An improved noninfectious murine skin model of organized granulomatous inflammation

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Summary. An improved model of granulomatous inflammation in skin was developed by second passage skin grafting of isolated, lyophilized skin granulomas, originally elicited in naive mice by inoculations of lyophilized hepatic schistosome egg granulomas. The tissue reaction is caused by a single exposure to a noninfectious, acellular granulomagenic stimulus and occurs in healthy mice free of systemic disease. The model should prove useful for isolation of granuloma initiation factor(s). Furthermore, because there is a time lag before new granuloma formation begins, a window exists for analytical dissection of the initiation process. In this study we described the responses of host cells by autoradiography, and light and electron microscopy. The activity of angiotensin-converting enzyme and proline-specific endopeptidase showed a modulation during granuloma formation. In addition we found that severe immunosuppression with high dose cyclosporine therapy did not alter granuloma formation, supporting the idea that initiation of organized granulomas is T-cell independent.
Key words. Skin granuloma model; granulomatous inflammation; T-cell independent; granuloma; angiotensin-converting enzyme; proline-specific endopeptidase.

Granulomatous inflammation is a characteristic pathological feature of certain diseases such as sarcoidosis, tuberculosis and leprosy. The most representative animal model has been hepatic lesions of experimental murine schistosomiasis, which has been widely used for morphological, immunological and biochemical studies[1,2]. Recently, Nishimura et al.[3] and Okamoto et al.[4] established a skin model by transplanting fresh and lyophilized hepatic granulomas, respectively, into the skin of naive mice. The skin model has distinct advantages over the hepatic model since the mice with skin granulomas do not suffer from systemic effects of the ongoing parasitic infection, and the granulomagenic stimulus is introduced only once, allowing examination of a time course of the changes. However, the lyophilized hepatic granulomas contain parasite products, such as soluble egg antigen, which might influence tissue reactions. We now report an improved model in which skin granulomas appearing after first passage in mice are isolated, lyophilized and grafted into the skin of a second generation of naive host mice.

Material and methods

Female C57BL/6 strain mice (5–6 weeks old) were infected with *Schistosoma mansoni* and hepatic granulomas isolated from mice after nine weeks by the method of Nishimura et al.[3,5]. The granulomas were frozen in an acetone/dry ice mixture and thawed in running tap water. After repeating the process for 2 cycles they were frozen and dried using a Unitorap II (Virtis, New York). The lyophilized sample was wet with distilled water and about 50 mg inoculated into first passage subcutaneous tissue of the dorsum of healthy naive mice. Five weeks later, granulomas which developed in the skin were separated from surrounding tissue, minced, lyophilized, and grafted into the skin of naive mice. The skin lesions were excised at different time intervals from both first and second passages and prepared for morphological and biochemical analysis. Tritiated thymidine (10 μCi in 0.1 ml saline) was locally injected into skin granulomas of some mice for autoradiographic study. In addition, using an earlier protocol[6], cyclosporine (150 mg/kg/day) was injected intramuscularly 5 times a week into some mice receiving second passage grafts 2 weeks prior to and 3 weeks after grafting in order to validate in this new model the previous finding that T helper/inducer cell function is not required for initiation of organized granuloma formation[6].

