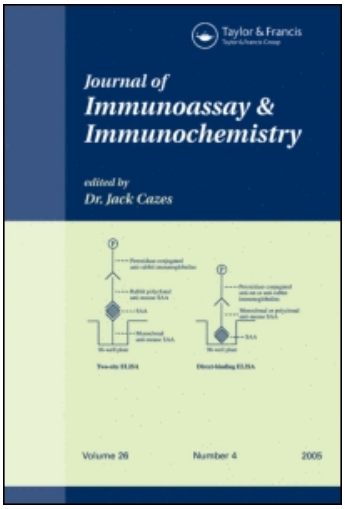

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

The Hy-Antigen: A Method for the Detection of Antibodies in Rat Serum

Carole P. McArthur[a]; Saroj Sengupta[b]
[a] Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, U.S.A. [b] Department of Surgery, University of Otago Medical School, Dunedin, New Zealand

To cite this Article McArthur, Carole P. and Sengupta, Saroj(1981) 'The Hy-Antigen: A Method for the Detection of Antibodies in Rat Serum', Journal of Immunoassay and Immunochemistry, 2: 2, 163 — 174
To link to this Article: DOI: 10.1080/15321818108056974
URL: http://dx.doi.org/10.1080/15321818108056974

JOURNAL OF IMMUNOASSAY, 2(2), 163-174 (1981)

THE HY-ANTIGEN: A METHOD FOR THE DETECTION OF ANTIBODIES IN RAT SERUM

Carole P. McArthur
Department of Microbiology and Public Health
Michigan State University, East Lansing,
Michigan 48824, U.S.A.
and
Saroj Sengupta
Department of Surgery, University of
Otago Medical School, Dunedin, New Zealand

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) using rat spermatozoa and thymocytes is described for the detection of anti-H-Y antibodies. This assay is highly sensitive and reproducible, and is particularly useful for the rapid screening of a large number of samples.

INTRODUCTION

The H-Y antigen was recognized when Eichwald and Silmser (1) demonstrated that highly inbred female mice rejected male skin grafts. Male → female skin grafting has been widely used to detect the male antigen in both rats and mice (2-8). The graft-versus-host reaction used by Lengerova and Chutna (9,10) is another *in vivo* test. The most popular serological method of detection has been Goldberg et al.'s (11) dye exclusion cytotoxicity test against rat or mouse sperm (6,12-15). Recently,

Gable et al. (16) reported a modification of this cytotoxicity assay using ^{86}rubidium release as a measure of antibody. Other tests described include the hybrid antibody method (17,18) and hemagglutination (19,20). A simpler method for the detection of H-Y using mouse lymphocytes and Staphylococcus aureus (SA) has been described by Tokuda et al. (21). This has been modified by Koo and Goldberg (22) into a rosetting assay using soluble protein A instead of intact SA. During the search in our laboratory for monoclonal antibodies against the H-Y antigen in the mouse system, we developed an enzyme-linked immunosorbent assay for the detection of anti-H-Y antibodies. We have now adapted this for the rat system and in our hands this method proved superior to the cytotoxicity test and satisfied the additional requirement of being able to screen a large number of samples (>400) quickly. This simple, sensitive, rapid and highly reproducible method is reported here for those in the field who, like us, have experienced difficulties with available assays such as cytotoxicity (16,23).

MATERIALS AND METHODS

Preparation of sperm and thymocytes

Epididymal sperm were prepared for the ELISA test from the sliced epididymis of HS adult rats and C57Bl/6(H-2^b) mice. The tissues were teased apart in a petri dish containing 10 ml Dulbecco's phosphate buffered saline supplemented with 5% heat inactivated fetal calf serum (Gibco), and passed through a steel mesh grid to separate the larger tissues from sperm. Sperm were

washed once, centrifuged and re-suspended in 0.15 M PBS-Tween 80 (0.05%, Sigma) (45 ml 0.5 M Na_2HPO_4, 15 ml 0.5 M NaH_2PO_4, 17 g NaCl, 2 L distilled water) to 15-20 x 10^6 per ml. For cytotoxicity testing, sperm were collected by cutting the epididymis into slices in 1/2 ml of phosphate-buffered saline (pH 7.0) containing 0.5% fructose and 5% fetal calf serum. Medium containing sperm was gently pipetted off after a few minutes at room temperature.

