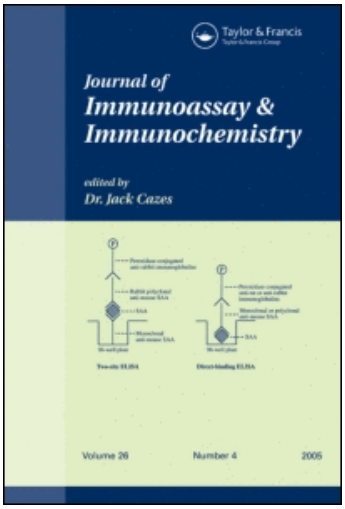

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PROCEDURES FOR *Sxs* ANTIGEN DETECTION BY ANTIBODY-MEDIATED CYTOTOXICITY TESTS. A COMPARATIVE ANALYSIS

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ABSTRACT

Biological reagents used in the serological detection of *Sxs* antigen by antibody-mediated cytotoxicity tests were compared in order to optimize the method. Our analyses showed that: (a) red cell-free spleen cells are the best target cells, (b) rabbit serum used as the complement source should be obtained from females, and absorbed with female spleen cells before use, (c) antiserum obtained by immunizing females with repeated injections of syngenic male spleen cells provides the highest anti-*Sxs* antibody titer, and (d) of the different biological fluids investigated, testis supernatant has highest concentration of *Sxs* antigen.

KEY WORDS: *Sxs*, Sex specific antigen, Cytotoxicity tests, Immunizing protocol.

INTRODUCTION

Male to female skin transplants in inbreed mouse strains allowed to demonstrate the existence of a mammalian male-specific antigen, known as histocompatibility Y antigen (H-Y) (1). Subsequently, this antigen was also demonstrated by tests of antibody-mediated (2) and cell-mediated (3) cytotoxicity. In all cases, the H-Y antigen detected was initially considered a single substance, although further investigations showed that different techniques could detect different antigens (see ref. 4).

Silvers et al. proposed the term SDM (serologically detectable male antigen) for the male-specific antigen revealed by serological methods (5). Furthermore, its association with the

heterogametic sex (males in mammals and females in birds) led Wiberg to propose the new term *Sxs* (sex specific) antigen (4).

A number of investigations have shown that this antigen is somehow implicated in the process of sexual differentiation. Wachtel et al. (6) suggested that the H-Y antigen would be the product of the mammalian testis determining gene (Tdy), although the hypothesis was subsequently ruled out (7). At present, the function and mode of action of these antigens remain unknown.

Several serological techniques have been used to detect the *Sxs* antigen, eg, cytotoxicity tests, hemadsorption, hemagglutination, ELISA, radioimmunoassay and fluorescence- or peroxidase-based tests. Cytotoxicity tests -the most used technique- are based on the ability of *Sxs*-positive cells to absorb anti-*Sxs* antibodies, thus reducing the cytotoxicity of *Sxs* antisera against target cells (usually, sperm or male epidermal cells).

However, *Sxs* antisera are difficult to obtain. Even after repeated immunizations, only some of the immunized females produce anti-*Sxs* antisera and the titers are generally very low. The poor quality of *Sxs* antisera makes most cytotoxicity assays subject to high backgrounds, with small differences between positive and negative results. The technical difficulties and differences in methodology regarding the immunizing protocols, type of target cell and serological assay used to detect this antigen, frequently make studies of the *Sxs* irreproducible, and frequently lead to contradictory results (8).

We analyzed the titer of different *Sxs* antisera obtained with several immunizing protocols, and investigated all the other biological reagents used in the cytotoxicity tests (target cells, and complement), in order to standardize and optimize the conditions of serological detection of this antigen. In addition, we analyzed the relative concentration of *Sxs* antigen in several fluids used as *Sxs* sources. The relation between the levels of *Sxs* and the immunizing capacity of different sources of this antigen is also discussed.

