Biochemical Pharmacology, Vol. 38, No. 1, pp. 121–131, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

IMMUNOSUPPRESSIVE AND ANTI-INFLAMMATORY PROPERTIES OF A MAJOR PROTEIN SECRETED FROM THE EPITHELIUM OF THE RAT SEMINAL VESICLES

SALVATORE METAFORA,*† GIANFRANCO PELUSO,* PAOLA PERSICO,‡ GIANPIETRO RAVAGNAN,§ CARLA ESPOSITO‖ and RAFFAELE PORTA‖

* CNR Institute of Protein Biochemistry and Enzymology, Via Toiano 6, Arcofelice (Naples); ‡ Department of Experimental Pharmacology, University of Naples, Naples; § CNR Institute of Experimental Medicine, Viale C. Marx 15, 00156 Rome; and ‖ Department of Biochemistry and Biophysics, University of Naples, Via Costantinopoli 16, 80138 Naples, Italy

(*Received* 20 *April* 1988; *accepted* 28 *July* 1988)

Abstract—The nonspecies specific immunosuppressive and anti-inflammatory properties of a major protein (SV-IV) secreted from the epithelium of rat seminal vesicles (SV) are described. To detect the immunosuppressive effect, peripheral blood lymphocytes (PBL) were pretreated for 2 hr at 37° with SV-IV, and the protein was maintained in the incubation medium during the whole culture time. We obtained evidence that, during preincubation of PBL with SV-IV the protein was transformed by a transglutaminase (TGase) released from PBL into modified low and high molecular weight forms able to bind to PBL surfaces. It is suggested that T lymphocytes are the possible targets of the immunosuppressive effect. SV-IV seems to inhibit only the early phase of the proliferative response of T lymphocytes to mitogens without having any direct effect on the enzymatic system involved in DNA synthesis. Moreover, the protein SV-IV was also shown to possess an anti-inflammatory property due to a block of the arachidonic acid cascade at the level of the enzyme phospholipase A_2 (PLA_2). The physiological significance of the immunosuppressive and anti-inflammatory properties of SV-IV are discussed in relation to different aspects of the mammalian reproduction.

The mammalian epididymal spermatozoon is highly immunogenic, possessing on the cell surface numerous nonself auto-antigens and allo-antigens, including those of the major histocompatibility system [1–3]. In the male and female genital tracts many cells belonging to the immune system (B lymphocytes, T lymphocytes, macrophages, etc.) are physiological residents [1–3] apparently serving the function of guarding these regions against the invasion of nonself alien elements or macromolecules. However, the seminal plasma immunocytes appear to be highly tolerant to ejaculated spermatozoa [1–3]. The mechanism of this phenomenon is still unclear. It has been suggested that various immunomodulatory substances of both high (proteins) and low (prostaglandins, polyamines, endorphins, etc.) molecular weight, normally present in the seminal plasma, could be responsible for this peculiar type of tolerance [1–3]. In fact, it has been demonstrated in rabbits that uteroglobin (UG),¶ a small acidic protein, synthesized and secreted under steroid hormone control by prostate, seminal vesicles (SV) and uterus, is transformed by the enzyme transglutaminase (TGase) in a molecular form able to suppress the immunogenicity of the epididymal spermatozoon [4, 5]. A protein structurally homologous to UG [6] is secreted in large amounts (about 10 mg/ml) in the lumen of adult rat SV [7]. This protein, named SV-IV (seminal vesicle No. 4) according to its mobility in SDS–PAGE [7], has a molecular weight of 9758 [8], an isoelectric point of 8.9 [9], very poor structural features and is thermostable (unpublished observations). Its known amino acid sequence [8] is coded for by at least 4 species of poly(A)$^+$mRNA [10] transcribed under strict androgen control [11, 12] from genes that have been recently isolated and sequenced [12–14]. Antigens related to SV-IV have been found in humans both free in seminal plasma and bound to the surface of ejaculated spermatozoa [15, 16]. The protein SV-IV, like UG, is transformed by TGase *in vitro* in different molecular forms as a consequence of inter- and perhaps intra-molecular crosslink formation [17]. The modified SV-IV species of high MW are able to suppress the strong immunogenicity of the rat male gametes by binding to the epididymal sperm surfaces [18, 19]. This biochemical event can be of crucial importance to avoid rejection of spermatozoa deposited in the female reproductive tract during coitus. Furthermore, we have recently demonstrated that the lymphocytes present in the human male genital tract do not seem to be appropriately equipped for a successful immune reaction against nonself elements [18]. Their surface, devoid of Ia antigens, suggests that they are not activated elements. In addition, these cells are unable to act as responder cells in mixed lymphocyte cultures [18]. The majority of the seminal lymphocytes functionally act as sup-

† To whom correspondence should be addressed.