Human sperm were washed, centrifuged and re-suspended to 15-20 x 10^6/ml. Rat thymocytes and mouse splenocytes were prepared by passing them through a series of needles of decreasing gauge before, in the case of spleen only (10 ml), adding 25 ml 0.83% NH_4Cl to lyse RBCS. Cells were washed 3 times in RPMI-1640 and re-suspended in 0.15 M PBS-Tween 80 (0.05%).

Rats and production of antisera

Anti-H-Y sera were produced in HS virgin females by immunizing them once intrapedally with 15-20 x 10^6 male splenocytes homogenized in FCA, and then 8 times subsequently with 20 x 10^6 splenocytes in PBS i.p. weekly. Sera for testing were diluted 1:4 and absorbed sequentially with half the volume of HS female splenocytes on ice for 1 h. To provide negative control serum, normal serum and immune serum were absorbed with HS male splenocytes to remove antibodies against the male antigen in the case of the immune serum or autoantibodies in the case of normal serum.

Enzyme-antibody conjugate

Sheep anti-rat IgG was affinity purified on IgG-sepharose by the method of Guimezanes et al. (24). The purified antibody was labelled with horse-radish peroxidase (type VI, Sigma Chemical Co., St. Louis, MO) by the method of Nakane and Kawaoi (25). The conjugate used in these experiments contained an average molar ratio of 3 mol of enzyme to 1 mol of Ig. The sterile conjugate was stored in 50% glycerol at 4°C and no loss of activity was observed over a 6 month period. With this antigen, the conjugate was used at dilutions ranging from 1:400 to 1:600 and dilutions were made in 0.5 molar NaCl containing 1% Tween 80 (Sigma), pH 7.5 (adjusted with K_2HPO_4).

Enzyme-linked immunoassay (ELISA)

The assay was carried out on polystyrene microtitre plates (Cooke Laboratory Products, Alexandria, VA; Cat. No. 18-29A). Plates were rinsed with distilled water and blocked by adding a 5% solution of gamma-globulin-free horse serum (Gibco) in saline to each well. After incubating for 15 minutes at room temperature the plates were rinsed carefully 4 times with distilled water. Dilutions of sera to be tested, including positive and negative controls, were made as follows: all wells received 0.025 ml PBS-Tween 80 (0.05%) and the first well in each row received 0.025 ml of the serum to be tested. These were diluted serially using 0.025 ml micro-diluters (Cooke) before 0.025 ml of sperm or male thymocyte suspension (15-20 x 10^6 per ml) was added to each well

and the plate incubated for 10 minutes at 37°C on a micro-mixer (Cooke). After incubating, excess antibody was removed by adding 0.2 ml of 0.15 M PBS-Tween 80 (0.05%) shaking for 30 seconds on the micro-mixer, centrifuging the plate at 250 G for 5 min and carefully aspiring the supernatants. Plates were washed twice more before 0.025 ml of the labelled diluted conjugate was added to each well and the plate incubated on the shaker for a further 10 minutes at 37°C. After incubation, the plate was washed and centrifuged 4 times to 0.15 M PBS-Tween 80 (0.05%) as previously. Next, 0.025 ml PBS-Tween 80 (0.05%) and 0.025 ml of substrate was added to each well and incubated in the dark for 15 minutes before stopping the reaction by adding 1 drop of 0.3% sodium azide to each well and stabilizing the reaction after 10 min by adding 0.025 ml of 4N H_2SO_4. The substrate was made up directly before use by dissolving 6 mg of O-phenylenediamine in 10 ml PBS and adding 0.6 ml of this solution plus 0.01 ml fresh H_2O_2 to a second 10 ml of PBS for use. We have found the substrate unstable after 6 hours, so recommend that this be made up freshly. Positive sera can be visualized by the appearance of the yellow color in the wells and scored appropriately, or readings to determine optical density can be made using an automatic scanner (Multiscan, Flow Laboratories) designed to screen microtitre plates.