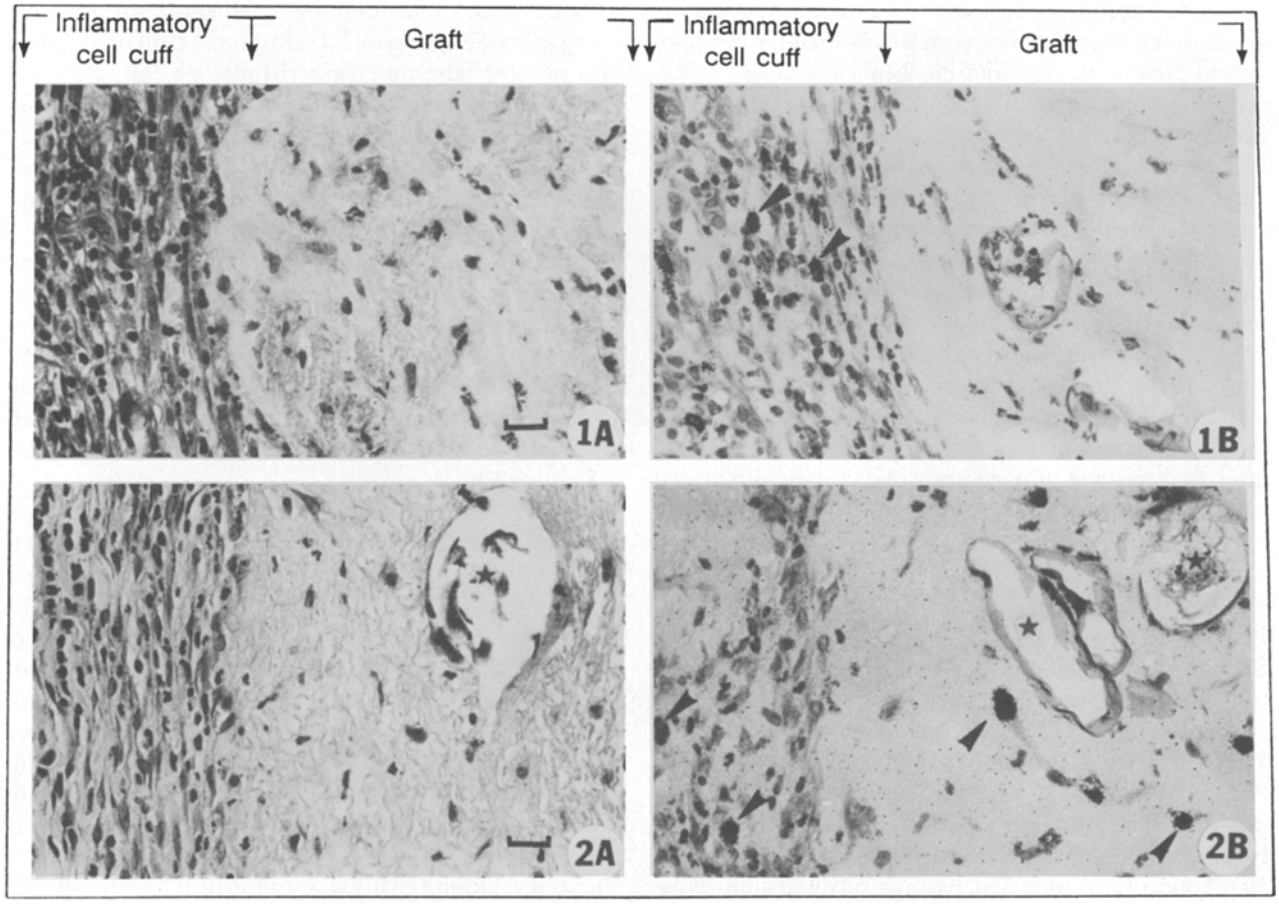
For light and electron microscopy, formalin-fixed, paraffin-embedded tissues were sectioned at 4 μm thick and stained with hematoxylin and eosin. Skin samples also were fixed in 3% glutaraldehyde, post-fixed in 2% osmium tetroxide and processed for routine transmission electron microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a Siemens Elmiskop IA.