¶ Abbreviations used: Con A, concanavalin A; DTT, dithiothreitol; IL2, interleukin 2; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MoAbs, monoclonal antibodies; PBL, peripheral blood lymphocytes; PG, prostaglandin; PHA, phytohemoagglutinin; PLA_2, phospholipase A_2; SV, seminal vesicle; SV-IV, protein no. 4 (secreted from rat seminal vesicle epithelium); TGase, transglutaminase; UG, uteroglobin.

pressor cells [20, 21] and are unexpectedly characterized by a surface antigenic phenotype resembling that of immature T lymphocytes (presence of T_6, T_9, T_{10}, etc.) [18]. However, the cause of the seminal lymphocyte quiescence is not known. The possibility that various immunomodulatory substances present in the seminal plasma [2, 3] could play a role in this phenomenon can be reasonably conceived, even though any direct evidence is lacking at present.

Considering these data in the light of both the great wealth of biochemical information on the structure of the protein SV-IV and on the gene(s) coding for it, and the ability of this protein to suppress the epididymal sperm immunogenicity, it seemed to us of great interest to thoroughly investigate the possible immunomodulatory and anti-inflammatory activities of the protein SV-IV. Our aim was not only to elucidate the physiological role played by this protein in seminal fluid, but also to understand the molecular mechanism underlying the peculiar lack of reactivity of the immunocytes present in the male genital tract and the predisposition to chronicity of the inflammatory pathologies so often affecting this anatomical region.

MATERIALS AND METHODS

Protein purifications. The protein SV-IV was purified to homogeneity from adult rat (Fisher–Wistar) SV secretion as described by Ostrowski *et al.* [7]. The purity of the protein was evaluated by 15% PAGE in denaturing and nondenaturing conditions [6], by fingerprint technique and by amino acid composition analysis [9]. The proteolyzed SV-IV was obtained by incubating, at 37° for 8 hr, 0.5 mg of the protein with 5 μg of bovine pancreatic trypsin (Warthington, TRTPCK) in 50 mM ammonium bicarbonate buffer, pH 8; an equal aliquot of the enzyme was subsequently added and the incubation continued for 12 hr. At the end of the treatment the reaction mixture was boiled for 10 min and then used in immunological experiments.

UG, purified to homogeneity from rabbit uterus [22], was a generous gift from Dr. T. Tancredi.

Purified guinea-pig liver TGase was from Sigma Chemical Co. (St. Louis, MO).

Protein concentration was determined by the method of Lowry *et al.* [23].

Preparation of human intact peripheral blood lymphocytes (PBL). Intact human PBL were obtained by Ficoll-Paque (Pharmacia) fractionation from buffycoats of normal, healthy subjects unaffected by viral diseases such as AIDS, hepatitis B, cytomegalovirus, or herpes. The isolated cells, washed three times in incomplete (without glutamine, FCS and antibiotics) RPMI 1640 medium, were suspended in the same medium at a final concentration (f.c.) of 3×10^6 cells/ml. The structural and functional integrity of the cells was verified, both at the beginning and at the end of the experiments, by phase contrast microscopy and Trypan blue exclusion test. The analysis of the mononuclear cell subsets present in the purified PBL population was performed by a fluorescence activated cell sorter (FACS) using fluorescent monoclonal antibodies (MoAbs) obtained from Ortho or from Becton-Dickinson. The results of this analysis demonstrated that approximately 65% of the whole population were T lymphocytes characterized by the presence on their surface of a typical set of antigens: T_3, T_4 (Leu 3), T_8 (Leu 2) and T_{11}; about 15% were B lymphocytes ($B2^+$, $B7^+$, DR^+) and about 20% were monocytes ($M3^+$, $M5^+$, DR^+).

