a limited degree of cross reaction. In the production of species specific antisera against ruminant tissue antigens, goat seems to be preferable to rabbit.

forensic medicine; boiled antigens; antisera.

The identification of species based on organs is of considerable importance in forensic medicine and the control of animal food products. In criminal cases it may be necessary to diagnose species origin of blood stains and tissue debris. Commercially available species antisera are, however, mostly produced by immunizing rabbits with whole blood or serum. These antisera seem to be of limited value in the detection of antigens in putrefied or heated tissue, especially in the problem of differentiating between the various ruminants. From our experience, it is almost impossible to make any conclusion about species origin when materials are denaturated.

In their study on organ specificity *Milgrom et al.* (1962, 1964a, 1964b), using porcine, equine, bovine and human tissues, made the interesting observation when immunizing rabbits that thermostable and ethanol insoluble tissue fraction antigens gave species specific antibodies in addition to organ specific reactions. They found that after boiling and ethanol precipitation, fractions from organs, rather than serum, gave antisera which could be considered as a "universal" reagent for detecting species specific antigens (*Milgrom et al.* 1964a).

The aim of the present study was to develop antisera which could differentiate between ruminant species and especially denaturated material.

MATERIALS AND METHODS

Preparation of antigens

The principles described by *Milgrom & Witebsky* (1962) were followed for the preparation of boiled and ethanol precipitated organ (BE organ) antigens. Kidney and spleen from cow, sheep and goat were supplied from an abattoir and the same organs from European moose, reindeer, deer and roe deer were kindly supplied by hunters from different places in Norway and stored at —20C°. According to *Milgrom et al.* (1964b), kidney and spleen should be the best source of very strong species specific antigens, but no organ specific antigens had been shown in either of them.

After cutting into small pieces and weighing, saline was added (approx. 35 % w/v) and the tissue homogenized in a homogenizer*. The organ suspensions were stored at —20C° and samples of approx. 50 ml were crushed in an X-press** at approx. —25C° after which they were placed in a boiling water bath for 15 min. Lost volume was replaced by distilled water and the boiled suspensions rehomogenized. After centrifuging for 10 min. at 12000 × g, the supernatant was collected, autoclaved for 30 min. at 120C° and recentrifuged for 30 min. at 48000 × g. The supernatant was again collected and diluted 1:4 with 96 % ethanol. The preparation was stirred overnight and the sediment dried by low pressure in a Rotavapor*** and weighed. The

* MSE Ltd., London, England.
** AB Biox, Nacka, Sweden.
*** Büchi, Flawil, Switzerland.

residue was redissolved in saline to a concentration of 0.5—1 %. In the present study a BE preparation of bovine kidney was used for immunization.

Immunization procedure

The antigen solution was mixed with an equal volume of Freund's adjuvant*. The dose was divided approx. 50 % between sub- and intracutaneous sites. Freund's complete adjuvant was used for the first injection and incomplete adjuvant for the second and subsequent inoculations. Antigen solutions prepared for i.v. injection were free of adjuvant. Rabbits weighing 3—4 kg and young goats weighing 20—24 kg were used.

From animals picked randomly from different sources, group A with 2 rabbits and 2 goats were inoculated with 3 mg and 25 mg antigen per injection, respectively; and in group B, 2 rabbits and 2 goats were inoculated with doses of antigen 10 times larger than the doses in group A. These injections were placed on the back of the animals and repeated once a week for 8 weeks. The animals were then rested for 6 weeks. Sera were collected and tested from the 4th week after the start, but not during the resting period.

After the resting period the animals in group A were inoculated 3 times with 4 weekly intervals, using the same dosing as before; and with great care intracutanously on the neck and back under anaesthesia (Hypnorm vet.®** for rabbits and Rompun VM®*** for the goats). Similarly, the animals in group B were inoculated with the same doses as before but by intravenous route after prophylactic antishock treatment (mepyramin maleat).

Immunological testing

Double diffusion gel precipitation test (*Ouchterlony* 1971) was used until a demonstrable reaction occurred against bovine BE kidney. Then the sera were tested against bovine BE spleen, BE kidney and BE spleen preparations from the other ruminants. Immunodiffusion testing was performed on photographic glass

---

* Difco Labs, Detroit, USA.