To assay for angiotensin-converting enzyme (ACE) and proline-specific endopeptidase (PSE) activity, approximately 50 mg of separated skin lesions were homogenized in 1 ml of cold 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.8 with a Polytron homogenizer, Model PT-10 (Brinkmann, Switzerland), for 1 min. The homogenate was adjusted to 2 ml by adding 2% Triton X-100 to a final concentration of 0.25% (volume per volume) in the same buffer and stirred at 4 °C for 90 min. The homogenate was centrifuged at 1000 × g for 10 min and supernate used for enzyme essay. ACE activity was measured using a method established by Hara et al.[7]. Enzyme solution (20 μl) was added to 0.23 ml of 0.1 M phosphate buffer, pH 8.3, containing 5.5 mM hippuryl-L-histidyl-L-leucine. After incubation at 37 °C for 10 min, the reaction was stopped by adding 1.45 ml of 0.28 M NaOH. Fluorescence was developed by reacting 0.1 ml of 2% *o*-phthaldialdehyde with the remaining sub-

strate, histidyl-leucine, for 10 min. The fluorescence measured with a fluorescence spectrophotometer Model 204A, (Perkin-Elmer, California) at ex = 360 nm and em = 500 nm. PSE activity was assayed by the method of Yoshimoto et al.[8]. Enzyme solution (0.1 ml) was mixed with 0.2 ml of 40 µM N-(benzyloxycarbonyl)-glycyl-L-prolyl-4-methylcoumarinyl amide in 4% dimethyl sulfoxide, 0.5 ml of 0.2 M phosphate buffer, pH 7.0 and 0.2 ml of distilled water. After incubation at 25 °C for 10 min, the reaction was stopped by adding 1.0 ml of 0.7 M acetate buffer, pH 4.0. The fluorescence of 7-amino-4-methylcoumarin was measured at ex = 370 nm and em = 440 nm. One unit of ACE and PSE was defined as the amount of enzyme that hydrolyzed 1 µmole of each substrate per min at 37 °C and 25 °C, respectively. Protein concentration was determined by the method of Lowry et al.[9] using bovine serum albumin as the standard. Statistical analysis was performed using the Student's t-test.

Results

Light microscopy and autoradiography. In the first passage skin sites an increase of cells inside the graft was observed within 2 weeks after grafting of freeze-dried hepatic granulomas as reported by Okamoto et al.[4]. In contrast, the second passage skin granuloma grafts provoked a slowly evolving tissue reaction. Two weeks after transplantation, the graft was identified in subcutaneous tissue as mainly acellular eosinophilic mass surrounded by inflammatory cells (fig. 1 A). Many of the mononuclear cells in the cuff incorporated tritiated thymidine demonstrating that host cells proliferate at the edge of the graft (fig. 1 B). Even 4 weeks after transplantation the graft remained as a discrete area surrounded by a cuff of infiltrating cells (fig. 2 A), though a number of cells appeared within the graft, and autoradiography displayed clusters of silver grains localized over almost half of the cells within the graft with a decrease of labeled cells outside of the graft (fig. 2 B). The findings indicated that



Figures 1 and 2. Early changes in the second passage skin sites. Hematoxylin and eosin-stained paraffin sections (1 *A* and 2 *A*) and autoradiographs prepared with tissues fixed at 40 min after injection of tritiated thymidine (1 *B* and 2 *B*) × 800. The tissues taken at 2 weeks after grafting (1 *A* and 1 *B*) show a clear demarcation of the grafted tissue surrounded by a cuff of inflammatory cells. Many of the infiltrating cells are labeled (▲) indicating that host cells undergo DNA synthesis at the edge of the graft. The nuclei scattered within the graft do not show labeling and many of them appear condensed or irregularly shaped. The skin lesion at 4 weeks after grafting (2 *A*) retains a similar histology as seen earlier, but DNA synthesis is now demonstrated (▲) both inside and outside the graft (2 *B*). Bar = 10 µm.

cells had begun to migrate into the graft and subsequently underwent DNA synthesis and proliferation. Granulomas reformed by 5–7 weeks after grafting and most areas of the grafts were replaced by organized granulomas while the number of cells surrounding the grafts became considerably reduced. Using serial sections we attempted to associate remnants of parasite eggs with each granulomatous foci, and while some eggs appeared at the center of tubercles (fig. 3 A), other tubercles organized around vessels (fig. 3 B), and in some tubercles we could not identify any foreign structure. As noted previously[6], cyclosporine injection did not alter the course or degree of tissue reaction at each time period after transplantation of skin granulomas during the second passage.