Quantitation of mitogen-induced blastogenesis. Intact resting PBL, washed three times with incomplete RPMI 1640 medium, were suspended, at a f.c. of 3×10^6 cells/ml, in the same medium containing 2 mM glutamine, 5% FCS, 100 I.U./ml penicillin and 100 μg/ml streptomycin. PBL stimulability by mitogens was evaluated in the flat-bottomed wells of microtiter tissue culture plates by (^{3}H) thymidine incorporation technique. The final volume of the assay was 200 μl (3×10^5 PBL were used per well). Concanavalin A (Con A; Sigma) was used at a f.c. of 12.5 μg/ml, Phytohemoagglutinin (PHA; Flow) at a f.c. of 4 μg/ml, SEB (the *Staphylococcus* enterotoxin was a generous gift from Prof. Dianzani) at a f.c. of 2 μg/ml, OKT_3 (Ortho) at a f.c. of 1–2 ng/ml, lipopolysaccharide (LPS; lipopolysaccharide W, extracted from 055:B5 *E. coli* strain and obtained from DIFCO) at a f.c. of 20 μg/ml, and calcium ionophor A23187 at a f.c. of 1 μM. The cells were incubated for 3 days at 37° in a 5% CO_2/95% air incubator, each culture being in triplicate. When the calcium ionophor A23187 was used, the cells were cultured for only 24 hr. Six hours before harvesting, 1 μCi of (^{3}H) thymidine (2 Ci/mmole, Amersham) was added to each well and the culture was continued for another 6 hr. Cell harvesting, washing, and counting were performed according to conventional procedures. The degree of lymphocyte stimulability by mitogens was expressed as cpm of (^{3}H) thymidine incorporated/3×10^5 cells.

Treatment of PBL with SV-IV. Aliquots of 3×10^6 PBL were treated for 2 hr at 37° in 1 ml of incomplete RPMI medium in the absence (control) or presence of 90 μg of purified SV-IV. Following incubation suitable amounts of the components required to complete the RPMI 1640 medium were added by using concentrated stock solutions to avoid dilution of the incubation mixtures. 0.1 ml of each sample (control and SV-IV-treated PBL) was mixed in the flat bottom wells of sterile microtiter plates with 0.1 ml of RPMI 1640 complete medium containing the appropriate amount of mitogen. The final concentration of SV-IV in the culture was 45 μg/ml. Quantitation of blastogenesis was performed as described in the previous paragraph.

Mixed lymphocyte reaction (MLR). 1.5×10^6 PBL from two different histoincompatible individuals (PBL 1 and PBL 2) were separately incubated for 2 hr at 37° in 1 ml incomplete RPMI 1640 medium in the presence or absence of 90 μg of SV-IV. At the end of incubation appropriate amounts of the lacking components were added to render the RPMI medium complete. Aliquots of 0.1 ml (1.5×10^5 cells) of SV-IV-treated PBL 1 suspension were mixed with 0.1 ml (1.5×10^5 cells) of SV-IV-treated PBL 2 suspension in flat-bottomed wells of microtiter plates and the cells cultured for five days. A similar procedure was carried out with PBL suspensions not containing SV-

IV. Further processing of samples was performed as described above in the paragraph "Quantitation of mitogen-induced blastogenesis".

Binding of SV-IV to human PBL. The (^{125}I) SV-IV binding assay mixture (final volume, 0.3 ml) contained 3×10^6 PBL, 50 mM Tris–HCl buffer, pH 7.5, 120 mM NaCl, 5 mM dithiothreitol (DTT), 1.25 μg (^{125}I) SV-IV (5×10^6 cpm) in the presence or absence of 2.5 mM $CaCl_2$ and/or 1 μg of purified guinea-pig liver TGase. Following incubation for 2 hr at 37° the binding mixtures were centrifuged (12,000 *g*, 30 sec at room temperature) and the sedimented cells were washed with RPMI 1640 medium until no radioactivity was detectable in the supernatant. The washed PBL were transferred before lysis in another polypropylene test tube to avoid contamination of the final cell lysate with radioactivity not bound to the cells but adsorbed to the test tube walls. PBL lysis was performed by suspending each cellular pellet in 40 μl of distilled water. After addition of 20 μl of a solution containing 15% SDS, 15% β-mercaptoethanol, 190 mM Tris–HCl buffer, pH 6.8, and 30% glycerol, the samples were boiled for 2 min and then analysed by both TCA-precipitable radioactivity counting and SDS–PAGE [24] with fluorography of the gel [25].