MATERIALS AND METHODS

Sources of *Sxs* Antigen

Several fluids obtained from male rats (male serum, epididymal fluid, testis supernatant, Sertoli cell-conditioned medium), and female chicken ovary supernatant were used as *Sxs* antigen sources. The methods used for the obtention of these sources are summarized in table 1.

TABLE 1

Methods Used for Obtaining the Sources of *Sxs* Antigen.

Source[a] nomenclature	Abbreviated	Method for obtention	Reference
Male serum[b]	*MS*	bleeding and centrifugation	standard
Epididymal fluid	*EF*	homogenization, diffusion in PBS and centrifugation	9
Testis supernatant	*TS*	homogenization, diffusion in PBS and centrifugation	10
Sertoli cell--conditioned medium	*SCM*	*in vitro* culture for three days in serum-free medium	11
Avian ovary supernatant	*AOS*	homogenization, diffusion in PBS and centrifugation	10

a) All fluids were cleaned by filtration through a 0.2 μm pore size filters, aliquoted and stored at -70°C until use.

b) Before use, MS samples were absorbed with female spleen cells at 4°C for 3 hours with continuous shaking.

Sxs Antiserum

Sxs antiserum was produced in female Lewis rats by using several immunizing protocols, in which fluids, cells or tissues from isogenic male Lewis rats were employed. Table 2 summarizes the procedures for the different immunizing protocols. All immunized female rats were bled 7 days after the last immunization, except in the procedures for **SF** and **MP** antisera, for which females were bled two weeks after immunization and one month after the birth of their last litter, respectively. In all cases, antisera were stored at -70°C until use.

Target cells

Table 3 summarizes the procedures for the obtention of several cell types used as target in cytotoxicity tests.

TABLE 2

Immunizing Protocols Used to Obtain Different *Sxs* Antisera.

Antiserum	Immunogen	Doses	Route	Reference
S	Spleen cells	Eight weekly 15×10^6	Intraperitoneal	9
MS	Male serum	Eight weekly 1 ml	Subcutaneous	this paper
EF	Epididymal fluid	Eight weekly 1 ml	Subcutaneous	this paper
TH	Testis homogenate	Eight weekly 1 ml	Subcutaneous	this paper
TS	Testis supernatant :Freund A. (v:v)	Eight weekly 0.5 ml	Subcutaneous	this paper
SF	Skin fragments	3 or 4 fragments (2 mm diameter)	Intrasplenic	12
SFTS[a]	Skin fragments	3 or 4 fragment	Intrasplenic	12
	TS:Freund A.	0.5 ml	Subcutaneous	this paper
TS1[b]	TS:Freund A.	0.2 ml	Intrasplenic	this paper
	TS:Freund A.	1 ml	Subcutaneous	this paper
MP	Male fetuses	5 to 7 litters	---	13

a) The second immunization with *TS*:Freund A. was done three weeks after immunization with skin fragments.

b) The second immunization was done four weeks after the first.

Freund A.: Complete Freund's Adjuvant.

Complement source

Because *Sxs* antigen is present in serum from all mammalian males (19, 20), the serum used as the complement source in these cytotoxicity tests should be obtained from females. Hence, as the complement source, we used female rabbit serum which was absorbed with female Lewis rat spleen cells in the absence of divalent cations according to a previously described method (15) prior to its use.

TABLE 3

Methods used for the obtention of the target cells used in antibody-mediated cytotoxicity tests.

Cell type	Source Tissue	Method for obtention	Reference
Sperm	epididymis	homogenization, suspension in PBS and centrifugation	2
spleen cells	spleen	homogenization, suspension in PBS and purification in Histopaque	this paper
epidermal cells	tail skin	enzymatic dissociation, suspension in PBS and centrifugation	14
Sertoli cells	testis	*in vitro* culture, trypsinization, suspension in PBS and centrifugation	11

Absorption should always be done with females cells, as male cells may contaminate the complement with *Sxs* antigen, reducing the cytotoxicity of the *Sxs* antiserum.