Electron microscopy. Figure 4 A shows a typical granulomatous focus in plastic embedded tissue observed in the skin lesions 7 weeks after second passage. Many of the cells appeared to be typical epitheloid cells with large oblong nuclei and distinct nucleoli. Other less differentiated macrophages also were seen and a considerable number of eosinophils were present. By electron microscopy, epitheloid cells contained variable amounts of rough endoplasmic reticulum, and were primarily of the secretory type (fig. 4 B). Eosinophils often appeared degranulated and parasite eggs consisted of the remnants of empty egg shells.

ACE and PSE activity. Specific activity and time course of ACE after grafting of hepatic granulomas in the first passage host were essentially the same as reported by

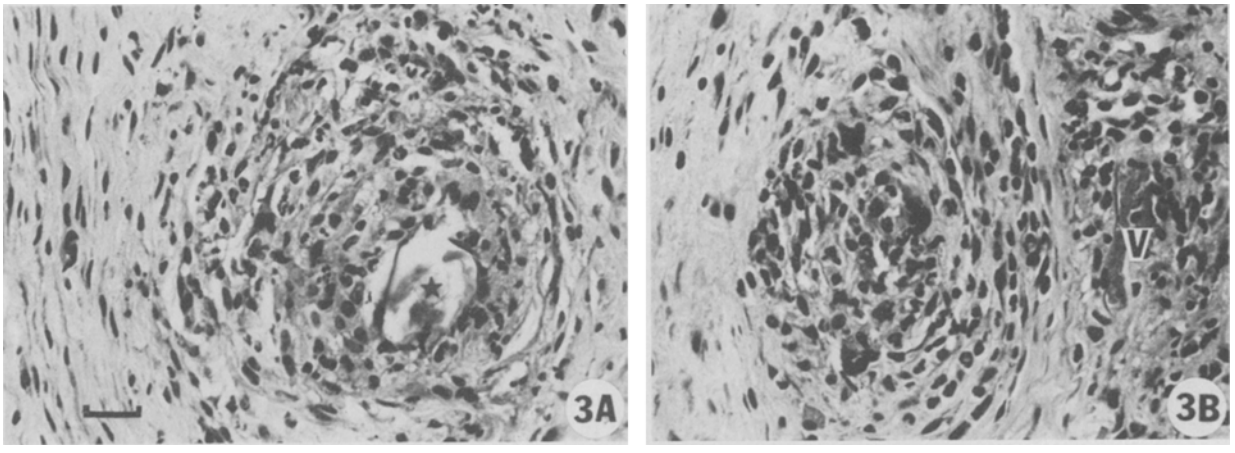


Figure 3. New granulomas which replaced the grafted tissues by 5 weeks after grafting. Granulomas formed around demolished eggs (*) (*A*) and vessels (v) (*B*), but no nidus was found in some granulomas. Hematoxylin and eosin-stained paraffin sections. × 800. Bar = 10 μm.