Assay of α-DNA polymerase. The activity of α-DNA polymerase in the presence or absence of either native SV-IV or TGase-pretreated SV-IV was measured essentially as described by Grippo *et al.* [26]. The standard reaction mixture, incubated for 20 min at 37°, contained in 300 μl of 50 mM Tris–HCl buffer, pH 8, 2.5 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM DTT, 0.165 mM each of dGTP, dATP, dCTP (Sigma), 2.4 μCi (^{3}H) dTTP (NEN; 2.4 μCi/nmole), 80 μg "gapped" salmon sperm DNA and 0.1–0.2 units (1 unit of α-DNA polymerase corresponds to that quantity of enzyme which catalyzes the incorporation in the "gapped" DNA of 1 nmole (^{3}H)dAMP at 37° in 20 min) of DNA polymerase α-DNA primase complex (a generous gift from Prof. P. Grippo and Dr. P. Orlando) purified from *Xenopus 1.* eggs. SV-IV (10, 50 and 200 μg) used in the DNA polymerase assay was preincubated with "gapped" salmon sperm for 2 hr at 37° in 10 mM Tris–HCl buffer, pH 8, before addition to the reaction mixture. In other experiments, 200 μg of SV-IV were pre-treated with purified guinea-pig liver TGase, for 2 hr at 37°, in 150 μl of Tris–HCl buffer, pH 8, containing 5 mM DTT and, where required, 2.5 mM $CaCL_2$ before addition to the DNA polymerase incubation mixture; EGTA was added at a final concentration of 5 mM to stop the SV-IV pretreatment. Preparation of "gapped DNA", termination of the standard DNA polymerase assay, TCA precipitation of the samples, and their counting in a Packard liquid scintillation counter were performed as previously described [26].

Carrageenin foot oedema induction. Hind-limb oedema was induced in groups of 10 male Wistar rats (150–200 g), lightly anaesthesized with diethyl ether, by plantar injection in the hind paw of 0.1 ml of 1% w/v carrageenin (Viscarin 402, from Marine Colloids) suspension prepared in saline. After 90 min some groups of animals received a superinjection of 0.1 ml saline containing 100 μg of arachidonic acid (Sigma). Dexamethasone (1 mg/kg) was subcutaneously administered 60 min before carrageenin injection. In contrast, SV-IV was injected together with carrageenin into the hind paw. Control rats received only saline injections. The volume of the paw was recorded at zero time and every 60 min thereafter up to 5 hr. The oedema area was calculated as Σ of the mean of oedema volumes at the 1st and the 3rd hr + ½ mean oedema observed at the 4th hr. A differential volume measuring instrument, a plethysmograph manufactured by U. Basile (Milan, Italy), was used in these experiments in order to quantitate the oedema volume after carrageenin injection.

Prostaglandin (PG) determination. The induction of prostanoid biosynthesis in phagocytosing leukocytes was carried out as previously described [27, 28]. Briefly, 8×10^6 resting peritoneal leukocytes (80% mononuclear and 20% polymorphonuclear), obtained from adult male Wistar rats and suspended in Krebs solution enriched with bovine serum albumin (100 μg/ml), were incubated for 120 min at 37° with heat-killed Bordetella pertussis (a gift from Borrough-Wellcome) in a ratio of 1000 bacteria per cell. At the beginning of incubation either dexamethasone, SV-IV, arachidonic acid, or saline was added. In some experiments the phagocytosis process was checked by staining with Gram's stain aliquots of cell suspensions collected at the end of incubation. After incubation the cells were removed by centrifugation and PGs, extracted from the supernatant, were bioassayed on the rat stomach strip by using synthetic PGE_2 (Sigma) as standard [27].

