found that high dose cyclosporine treatment (data not shown) which severely reduces murine T helper/inducer cell numbers and functions[6,23], did not affect the granulomatous response in this model. This confirms several earlier experimental studies which showed that induction of organized granuloma formation does not require an intact cell-mediated immune system[6,24–26], in keeping with increasing clinical reports of sarcoidosis and other granulomatous reactions in patients with AIDS and combined immunodeficiency states[27–30].

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Identification and possible biological relevance of spermatozoal transglutaminase

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Summary. Normal human spermatozoa were demonstrated by dot immunoblot analysis and immunohistochemistry to possess transglutaminase (TGase). The immunological identification of spermatozoal TGase is consistent with reports by others of its biochemical identification and suggested role in sperm motility, *and* provides, in view of the immunoregulatory properties of seminal plasma TGase, presumptive identification of a means whereby spermatozoa, under normal physiological conditions, may possibly be protected from immunological 'attack' within the female reproductive tract.
Key words. Insemination; motility; spermatozoa; transglutaminase.

Two essential properties of inseminated spermatozoa to successful normal mammalian reproduction are their 1) motility, and 2) ability to avoid destruction by immunocompetent cells within the female reproductive tract. These properties may be inherent to spermatozoa, and/or given the numerous constitutive immunoregulatory macromolecules within the male accessory sexual glands (MASG) tissues and secretions, may become adsorbed therefrom by their avidity to spermatozoa[1]. Of the numerous MASG immunoregulatory molecules identified, transglutaminase (TGase) appears to play a prominent role[2].

TGases are Ca^{2+}-dependent peptide ligases which catalyze the post-translational covalent cross-linking of proteins and incorporation of amines into proteins[3]. Recent studies suggest that in addition to their established physiological functions in cross-linking of fibrin and clotting of rodent seminal plasma (SePl)[3], TGases

(which are present in multiple forms and ubiquitous among tissues and secretions) may play a fundamentally multifunctional regulatory role in cellular phenomena[4,5].

As part of our continuing studies of the immunoregulatory factors within the MASG tissues and secretions[2], notably of SePl-uniquely representative of an admixture of MASG secretions, and the specificity of an antibody to human SePl TGase[6], we examined human spermatozoa for the presence of TGase.

Materials and methods

Normal human spermatozoa, as defined by sperm count (density), $\geq 50 \times 10^6$/ml; motility, $\geq 50\%$ at 2 h and morphology, $\geq 50\%$ normal, were obtained (courtesy of Dr Richard Bronson, SUNY, Stony Brook, NY) from ejaculates of three males attending a fertility clinic; washed 4 times with Hanks' balanced salt solution by centrifugation; adjusted to a concentration of 65×10^6/ml, and divided into two aliquots. One aliquot was extracted with 0.5% Triton X-100 (US Biochemical Corp., Cleveland, OH) in phosphate buffered saline (PBS) (0.15 M NaCl, 0.05 M Na_2HPO_4, pH 7.4) at 4 °C for 10 min, centrifuged (Beckman-Spinco Microfuge, Beckman, San Ramon, CA) at $8000 \times g$ and the supernatant, hereafter referred to as the Triton soluble fraction, saved. The second aliquot was used as substrate for the preparation of slides by dispensing 50-µl suspensions onto a glass slide, air drying and fixing in methanol. These respective preparations were then used for the evaluation of TGase by 1) dot immunoblot analysis and 2) immunohistologically by indirect immunofluorescent (IF) and immunoperoxidase staining.

Dilutions of the Triton solubilized fraction of spermatozoa in PBS were spotted onto nitrocellulose strips (0.45 µm pore size, Bio-Rad Laboratories, Richmond, CA) and blocked with 5% powdered milk in 50 mM TrisHCl, 0.2 M NaCl, pH 8.0. Nitrocellulose strips were then incubated with dilutions of a rabbit antibody to human SePl TGase [reactive with an 83 kDa protein identified and characterized as a tissue type TGase[6]], and controls as delineated in the table for 2 h at room temperature; washed in three changes of PBS over 30 min, and then incubated with biotinylated horse anti-rabbit immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA, Lot 50806) and horseradish peroxidase conjugated avidin (Vector Laboratories, Burlingame, CA, Lot 60114) for 1 h. Following washing, nitrocellulose strips were treated with 1.3 mM 3,3'-diamino-benzidine and 0.02% H_2O_2, and the reaction read vs concomitantly run reactions of the Triton soluble fraction of spermatozoa and saline; normal rabbit serum and anti-plasma FXIII *a*.

Smears of spermatozoa-containing slides were rehydrated with PBS and evaluated by indirect IF and immunoperoxidase staining. In the case of indirect IF, slides were treated with rabbit anti-human SePl TGase and a fluorescein conjugated goat anti-rabbit Ig (Calbiochem-Behring, LaJolla, CA, Lot 011080). For immunoperoxidase staining, application of the anti-SePl TGase was followed by incubation with a biotinylated horse antibody to rabbit IgG (Vector Laboratories, Burlingame, CA, Lot 50806) and horseradish peroxidase conjugated avidin (Vector Laboratories, Burlingame, CA, Lot 60114).