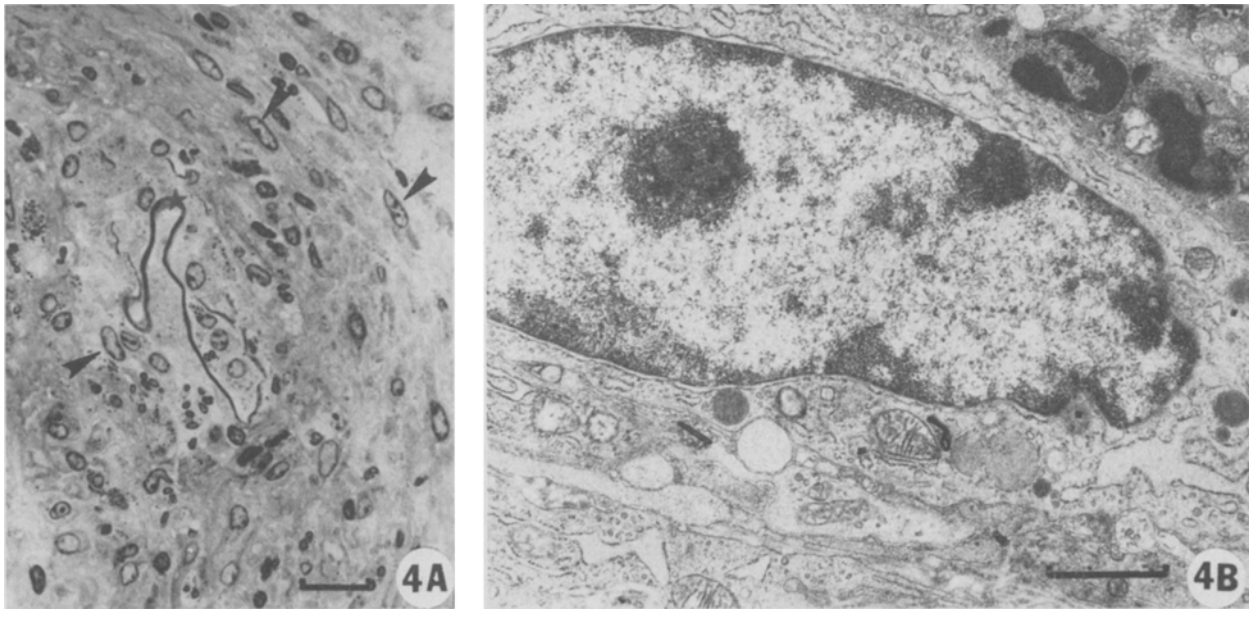


Figure 4. Light and electron microscopy of 7-week-old second passage skin granuloma. *A* 1-μm thick section demonstrates a parasite egg shell embedded in the center of cells organized in the form of a granuloma. Many cells show the characteristic appearance of epithelioid cells (▲) and eosinophils also are evident. × 1120. Bar = 10 μm. *B* An epithelioid cell containing well-developed rough endoplasmic reticulum in the cytoplasm. × 16,000. Bar = 1 μm.

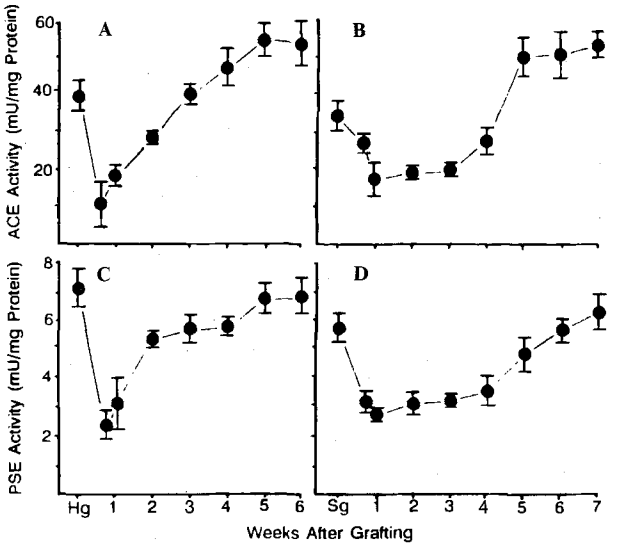


Figure 5. The activity of angiotensin-converting enzyme (*A* and *B*) and proline-specific endopeptidase (*C* and *D*) measured weekly after grafting of hepatic (*A* and *C*) or skin (*B* and *D*) granulomas. Each point is the mean specific activity calculated with the values from 3–5 mice (vertical bars represent ± standard error). Hg and Sg indicate enzyme activity grafted into the skin of the first and second passage, respectively.