Establishing whether a sample of low titre was positive or negative required experimentation with several known positive and negative sera, and the appropriate adjustment of the time for the development of the substrate. For our use, visible assessment of

the color proved adequate. The last dilution of test serum showing a color more intense than that of the absorbed negative control was considered the titre of that sample.

Antisera from rats immunized with splenocytes were first tested for autoantibodies with rat sperm. Autoantibodies were then removed by absorption with female rat splenocytes. Antisera were then tested by ELISA with rat sperm to show the presence of the H-Y antigen(s) shared by male rat splenocytes and rat sperms.

To examine the cross-reactivity of putative rat anti-H-Y sera they were first absorbed with female mouse or human lymphocytes to remove possible naturally occurring anti-species activity and then tested by ELISA for binding with mouse and human sperm. When the volume of antiserum permitted supernatants removed at this point (anti-H-Y absent) from microtitre plates were retained and again tested by ELISA for binding with rat sperm. In our experience mouse or human sperm removed all anti-rat sperm activity.

Cytotoxicity test

This was carried out according to the method of Goldberg et al. (11) with modifications. Briefly, antisera were diluted from 1:4 after absorption, to 1:32 and equal volumes (0.05 ml) of diluted antiserum, sperm suspension (5×10^6) and absorbed rabbit serum (complement source) diluted 1:4 were incubated for 45 min at 37°C. Live and dead sperm were counted in a hemocytometer under a light microscope after adding 0.1 ml of a freshly prepared solution of trypan blue (stock solution of 1% trypan blue) in

distilled water, diluted prior to use with 42.3 g/l of NaCl, one part in five for isotonicity. Considerable care was taken to eliminate bias by the person making the observations, so preparations were numbered.

RESULTS

Table 1, column 1, shows the results of the ELISA test on antisera from 19 immunized rats. Antisera were scored as clearly positive, equivocal or clearly negative. Nine of these indicated the presence of autoantibodies by a reduction in titre of antisera in column 2 following absorption with female rat splenocytes. Several of the antisera showed clear cross-reactivity (Rat no. 2, 7, and 13) with mouse and human sperm (columns 3 and 4). Absorption of positive antisera with male cells removed this activity. Serum from normal rats absorbed and tested was negative, although unabsorbed sera frequently contained autoantibodies.

DISCUSSION

The differences in titres of a particular serum recorded probably reflects either slight differences in sperm numbers, and/or amount of H-Y antigen present on the sperms of different species. The apparent lower sensitivity of mouse sperm may be attributed to the fact that in this case sperm were obtained from very young mice and cells may have been immature.

In our hands ELISA proved superior to the cytotoxicity test (11) as well as the rosetting assay (22). We found dye exclusion

TABLE 1

Titers of 19 Rat H-Y Antisera Determined by Binding in the ELISA Test With Rat, Mouse and Human Sperm. Antisera Were Absorbed With ♀ Cells Prior to Testing and the Signs in Parenthesis Refer to the Results Obtained by the Sperm Cytotoxicity Test. (+) Indicates >15% Cytotoxicity Above the Control. (±) Indicates 5-10% Cytotoxicity Above the Control. The Cytotoxicity Test Included a Control in Which Complement was Omitted and Another Control Without Antiserum but with Absorbed Complement. Cytotoxicity Varied Between 15-20% in such Controls.