** Mekos, Helsingborg, Sweden.

*** Bayer, Leverkusen, Germany.

plates with a gel of 1 % agarose* in veronal buffer pH 8.6 (*Cullifford* 1964). The thickness of the gel was adjusted to 2 mm, and wells 3 mm in diameter and 7 mm distant (from centre) were punched. Readings were made after incubation for 24 and 48 hrs. at 4C°.

RESULTS

After the first period of immunization the sera from group A gave only weak reactions. One week after the start of the second immunization period, more significant precipitation reactions were observed and during the following 8 weeks, the reactions seemed to develop a satisfactory stable precipitation reaction pattern. The rabbits gave different reactions as shown in Fig. 1.

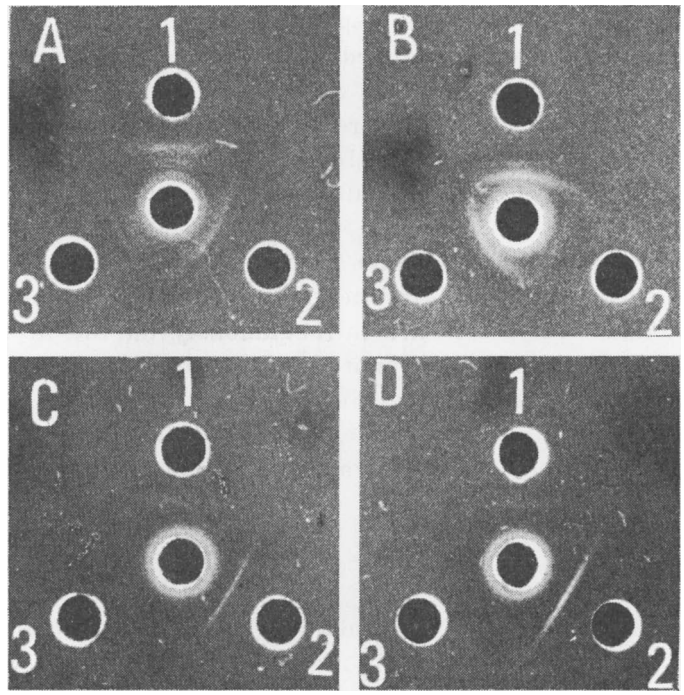

Figure 1. The precipitation test of sera from group A. In the central wells: A. Rabbit I, B. Rabbit II, C. Goat 08 and D. Goat 98. The peripheral wells: 1. Bovine BE kidney, 2. Bovine BE spleen, 3. Ovine BE kidney.

* L'Industrie Biologique Francaise, Gennevilliers, France.

It can be seen that 1 of the rabbit sera gave a distinct species specific reaction. The other rabbit serum exhibited an obvious organ specific reaction in addition to a weaker species specific reaction. Both sera showed a limited degree of cross reactions. The caprine antisera showed a clear species specific reaction against BE preparation of bovine organs compared with the other ruminants as shown in Table 1, which summarizes the results

Table 1. Reactions of testing sera from group A animals against BE kidney and BE spleen from 7 ruminant species in double diffusion gel precipitation.

Antigens / Antisera	Cow		Sheep		Goat		Reindeer		Roe deer		European moose		Deer	
	kidney	spleen	kidney	spleen	kidney	spleen	kidney	spleen	kidney	spleen	kidney	spleen	kidney	spleen
Rabbit I	+	+	+	—	+	+	—	—	+	+	—	—	(?)	—
Rabbit II	+	+	+	—	+	+	+	+	+	+	+	—	+	—
Goat 08	+	+	—	—	—	—	—	—	—	—	—	—	—	—
Goat 98	+	+	—	—	—	—	—	—	—	—	—	—	—	—

Reaction symbols:
distinct reaction +
no reaction —
uncertain (?)

from titrations of 4 sera from group A against BE-kidney and spleen of the 7 ruminant species in order to optimize concentrations for antibody/antigen reactions. In cross-wise titration sera were diluted 2-fold from 1:1 to 1:16, and 1 % antigen solutions were used in 2-fold increasing dilutions from 1:1 to 1:32. Under the conditions of the experiment, serum dilutions 1:1 or 1:2 and antigen dilution 1:2 were found to be optimal for precipitation reaction. In group B, the precipitating reaction development was good with the rabbit sera and with the serum from 1 of the goats. After 8 intravenous injections the reaction seemed to be satisfactory, and after a booster dose blood was drawn 7 days later. Sera from all the animals in this group were then tested but none of them gave precipitation reactions with the test antigens.