Kanazawa et al.[10] (fig. 5A). The activity of grafted freeze-dried hepatic granulomas (41.4 ± 4.6 mU/mg protein) reduced to 17.7 ± 9.3 mU/mg protein by 4 days after grafting, but the activity recovered to achieve higher levels than the original graft as new skin granulomas formed 4–6 weeks later. The activity (56.4 ± 6.0 mU/mg protein) detected in 5-week skin granulomas was decreased to 35.1 ± 2.1 mU/mg protein during lyophilization and preparation for second passage grafting (fig. 5B). The activity further reduced in the skin sites up to 3 weeks after grafting. Between the 4th and 5th week, a significant ($p < 0.001$) increase in the activity (51.6 ± 4.0 mU/mg protein) was detected when maturation of organized granulomas took place. ACE activity in 6-week skin granulomas of first and second passages were comparable. ACE activity in normal dermis and skin sites inoculated with 8000 Polybead-Polystyrene Microspheres latex beads (Polysciences, Pennsylvania) suspended in saline were 6.2 ± 0.3 and 8.2 ± 0.6 mU/mg protein, respectively.

PSE activity detected in 9-week-old hepatic granulomas was 7.37 ± 0.56 mU/mg protein, while the value of normal liver was 2.33 ± 0.26 mU/mg protein, indicating elevated enzyme activity associated with granulomatous inflammation. Changes in the activity of PSE in the skin sites of first (fig. 5C) and second (fig. 5D) passages showed a similar tendency to ACE activity in the skin sites. Activity decreased during the early time periods after grafting, but returned to higher levels compatible with those present in grafted hepatic or skin granulomas and were significantly ($p < 0.01$) higher than normal dermis (1.50 ± 0.23 mU/mg protein) and skin sites inoculated with latex beads (1.59 ± 0.02 mU/mg protein) as a control.

Discussion

Freeze-dried skin granulomas elicited new organized granuloma formation in skin that morphologically mimicked those reported by Nishimura et al.[3] and Okamoto et al.[4], except the difference between the grafts used for first and second skin passages was the amount of contaminating parasite components. In hepatic schistosome egg granulomas adult worms continue to lay eggs which elicit new granuloma formation. The isolated granulomas used for first passage grafts contain egg and parasite products such as structural proteins of egg shell[11], enzymes[12,13], and soluble egg products known to be antigenic[14–17] and chemotactic[18,19]. Although we have repeatedly confirmed that inoculation of isolated eggs alone does not cause granuloma formation in the skin of naive mice, it is conceivable that egg products preexisting in granulomas may influence the inflammatory response. The second passage skin grafts should be virtually devoid of parasite products. The result of this 'cleaning-up' process seems to be mainly a delay in the time for granuloma reformation to begin.

The biochemically measured activity of two enzymes correlated well with the skin tissue reaction analyzed morphologically, including the lag time after second passage grafting. ACE is a classic enzyme marker for granulomatous inflammation, and its activity has been shown to increase as granulomas grow in either liver[20] or skin[10]. In the present study we measured another endopeptidase, PSE [EC 3.4.21.26] which cleaves biologically active peptides at the carboxyl side of prolyl residues, but does not hydrolyze high molecular weight proteins[21]. Since PSE was found to hydrolyze angiotensin II[22], we anticipated that modulation of PSE activity would occur during granulomatous reactions. The enzyme activity showed an initial depression after grafting and subsequent recovery as new granulomas formed, mimicking the time course of ACE activity. Whether PSE will prove useful as a second granuloma marker enzyme requires further investigation.

This improved skin granuloma model embodies the advantages of the earlier one[3,4], namely a one-time stimulus, causing a local granulomatous response in a healthy host that does not become systemically ill. It adds the circumstance where the inciting granulomagenic agent has been separated from contaminating, potentially toxic or interfering foreign material. So now we can seriously attempt to isolate and purify the elusive 'granuloma initiating factor' which has been shown to be a nonviable subcellular substance[4] and T-cell independent[23]. Additionally, the improved skin granuloma model provides a time lag before new granulomas reform. This allows a window of time for pharmacologic or therapeutic intervention to better analyze the induction process of granulomatous inflammation. As an example, in this study we