	Column 1 rat sperm		Column 2 rat sperm		Column 3 mouse sperm		Column 4 human sperm	
1	-	(+)	-	(-)	-	(-)	-	(-)
2	1:8	(+)	1:8	(+)	1:2	(+)	1:4	(+)
3	-	(-)	-	(-)	-	(-)	-	(-)
4	1:4	(-)	1:2	(-)	1:2	(-)	+	(-)
5	1:2	(-)	-	(-)	-	(-)	-	(-)
6	-	(+)	-	(-)	(N.D.)		-	(-)
7	1:8	(+)	1:4	(+)	1:2	(-)	1:2	(±)
8	1:2	(-)	-	(-)	-	(-)	-	(-)
9	1:2	(+)	1:2	(-)	-	(-)	±	(-)
10	-	(-)	-	(-)	-	(-)	-	(-)
11	1:4	(+)	1:2	(+)	-	(-)	±	(-)
12	-	(-)	-	(-)	-	(-)	-	(-)
13	1:4	(+)	1:4	(+)	1:2	(-)	1:4	(+)
14	1:2	(+)	-	(-)	-	(-)	-	(-)
15	1:2	(-)	1:2	(-)	-	(-)	±	(-)
16	1:2	(+)	-	(-)	-	(-)	-	(-)
17	1:4	(+)	1:2	(-)	-	(-)	±	(-)
18	1:2	(±)	-	(-)	-	(-)	-	(-)
19	-	(-)	-	(-)	-	(-)	-	(-)

The results in columns 1 and 2 were obtained by one worker on the same day using sperm from that pooled from 3 rats. Those in columns 3 and 4 were obtained on 2 different days 10 and 11 days subsequently (by the same experimenter). N.D. = not tested.

cytotoxicity and rosetting to lack reproducibility, be technically laborious and very subjective. We found that the results obtained by one person using the cytotoxicity test were often different from results obtained by another worker. This was not an obvious problem with ELISA. For example, a serum tested with sperm from two different rats would frequently yield different results so all sera had to be tested with sperm from the same rat or a pool of sperm. It has been suggested that sperm differ also depending on the time of the year (26). Cytotoxicity requires excellent cell suspensions and has the added problem of screening for suitable complement. Cytotoxicity also depends upon lysis of the cell and so overlooks antibody which merely binds to the cell without actually damaging the membrane. With the rat we experienced the added difficulty with rosetting using Staphylococcus protein-A (PA) in that rat Ig did not appear to bind as effectively to PA as did mouse Ig.

The ELISA is a highly sensitive, reproducible, simple, fast, and relatively inexpensive assay. A large number of samples can be screened in a few hours, and only 0.025 ml of rat serum is required. The use of whole cells and centrifugation avoids the added complexity of preparing antigen to be fixed as the solid phase onto microtitre plates. Compared with solid phase radio-immunoassays (RIA), which have been widely used to detect antibodies to soluble antigens (27), the ELISA has obvious advantages. The limited shelf life of reagents and the health hazards are among the disadvantages of utilizing radio-isotope. Additionally,

the results obtained by ELISA are rapidly visible and do not require counting as in the case of RIA.

We are presently modifying this method to provide a clinical test for the H-Y antigen in the human system.

ACKNOWLEDGEMENTS

The financial support of the Medical Research Council of New Zealand is gratefully acknowledged.

The useful suggestions of Dr. Stephen Wachtel at the Memorial Sloan-Kettering Cancer Center, New York, NY, and his advice in solving our complement problems, are very much appreciated.

REFERENCES

1. Eichwald, ES, Silmser, CR. Skin communication. Transplant. Bull. 1955; 2:148.

2. Gasser, DL, Shreffler, DC. Genetic control of the immune response in mice: V minor genes involved in the response to H-Y and Ea-1 antigens. Immunogenetics 1974; 1:133-140.

3. Hildemann, WH, Cooper, W. Transplantation genetics: unexpected histoincompatibility associated with skin grafts from F2 and F3 hybrid donors to F_1 hybrid recipients. Transplant. Bull. 1967; 5:707-720.

4. Heslop, BF. The male antigen in the rat. Transplant. 1973; 15:31-35.

5. Heslop, BF, Lyttle, V. Hyperplasia of donor cells in rejecting weakly antigenic skin grafts. Proc. R. Soc. Lond. B. 1976; 193:209-214.

6. Krupen, BK, Wachtel, SS. Cytotoxic and agglutinating H-Y antibodies in multiparous female mice. Transplant. 1979; 27:406-409.