Phospholipase A_2 (PLA_2) assay. The enzyme assay was performed as previously described [28, 29] with minor modifications. The reaction mixture (final volume, 0.5 ml) contained 20 mM Tris–HCl buffer, pH 8, 2 mM $CaCl_2$, 0.1 unit of porcine pancreatic PLA_2 (Sigma) and variable amounts of SV-IV. After a preincubation at 4° for 60 min, 0.04 μCi of 1-palmitoyl-2-(1-^{14}C) oleyl phosphatidylcholine (sp. act., 57 mCi/mmole; Amersham) were added to the assay mixture together with 50 nmoles of the unlabelled compound (Sigma). At the end of incubation (10 min at 37°), the reaction was terminated by adding 0.1 ml of 50 mM EDTA. *n*-Hexane (2 ml), containing tracer amounts of (^{3}H)oleic acid for recovery evaluation, was then added in order to extract the (^{14}C)oleic acid release from the substrate during the enzymatic reaction. Aliquots of the organic phase were taken and their radioactivity measured in a liquid scintillation spectrometer. The PLA_2 activity was expressed as nmoles of oleic acid released (±SEM) from the substrate during 10 min of incubation at 37°.

RESULTS

SV-IV inhibits both the stimulation of human PBL by different types of mitogens and the mixed lymphocyte reaction (MLR)

In Table 1 are reported data showing that microgram amounts of SV-IV (90 μg/ml during the PBL preincubation and 45 μg/ml during the 3 day culture) suppress with varying degrees of efficiency PBL blastogenesis induced by different types of mitogens, such as Con A, PHA, OKT_3 and SEB. Nonsignificant inhibition was observed by using the calcium ion-

Table 1. Inhibition of human PBL response to various mitogens by treatment of the cells with SV-IV*

Mitogen	(^{3}H)Thymidine incorporation into lymphocytes (cpm ± SEM)		
	−SV-IV	+SV-IV	(% of inhibition)
None	250 ± 50	280 ± 31	(0)
ConA	30,120 ± 1800	5393 ± 169	(82)
PHA	88,600 ± 2430	45,830 ± 1490	(48)
SEB	23,080 ± 1200	9981 ± 820	(57)
OKT3	25,112 ± 1100	7990 ± 700	(68)
LPS	12,325 ± 839	10,243 ± 1072	(17)
A23187	12,100 ± 1090	11,500 ± 1200	(5)

* Experimental details are described in Materials and Methods.

Table 2. Effect of different concentrations of SV-IV on the response of human PBL to Con A and PHA

SV-IV concentrations* (μg/ml)	(^{3}H)Thymidine incorporation into lymphocytes (cpm ± SEM)	
	Con A (% of inhibition)	PHA (% of inhibition)
0 (Control	27,627 ± 2107 (0)	84,540 ± 2113 (0)
0.5	24,227 ± 1603 (12)	75,913 ± 1910 (10)
5	21,883 ± 1099 (21)	76,980 ± 1963 (9)
25	18,606 ± 1123 (33)	71,775 ± 1645 (15)
50	8168 ± 1072 (70)	49,650 ± 1070 (41)
200	8309 ± 985 (70)	47,903 ± 1011 (43)
500	16,600 ± 1220 (40)	66,055 ± 2003 (22)
2000	27,476 ± 2201 (0)	71,260 ± 2125 (16)

* The reported values refer to SV-IV concentrations in the cell culture. Further experimental details are described in Materials and Methods.

ophor A 23187 and LPS as mitogens. Similar results (data not shown) were obtained by stimulating either PBL or splenocytes from rats of different species (Wistar, Fisher, Wistar–Fisher hybrids) with Con A or PHA. The immunosuppressive effect of SV-IV was highest by using Con A as mitogen and progressively lower when PBL were stimulated by OKT_3, SEB or PHA. Moreover, no significant difference was found in the SV-IV-induced immunosuppressive effect when the protein was prepared either from animals of the same weight belonging to different rat strains or from animals of the same strain having different weights (150–400 g).

The effect of different amounts of SV-IV on the response of human PBL to Con A or PHA are reported in Table 2. The highest immunosuppression was obtained when SV-IV was present in the incubation mixture at concentrations ranging between 50 and 200 μg/ml. At higher concentrations, the SV-IV effect progressively decreased to reach a null effect when the protein was used at about 2 mg/ml. Mandatory requirements to observe the immunosuppressive effect were both a preincubation of PBL at 37° with the protein before mitogen addition, and maintenance of the protein in the incubation mixture during the whole period of culture. In fact, PBL were normally stimulated by the mitogens when SV-IV was added either together with the mitogen or 24 or 72 hr after (data not shown). Moreover, the washing of the SV-IV pretreated cells with RPMI, performed to eliminate the protein from the culture medium before mitogen addition, effectively prevented the inhibition of blastogenesis. This effect could be due to endocytosis of the protein bound to lymphocytes during the preincubation period.