Cytotoxicity tests

Direct cytotoxicity tests were performed according to Goldberg et al. (2). Fifty microliters of different dilutions of heat-inactivated antiserum in serum-free RPMI medium were incubated with 50 μl of cellular suspension ($7x10^6$ cells/ml) for 15 min at room temperature. Then 50 μl of complement was added, and the mixture incubated for 1 h at 37°C. The cells were then stained with trypan blue and fixed with formaldehyde. The cytotoxicity level was estimated by calculating the percentage of dead cells (stained).

A tube lacking the antiserum (complement control) and another tube lacking both antiserum and complement (negative control) were also prepared.

For indirect cytotoxicity tests, 40 μl of different antiserum dilutions were absorbed for two h at 4°C with 30 μl of the different *Sxs* sources, before the addition of 70 μl of cellular suspension. The cytotoxicity level of the absorbed antiserum was determined as described for the direct tests.

In both direct and indirect cytotoxicity tests, dead and live cells were scored in a Neubauer chamber, and the percentage of cells that died due to the antiserum (X) was calculated as:

TABLE 4

Results of the use of different types of target cells in *Sxs* antibody-mediated cytotoxicity tests.

Cell type	Advantages (Ref.)	Disadvantages (Ref.)
Sperm cells	-easy obtention (2)	-unreliable scoring (8, #) -affinity to heterophilic antibodies (8) -variable *Sxs* content (16, 17) -imprecise antisera titration (17, #)
Dissociated epidermal cells	-sensitivity to *Sxs* antisera (14, 18) -easy recognition of dead cells (#)	-elaborate obtention method (14, 18, #) -reduced viability (#) -other dead cell types present (#) -high unspecific lysis (#) -high sensitivity to complement (#) -undetectable *Sxs*-specific lysis (#)
Cultured Sertoli cells	-sensitivity to *Sxs* antisera (#) -easy recognition of dead cells (#)	-frequent formation of aggregates (#) -very difficult scoring (#) -low viability after trypsinization (#)
Spleen cells	-sensitivity to S-type antiserum (#) -low unspecific lysis (#) -easy recognition of dead cells (#) -reliable scoring (#) -accurate antisera titration (#)	-Gradient density (Histopaque) purification required (#)

(#): observed in this study

$$X=\frac{a-b}{100-b}100$$

where **b** is the percentage of dead cells in the negative control and **a** the percentage of dead cells in each tube.

RESULTS

Target Cells

We have evaluated the advantages and disadvantages of several cell types to be used as target cells in antibody-mediated cytotoxicity tests. Table 4 summarizes our results in this respect and

includes some data previously obtained by other authors. These results clearly demonstrate that purified spleen cells show the most favourable features and, hence, they were used as target cells in the rest of experiments.

Complement

According to the findings of Boyse et al. (15), our results showed that absorption of this serum with female Lewis rat spleen cells in the absence of divalent cations removed natural heteroantibodies, which reduces the unspecific lysis of target cells due to complement in the absence of antiserum, whereas specific lysis due to *Sxs* antiserum remained unaffected.

Furthermore, the exposition of target (spleen) cells to the antiserum of choice (S antiserum, see below), produced a very low percentage of cell death (less than 10%), indicating that heat-inactivated antiserum does not lyse cells in the absence of complement.

Sxs Antisera

A number of cytotoxicity tests were performed to titer the different *Sxs* antisera obtained. According to the above results, spleen cells were used as target cells, and absorbed female rabbit serum was used as the complement source. Furthermore, all antiserum stocks were tested before use, in order to select those showing the highest cytotoxicity against male cells, and the lowest against female cells.

Cytotoxicity tests performed under these conditions showed that antisera from different immunizing protocols displayed a similar degree of cytotoxicity against female cells, but variable degrees of cytotoxicity against male cells. The S serum, which we obtained by immunizing females with repeated intraperitoneal injections of male spleen cells, produced the highest levels of specific cell death (Fig. 1).