7. Silvers, WK, Murphy, G, Poole, TW. Studies on the H-Y antigen in rats. Immunogenetics 1977; 4:85-100.

8. Weissman, IL. Failure to demonstrate postnatal testicular-dependent expression of the male specific transplantation antigen in mice. Transplant. 1973; 16:122-125.

9. Lengerova, A, Chutna, J. Therapeutic efficiency of isologous bone marrow cells in lethally irradiated C57 black mice in different donor recipient sex combinations. Folia Bol. (Praha) 1959; 5:24-28.

10. Kuroiwa, A, Nagino, N, Miyazaki, S, Nomoto, S, Goya, N. Graft versus host reactions against H-Y antigen. Int. Arch. Allergy Appl. Immun. 1980; 62:235-239.

11. Goldberg, EH, Boyse, EA, Bennett, D, Sheid, M, Carswell, EA. Serological demonstration of H-Y (male) antigen on mouse sperm. Nature 1971; 232:478-480.

12. Sheid, M, Boyse, EA, Carswell, EA, Old, LJ. Serological demonstrable alloantigens of mouse epidermal cells. J. Exp. Med. 1972; 135:938-955.

13. Ohno, S, Wachtel, SS. On the selective elimination of Y-bearing sperm. Immunogenetics 1978; 7:13-16.

14. Simpson, W, Brunner, C, Hetherington, C, Chandler, P, Brennan, M, Dagg, M, Bailey, DW. H-Y antigen: no evidence for alleles in wild strains of mice. Immunogenetics 1979; 8:213-219.

15. Sharp, AJ, Wachtel, SS, Benirschke, K. H-Y antigen in a fertile XY female horse. J. Reprod. Fert. 1980; 58:157-160.

16. Gable, RJ, Levinson, JR, McDevitt, HO, Goodfellow, PN. Assay for antibody cytotoxicity of mouse spermatozoa by ^{86}rubidium release. Tissue Antigens 1979; 13:177-182.

17. Hammerling, U, Aoki, T, Harven, E, Boyse, EA, Old, LJ. Use of hybrid antibody with anti-γG and anti-ferritin specificities in locating cell surface antigens by electron microscopy. J. Exp. Med. 1968; 128:1461-1473.

18. Wachtel, SS, Koo, GC, Zuckerman, EE, Hammerling, U, Sheid, MP, Boyse, EA. Serological cross reactivity between H-Y (male) antigens of mouse and man. Proc. Natl. Acad. Sci. 1974; 71:1215.

19. Furasawa, M, Kototank, M, Takeuchi, H. Male specific hemagglutination in mice. Nature 1963; 200:182.

20. Shalev, A, Berczi, I, Hamerton, JL. Detection and cross reaction of H-Y antigen by hemagglutination. J. Immunogenetics 1978; 5:303-312.

21. Tokuda, S, Arrington, T, Goldberg, E, Richey, J. Simpler technique for serological detection of H-Y antigen on mouse lymphocytes. Nature 1977; 267:433-434.

22. Koo, GC, Goldberg, CL. A simplified technique for H-Y typing. J. Imm. Methods 1978; 23:197-201.

23. Jones, HW, Jr, Rary, JM, Rock, JA, Cummings, D. The role of H-Y antigen in human sexual development. The Johns Hopkins Med. Jl. 1979; 145:33-43.

24. Guimezanes, A, Fridman, WH, Gisler, RH, Kourilsky, FM. Reversible binding of a T cell factor on IgG coated sepharose beads. Eur. J. Imm. 1976; 6:69-72.

25. Nakane, PK, Kawaoi, A. Peroxidase labelled antibody: a new method of conjugation. J. Histochem. Cytochem. 1974; 22: 1084.

26. Wachtel Stephen, Memorial Sloan Kettering Cancer Center, New York, NY. 1981. Personal communication.

27. Hunter, WM. In: Applications of Immunological Methods, 3rd ed., 1978; Vol. 3, ed. D. M. Weir.