Finally, when suitable aliquots of human PBL, obtained from two different histoincompatible individuals, were preincubated with or without SV-IV and then mixed in culture, a marked inhibition of the (^{3}H)thymidine incorporation was observed when SV-IV was present in the preincubation medium and maintained in the culture medium during the whole incubation time (Table 3).

Uteroglobin inhibits human PBL response to Con A

On the basis of a significant structural homology between SV-IV and UG [6] it was interesting to investigate whether also UG was able to inhibit the human PBL response to Con A. We found that both native and reduced and carboxymethylated UG were able, at a concentration of about 50 μg/ml, to suppress about 65% of (^{3}H)thymidine incorporation in the PBL stimulated by Con A (12.5 μg/ml f.c.). As with SV-IV, UG also required preincubation (2 hr at 37°) of

Table 3. Inhibition of mixed lymphocyte reaction by SV-IV*

Addition	(^{3}H)Thymidine incorporation into lymphocytes (cpm ± SEM)
None	9986 ± 1235
SV-IV	583 ± 104

* The viability of human PBL obtained from two different histo-incompatible individuals (PBL 1 and PBL 2) was evaluated by Con A stimulation and (^{3}H)thymidine incorporation (PBL 1, 382 ± 60 cpm; PBL 1 plus ConA, 34,600 ± 543 cpm; PBL 2, 435 ± 91 cpm; PBL 2 plus Con A, 31,375 ± 730 cpm). Further experimental details are reported in Materials and Methods.

Table 4. Effect of different experimental conditions on the binding of (^{125}I) SV-IV to the surface of human PBL

Experiment	Composition of binding assay mixture	With Ca^{2+} (cpm ± SEM)	Without Ca^{2+} (cpm ± SEM)
1	PBL + (^{125}I) SV-IV	3280 ± 315	815 ± 90
2	PBL + (^{125}I) SV-IV + TGase	6052 ± 600	800 ± 85
3*	PBL + (^{125}I) SV-IV + TGase + EGTA	—	1400 ± 120
4*	PBL + (^{125}I) SV-IV + TGase + EGTA	—	45,220 ± 3900

* The SV-IV binding assay was performed as described in Materials and Methods after a previous incubation of (^{125}I) SV-IV in 30 mM Tris–HCl buffer, pH 7.5, containing 5 mM dithiothreitol, 130 mM NaCl and 1 μg of purified guinea-pig liver TGase in the absence (Exp. 3) or presence (Exp. 4) of 2.5 mM $CaCl_2$. At the end of the incubation (2 hr at 37°) and before PBL addition, 5 mM EGTA was added to the samples to inhibit TGase activity during the binding assay.

PBL with the protein, and constant incubation of the protein during the whole period of culture, in order to observe the immunosuppressive effect.

SV-IV specifically inhibits the mitogen-induced proliferation of T lymphocytes

To verify whether the protein SV-IV was able to inhibit the proliferation of cells not belonging to the immune system, both morphology and growth characteristics of some normal or transformed mammalian cells were studied in the presence or absence of purified SV-IV in the culture medium. Doubling time, saturation density and morphology of the cell lines tested (primary cultures of human normal fibroblasts, human liposarcoma cells, human thyroid epithelial cells (FTRL line) transformed by SV-40, and rat seminal vesicle epithelial cell line transformed by Harvey MSV) did not change at all in the presence of concentrations of SV-IV (40 μg/ml) clearly immunosuppressive for the T lymphocytes (data not shown). Similar results were obtained by preincubating the cells with SV-IV for 2 hr at 37° in their culture medium before seeding, and continuing the culture in the presence of the protein.

The immunosuppressive effect of SV-IV is neither caused by decrease in (^{3}H) thymidine uptake nor by inhibition of the enzymes involved in DNA synthesis

To rule out the possibility that the SV-IV immunosuppressive effect on T lymphocytes was due to an inhibition of either labelled thymidine uptake or of the activity of enzymes involved in the normal process of DNA replication, SV-IV (45 μg/ml f.c.) was added, together with (^{3}H)thymidine, to Con A-activated PBL after three days of culture. The results of this experiment indicated that (^{3}H)thymidine incorporation in the nuclei of activated T lymphocytes did not change when SV-IV was present in the solution containing the labelled material added to the activated cells (data not shown).