Sources of *Sxs* Antigen

We analyzed the *Sxs* antigen content in the different source fluids by using indirect cytotoxicity tests, according to the method of Müller et al. (9). Increasing dilutions of S antiserum were absorbed with the different fluids, and their residual cytotoxicity was tested against male spleen cells. Figure 2 shows the absorption capacity of several male fluids when antiserum dilutions were incubated with a volume of each of them. In the case of male serum, this was previously heat-inactivated to eliminate the complement, and then absorbed with female spleen cells to eliminate the residual cytotoxicity caused by autoantibodies. Otherwise, both complement and autoantibodies could skew the estimated *Sxs* content.

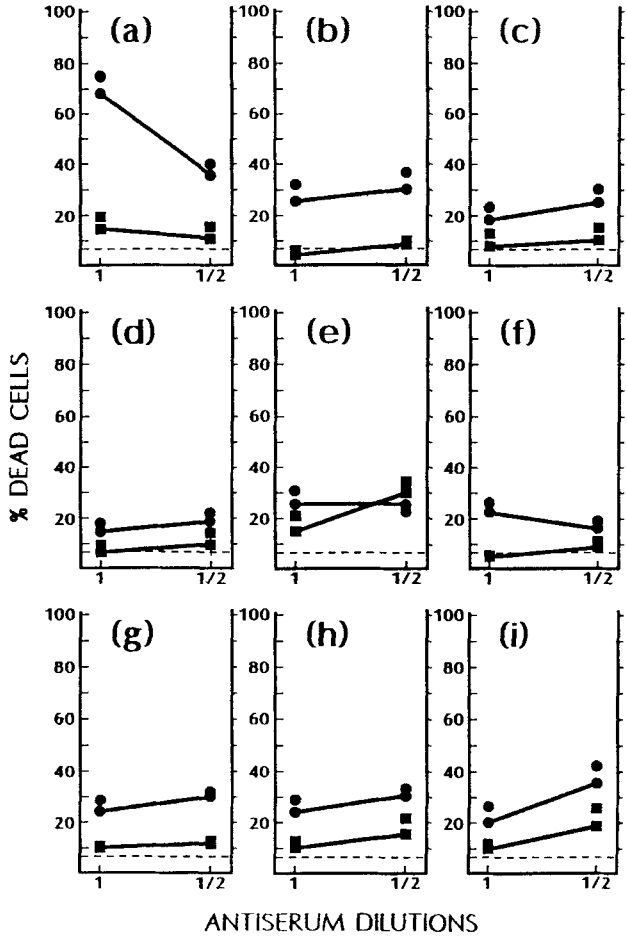

Fig. 1. Summary of cytotoxicity tests on male (O) and female (■) Lewis spleen cells using anti-*Sxs* antisera raised by different immunizing protocols (see materials and methods): **S** in a, **EF** in b, **TH** in c, **MS** in d, **MP** in e, **SF** in f, **TS1** in g, **SFTS** in h and **TS** in i. (- - -) indicates the cytotoxicity level for complement control. Each point represents average values of three separate tests. Upper and lower points accompanying each line represent standard deviation.