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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1 Phillips, S. M., and Colley, D. E., Progr. Allergy *24* (1978) 49.
2 Boros, D. L., Clin. microbial Rev. *2* (1989) 250.
3 Nishimura, M., Higuchi, M., Fukuyama, K., and Epstein, W. L., Archs Dermat. Res. *278* (1985) 61.
4 Okamoto, M., Epstein, W. L., Suya, H., Kanazawa, K., and Fukuyama, K., Exp. Cell Biol. *55* (1987) 173.
5 Nishimura, M., Epstein, W. L., and Fukuyama, K., J. Invest. Dermat. *79* (1982) 153.
6 Suya, H., Fujioka, A., Pincelli, C., Fukuyama, K., and Epstein, W. L., J. Invest. Dermat. *90* (1987) 430.
7 Hara, A., Fukuyama, K., and Epstein, W. L., Biochem. Med. *26* (1981) 199.
8 Yoshimoto, T., Ogita, K., Walter, R., Koida, M., and Tsuru, D., Biochim. biophys. Acta *569* (1979) 184.
9 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 264.
10 Kanazawa, K., Higuchi, M., Okamoto, M., Fukuyama, K., and Epstein, W. L., J. Cell Biochem. *34* (1987) 61.
11 Rodrigues, V., Chaudhri, M., Knight, M., Meadows, H., Chambers, A. E., Taylor, W. R., Kelly, C., and Simpson, A. J. G., Molec. biochem. Parasit. *32* (1989) 7.
12 Kasschau, M. R., and Mansour, T. E., Nature *296* (1982) 66.
13 Zerda, K. S., Dresden, M. H., and Chappell, C. L., Exp. Parasit. *67* (1988) 238.
14 Boros, D. L., and Warren, K. S., J. exp. Med. *132* (1970) 488.
15 Boros, D. L., and Lande, M. A., Am. J. trop. Med. Hyg. *32* (1983) 78.
16 Haran, D. A., Mitsuyama, M., and David, J. R., J. exp. Med. *159* (1984) 1371.
17 Freedman, D. O., and Ottesen, E. A., J. infect. Dis. *158* (1988) 556.
18 Tsuda, S., Fukuyama, K., and Epstein, W. L., J. Immun. *122* (1979) 2554.
19 Owhashi, M., Maruyama, H., and Nawa, Y., Infect. Immun. *54* (1986) 723.
20 Hara, A., Fukuyama, K., and Epstein, W. L., Exp. molec. Path. *35* (1981) 199.
21 Walter, R., and Yoshimoto, T., Biochemistry *17* (1978) 4139.
22 Yoshimoto, T., Simmons, W. H., Kita, T., and Tsuru, D., J. Biochem. *90* (1981) 325.
23 Fujioka, A., Pincelli, C., Hashimoto, A., Fukuyama, K., and Epstein, W. L., Int. Archs Allergy appl. Immun. *90* (1989) 313.
24 Epstein, W. L., Okamoto, M., Suya, H., and Fukuyama, K., Immun. Lett. *14* (1986) 59.
25 Tanaka, A., Emori, K., Nagao, S., Kushima, K., Kohashi, O., Saitoh, M., and Kataoka, T., Am. J. Path. *106* (1982) 165.
26 Pincelli, C., Fujioka, A., Fukuyama, K., and Epstein, W. L., Exp. Cell Biol. *56* (1988) 229.
27 Jagadha, V., Andavolu, R. H., and Huang, C. T., Am. J. clin. Path. *84* (1985) 598.
28 Burmester, G. R., Gramatzki, M., Gernler, J., Bartels, O., and Kalden, J., Clin. Immun. Immunopath. *37* (1985) 406.
29 Vilalta-Castel, E., Valdes-Sanchez, M. D., Guerra-Vales, J. M., Teno-Estebans, Garzon, A., and Lopez, J. I., Eur. J. Hemat. *41* (1988) 12.
30 Blumenfeld, W., Basgoz, N., Owen, W. F., and Schmidt, D. M., Annls int. Med. *109* (1988) 505.

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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