Additional experiments showed that 200 μg of native or TGase-modified SV-IV (for experimental details see Materials and Methods) were unable to influence the activity, assayed *in vitro*, of α-DNA polymerase purified from *Xenopus laevis* eggs (data not shown). This finding suggests that SV-IV did not exert the immunosuppressive effect through inhibition of the main enzyme (α-DNA polymerase/DNA primase complex) involved in the metabolic pathway of DNA synthesis.

Only few receptors for interleukin 2 (IL 2) and MHC Class II antigens appear on the surface of SV-IV-treated human T lymphocytes following addition of Con A to the culture medium

One of the more important and meaningful biochemical events occurring in the T lymphocyte mitogen-induced blastogenesis is the appearance of the receptors for IL2 and DR antigens on the plasma membrane of the stimulated immunocytes. Data from FACS analyses using MoAbs showed that after three days of culture both receptors appeared at very low density only on 20–30% of the T lymphocytes treated with SV-IV and activated by Con A (data not shown).

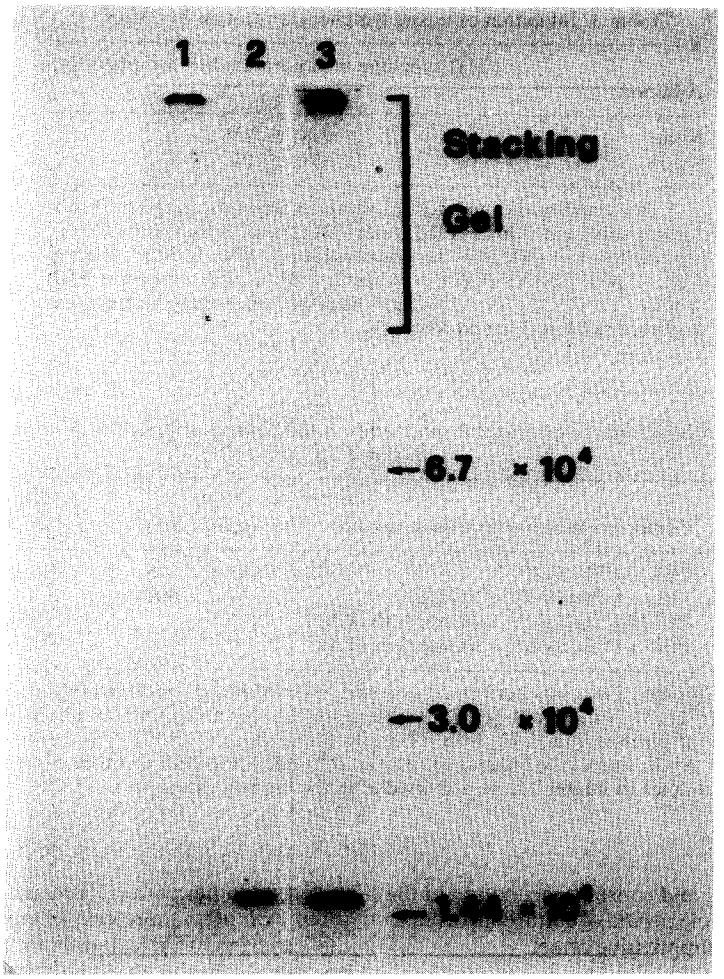

Fig. 1. Fluorography of the 15% polyacrylamide gel after SDS-electrophoresis showing the influence of TGase in the binding of (^{125}I) SV-IV to PBL. Radioactive SV-IV was incubated with PBL and $CaCl_2$, under the experimental conditions described in Materials and Methods, in the presence (lane 1) or absence (lane 2) of purified guinea-pig liver TGase. Lane 3 refers to the SV-IV binding assay performed after pretreatment of the radioactive protein with TGase and $CaCl_2$ (see Exp. 4 in Table 4). Equal volumes of samples were loaded onto the gel.

Primary structure of SV-IV is responsible for the generation of the immunosuppressive effect

The immunosuppressive properties of SV-IV were retained after boiling the protein for 15 min in 50 mM Tris–HCl at pH 7.5, whereas they were lost after trypsin digestion plus 15 min boiling (data not shown). A PBL sample treated with trypsin and without SV-IV was run at same time to rule out any effect of trypsin on PBL viability. These data strongly suggest that the immunosuppressive activity of SV-IV did not depend on peculiar tridimensional conformation of the protein, but more probably on specific amino acid sequence(s) located in particular segment(s) of SV-IV.