DISCUSSION

The use of rabbits is well known in the production of antibodies for many purposes. In the present study, however, better specificity reactions were found by using goats to produce antisera against another ruminant, although the development of antibodies in the goats was rather slow when compared with the reactions of the rabbits. The ruminants probably have several common organ antigens. The specific antigens should, however, mobilize only specific antibodies in another ruminant species.

The unspecific Forssman antigen should not constitute a problem when goat and possibly sheep are chosen for antibody production as these species are Forssman positive (*Forssman* 1930). The 2 rabbits used were siblings, and yet dissimilar antigen/antibody reactions were seen. It would therefore be of interest to further study the mechanisms of this reaction.

It is difficult to explain the depression of the precipitating reaction which occurred to the sera from group B. *Milgrom et al.* (1964b) inoculated animals for a similar period and with similar doses, using organ specific brain tissue BE fraction. The most distinct difference between the experiments of *Milgrom et al.* (1964 b) and the present study seems to be the choice of organs used as antigen sources. It is not clear, however, which tissue has the highest antigenic potency. The reason for the development of an immunological tolerance may be that BE kidney is especially potent. It could also be that there was total blocking of antibodies by the large doses of antigen.

REFERENCES

Culliford, B. J.: Precipiting reaction in forensic problems. Nature (Lond.) 1964, *201*, 1092—1094.

Forssman, J.: Die heterogenetischen Antigene, besonders die sog. Forssman-Antigene und ihre Antikörper. (The heterogenetic antigens, especially the Forssman Antigens and their antibodies). Handbuch der Pathogenen Mikroorganismen. Band III, 469—526, 3. Aufl. Fischer-Jena 1930.

Milgrom, F. & E. Witebsky: Immunological studies on adrenal glands. I. Immunization with adrenal of foreign species. Immunology 1962, *5*, 46—66.

Milgrom, F., Walter A. Campbell & E. Witebsky: Studies on immunization with organ-specific haptens. Proc. Soc. exp. Biol. (N.Y.) 1964a, *115*, 165—169.

Milgrom, F., Z. M. Tuggac & E. Witebsky: Studies on species specificity. J. Immunol. 1964b, *93*, 902—909.

Ouchterlony, Ö.: Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publ. Inc. London 1971.

SAMMENDRAG

Artsspesifisitet i termostabile og etanoluløselige vevsantigener.

I. Immunisering av kanin og geit med storfeantigen.

Nyre- og miltantigen fra storfe, sau, geit, elg, reinsdyr, hjort og rådyr ble preparert ved koking og alkoholpresipitering og testet mot antistorfenyresera fra kaniner og geiter. To ulike antigenkonsentrasjoner ble brukt ved immuniseringen av dyregruppene. I gruppen som ble podet med høy antigenkonsentrasjon, ble det først observert presipitasjonsreaksjon, men denne forsvant etter siste poding, sannsynligvis p.g.a. immunologisk toleranse. Sera fra dyrene som ble podet med lav dose antigen viste forskjellig immunrespons. Antistorfesera fra geit ga tydlig artsspesifikk reaksjon, mens antistorfesera fra kanin viste enten artsspesifikk eller organspesifikk reaksjon og i noen grad kryssreaksjon. Ved produksjon av antisera mot vevsantigen fra drøvtyggere synes geit å være bedre egnet enn kanin.

(Received December 6, 1974).

Reprints may be requested from: Arne E. Andersen, Veterinary College of Norway, Postboks 8146, Oslo Dep., Oslo 1, Norway.