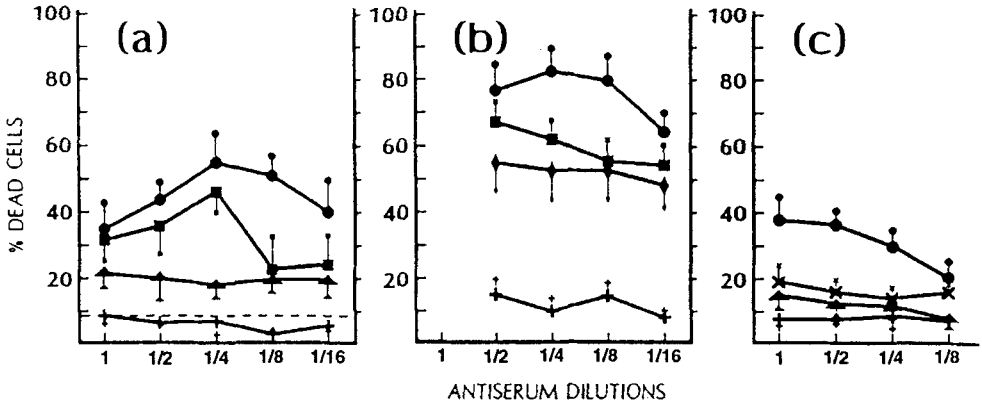

Fig. 2. Cytotoxicity of anti-*Sxs* antiserum (S) to male Lewis spleen cells after absorption with: (+) *TS*, testis supernatant; (▲) *EF*, epididymal fluid; (◆) *AOS*, avian ovary supernatant; (I) *MS*, male serum; (■) *SCM*, Sertoli- cell conditioned medium. a) test for *TS*, *EF* and *SCM*; b) test for *TS*, *AOS* and *SCM*; c) test for *TS*, *EF* and *MS*. *TS* has been included in the three experiments to permit comparison between them. (O): FCS free RPMI medium (as absorption control); (- - -): cytotoxicity level for complement control. Each point represents average values of three separate tests. Upper and lower points accompanying each line represent standard deviation.

In all cases, *TS* led to the greatest reduction in *Sxs* antiserum cytotoxicity, indicating the highest content in *Sxs* antigen.

DISCUSSION

The choice of target cell to be used in antibody-mediated cytotoxicity tests is a key decision. Ideally, these cells should show the following features:

a) They should be easily individualized to obtain a cellular suspension that facilitates scoring.

b) They should be able to withstand the preparatory procedures, with maximum survival before the cytotoxicity tests are performed.

c) They should show low percentages of unspecific cell death in controls.

Cells from many different sources have been used as target cells, eg, sperm (2), dissociated brain cells (21), Raji cells (22), red blood cells (23), white blood cells (24) and dissociated epidermal cells (14).

We have tested the advantages and disadvantages shown by several cell types when they are used as target cells in cytotoxicity tests, demonstrating that purified spleen cells are the most useful if they are used in combination with *Sxs* antiserum obtained by immunization with spleen cells (the **S** antiserum). This is consistent with the presence of *Sxs* antigen on the surface of these cells as suggested by several fact: a) their ability to absorb *Sxs* antibody from immune sera, reducing its cytotoxic capacity (25); b) their ability to immunize females against this antigen (9) and c) the presence of *Sxs* antigen on human peripheral blood cells, as shown by indirect immunofluorescence (26). On the other hand, these data seems to be in disagreement with several findings (3, 14, 18) demonstrating that these cells are not sensitive to lysis by *Sxs* antiserum in direct cytotoxicity tests. However, it is important to note that in these experiments *Sxs* antisera were obtained after immunization of receptor females with male skin grafts and not with spleen cells (see below).

We have shown in addition that elimination of red cells from the spleen cell suspension is very important to obtain good results, which is probably due to the fact that red cells may unspecifically bind the antigen on their membrane, and consequently may be unspecifically lysed by the *Sxs* antiserum (23), thus interfering with the specific lysis of spleen cells.

Obtaining antisera is probably the most difficult step in the preparation of biological reagents used in serological assays for *Sxs* antigen detection. A variety of immunological protocols have been used, but, as noted above, they contain low titers of antibodies, and frequently have auto- and heteroantibodies that cross-react with target cells. Consequently, the results from different laboratories, different serological tests, or even different assays using the same serological technique, are generally not comparable.

Is it noteworthy that the antisera obtained by immunizing females with epididymal fluid (**EF** serum), testis supernatant (**TS** serum) and testis homogenate (**TH** serum) showed very low antibody titers, whereas epididymal fluid (*EF*) and testis supernatant (*TS*) showed very high levels of *Sxs* antigen, as demonstrated by the reductions in cytotoxic capacity of the **S** antiserum after absorption with these sources of antigen (see below). In addition, the *Sxs* antigen of mammals is produced exclusively by Sertoli cells of the testis (27), which makes it difficult to explain the lack of immunogenic capacity of these *Sxs* antigen sources. However, our results agree with those of Shearer and Hunterbach (28), who showed that the injection of syngenic sperm caused immunosuppression in receptor females.