To further evaluate the molecular specificity of the TGase identified, i.e., plasma vs tissue type, spermatozoa were reacted with rabbit anti-human factor (F) XIII *a* (Calbiochem-Behring, LaJolla, CA, Lot 60114) – an antibody to plasma TGase, i.e., the thrombin-dependent type, and rabbit anti-rat liver TGase (a gift of Dr Rozá Ádány, University School of Medicine, Debrecen, Hungary) – an antibody to tissue TGase, i.e., the thrombin-independent type, respectively.

Results

A summary of the reactivity of human spermatozoa obtained with various antisera to plasma and tissue type TGases as evaluated by dot immunoblot analysis and indirect IF and immunoperoxidase staining is presented in the table.

As shown in the table, a 1:64 dilution of the Triton solubilized fraction of spermatozoa in PBS (predetermined as the optimal dilution from a chess board titration of Triton soluble fractions of spermatozoa vs the respective antisera named in the table) gave titres of 1:1024 and 1:64 with antisera to SePl TGase and rat liver TGase (a tissue type TGase), respectively, by dot immunoblot analysis. No reactivity was seen with saline, normal rabbit serum or anti-plasma FXIII *a* (the latter two being negative at 1:8, the lowest dilution tested).

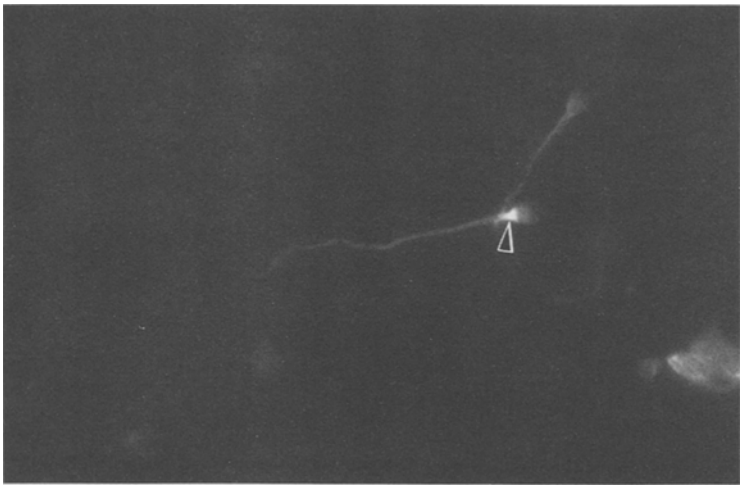
IF and immunoperoxidase staining patterns of the neck and tail of mature spermatozoa were, as noted in the table, seen with anti-SePl TGase and anti-rat TGase at

Evaluation of solubilized and intact human spermatozoa for immunological reactivity with antibodies to plasma and tissue type transglutaminases

Antibody	Reactivity with human spermatozoa by:		
	Dot immunoblot analysis[a] (reciprocal of titre)	Indirect immunofluorescence and immunoperoxidase staining[b]	
		Localization	Reciprocal of titre
(Saline)	–	–	–
Normal rabbit serum	<8	–	<8
Seminal plasma TGase	1024	Neck, tail	500
Plasma factor XIII *a*	<8	–	<8
Tissue TGase	64	Neck, tail	64

[a] Obtained with 1:64 dilution of Triton solubilized fraction of human spermatozoa (Donor: L. V.).

[b] Positive controls for antisera to seminal plasma (SePl) TGase; plasma factor (F) XIII *a* and tissue TGase were their concomitant reactivity with frozen sections of human prostate (SePl TGase and plasma FXIII *a*) and rat liver (tissue TGase).



Photomicrograph of indirect immunofluorescent staining reaction obtained on normal human spermatozoa with rabbit antibody to human seminal plasma transglutaminase. Localization of the antibody at a dilution of 1:500 to the neck and tail are shown by the arrow. × 5000 with oil.

titres of 1:500 and 1:64, respectively. A photomicrograph representative of these patterns as seen by indirect IF staining is shown in the figure. An occasional reaction (not shown) was seen between anti-FXIII *a* and what, initially appearing as immature spermatozoa, were determined from further microscopic examination, to be mononuclear cells.

Although spermatozoa were rigorously washed, the known avidity of numerous SePl proteins for spermatozoa and the current methodology, preclude determination of the origin of the sperm-bound TGase.

Discussion

The present immunological identification of spermatozoal TGase is consistent with identification of its biochemical activity[2,6,7] and suggested role in sperm motility[7], and provides, in view of the immunoregulatory properties of SePl TGase[2,6], presumptive identification of a means whereby spermatozoa may be protected from immunological 'attack' within the female reproductive tract. As such, the presence of sperm-bound TGase may possibly provide two of the essential properties of inseminated spermatozoa for successful reproduction. The genetic variability in sperm-bound TGase may be a contributing factor to infertility, wherein, e.g., its absence or decrease exposing foreignness ('non-selfness') of the male gamete, may permit the development of anti-sperm antibodies and other possible sequelae in the female.

In terms of protection, a similar role for TGase (in conjunction with uteroglobin) has been suggested to be responsible for suppressing sperm antigenicity in the rabbit[8]. Nonetheless, comparison from the examination of spermatozoal TGase on normospermic (evaluated in this study) vs oligospermic samples, and the incidence and relationship of fertility and anti-sperm antibodies in fertile and infertile couples to the staining patterns obtained with anti-SePl TGase, must be completed before the clinical significance of the present observations may be realized. In this regard, it is of interest to note the reported significant association of decreased immunosuppressive activity of SePl and the presence of anti-sperm antibodies in recurrent aborting couples[9].

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