Ohno et al. (29) postulated that recognition of *Sxs* antigen by antibodies may be restricted by the major histocompatibility complex. According to this idea, some *Sxs* positive cells with a specific H-2 haplotype could not bind *Sxs* antibodies. It has been also demonstrated (14) that *Sxs* antisera obtained from female mice with the H-2^b haplotype lysed cells with the H-2^b and H-2^d, but not with the H-2^k haplotype. *Sxs* antibodies may recognize a concrete association between *Sxs* antigen and major histocompatibility complex antigens.

According to this hypothesis, a given *Sxs* antiserum would be more cytotoxic against the cells used in the immunizing protocol than against other cell types. Recognition may depend on the conformation of the *Sxs*-H-2 (mouse) or *Sxs*-HLA (human) complex and may vary depending on characteristics of the cell on whose surface complex is located. This may explain the fact that lymphoid cells are not lysed by *Sxs* antisera obtained by immunization with skin transplants (epidermal cells), but are lysed by antisera raised using these same cells as the immunogen. This could explain in turn the low titers of antisera raised by immunization with skin transplants, male fetal cells, or testicular fluid, when spleen cells are used as target cells.

The low titers of **SF** antisera, obtained by intrasplenic immunization with skin transplants, contrasts with the results of Bradley and Heslop (12), who obtained the highest titers reported to date for *Sxs* antisera, with this immunization protocol. However, they found wide variation between different strains of rat with regard to the time to appearance of antibodies and antibody titers, and pointed out that unlike other immunizing protocols, antibody production with this method was transient, and rapidly declined, making it difficult to bleed the animals precisely at the time of peak antibody yield. These factors may account for this apparent discrepancy.

With regard to serum from multiparous female rats (**MP**), our results also differ from those of Kraupen-Brown and Wachtel (13), who reported the presence of *Sxs* antibodies in multiparous female mice. However, all female rats analyzed in our laboratory lacked these antibodies. This may be due to possible existence of differences between mice and rats, or between their different strains, with regard to their capacity of respond to fetal *Sxs* antigen.

Different fluids such as testis supernatant (30, 31, 32), epididymal fluid (9), avian ovary supernatant (10), male serum (33, 20), Sertoli cell-conditioned medium (30, 32) and Daudi cell-conditioned medium (34) have been used as sources of *Sxs* antigen, but no study has reported up to date the relative amount of *Sxs* antigen in each of them. In this respect, our results have shwon that testis supernatant (*TS*) has the highest content in *Sxs* antigen, which is consistent with the fact that only Sertoli cells produce it in mammals (27, 35, 30). Consequently, it could be recommended the use of *TS* in experiments in which a potent source of *Sxs* antigen is needed. However, although

this source is certainly *Sxs*-rich, it is a very heterogeneous fluid whose composition is not defined at all. Because of this, other sources such as Sertoli cell-conditioned medium (*SCM*), which lead to lesser reduction in the cytotoxicity of *Sxs* antiserum, indicating a lower antigen content, may represent however purer forms of the antigen. In fact, the molecular nature of the *Sxs* antigen was investigated in antigen samples produced by cultured Sertoli and Daudi cells (34, 36). Thus, *Sxs* antigen from cultured Sertoli and Daudi cells could be considered the source of choice in studies on the role of this antigen in the process of sexual differentiation. So, we have demonstrate that rat *SCM* may induce the sex reversal of embryonic chicken gonads *in vitro* (37).

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Abbreviations: Sxs, sex specific antigen; Tdy. testis determining gene; PBS, calcium- and magnesium-free phosphate-buffered saline; FCS, fetal calf serum; BME, basal medium eagle; RPMI, RPMI 1640 medium; DME, Dulbecco's modification of eagle's medium; EDTA, ethylenediaminetetraacetic acid.

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