The effect of SV-IV on Con A-stimulated PBL is due to the binding of the protein to specific sites of PBL plasma membrane

The SV-IV-induced immunosuppressive effect in a Con A assay could be due to the binding of SV-IV to Con A specific binding sites on T lymphocytes. This possibility was ruled out because no difference was observed in the binding of fluoresceinated Con A to resting PBL before and after treatment (2 hr at 37°) of these cells with 100 μg/ml SV-IV (data not shown).

In addition, the immunosuppressive effect of SV-IV could be due to the formation of a homogeneous coat of SV-IV on the surface of T lymphocytes. This was also ruled out by the observation of unchanged binding of numerous specific fluorescent MoAbs (T_{11}, T_3, T_4, T_8, DR (MHC Class II), B_7, M_3, β_2-microglobulin, A-B-C (MHC Class I)) to human PBL before and after cell treatment for 2 hr at 37° with 100 μg/ml SV-IV (data not shown).

These results strongly suggest that the immunosuppressive effect is a consequence of the binding of the protein to specific sites of PBL plasma membrane.

SV-IV Binding to PBL is Ca^{2+}*- and TGase-dependent*

SV-IV is an effective TGase substrate [17] and

Table 5. Inhibition of carrageenin rat foot oedema by SV-IV administration

Treatment	Oedema area (cm^2)*	% of control
None (control)	7.62 ± 0.8 (10)	100
Dexamethasone, 1.0 mg/kg	4.03 ± 0.5 (5)	53
SV-IV, 0.5 mg/kg	5.98 ± 0.4 (5)	78
SV-IV, 1.0 mg/kg	4.67 ± 0.7 (5)	61
SV-IV, 1.5 mg/kg	3.48 ± 0.3 (5)	46

* Values are expressed as means ± SEM; the number of animals used in each group are reported in parentheses. For further details see Materials and Methods.

TGase is both produced and released in the extracellular environment by T lymphocytes and monocytes [30]. On the basis of these observations we performed experiments to measure the binding of radioactive SV-IV to intact human PBL in the presence or absence of purified guinea-pig liver TGase. The data shown in Table 4 clearly indicate that the binding of SV-IV to PBL required the presence of Ca^{2+} in the assay mixture. Calcium ions possibly serve the function of TGase cofactor in the production of TGase-dependent structural modifications of SV-IV, necessary for an effective binding of the protein to PBL surface. This point of view is supported by experiments showing that a marked stimulation of SV-IV binding was obtained when the protein was previously treated with Ca^{2+}-activated TGase (Table 4, Expts 3 and 4). The SDS-PAGE pattern shown in Fig. 1 indicates that (^{125}I) SV-IV was able to bind to PBL surface both as high molecular weight form(s) as well as low molecular weight form(s). The latter, most probably, are also TGase-modified molecular species. In fact, lane 3 in Fig. 1, shows that a marked binding of low molecular weight form(s) of SV-IV occurred in the absence of Ca^{2+}, but only if the protein was previously treated with active TGase. The binding of SV-IV, observed in the presence of Ca^{2+} but in the absence of exogenously added TGase, was probably a consequence of the structural modification of SV-IV by a TGase released from the immunocompetent cells in the medium. In conclusion, all these data seem to indicate that SV-IV acquired the ability to bind to PBL only after being structurally modified by a Ca^{2+}-activable TGase.

The protein SV-IV has anti-inflammatory properties in vivo

The test used to evaluate the anti-inflammatory properties of SV-IV was based on the ability of the substance to inhibit the intensity of the oedema induced in the rat hind limb by plantar injection of an appropriate volume of carrageenin suspension in saline. Table 5 shows that SV-IV, at a dose of 1.5 mg/kg, was able to decrease the carrageenin-induced oedema by about 54%. A similar result (47% inhibition) was obtained by plantar injection of dexamethasone at a dose of 1 mg/kg. SV-IV had a marked inhibitory effect on the carrageenin-induced oedema even when it was intravenously administered at a dose of 1.5 mg/kg. No effect was observed when

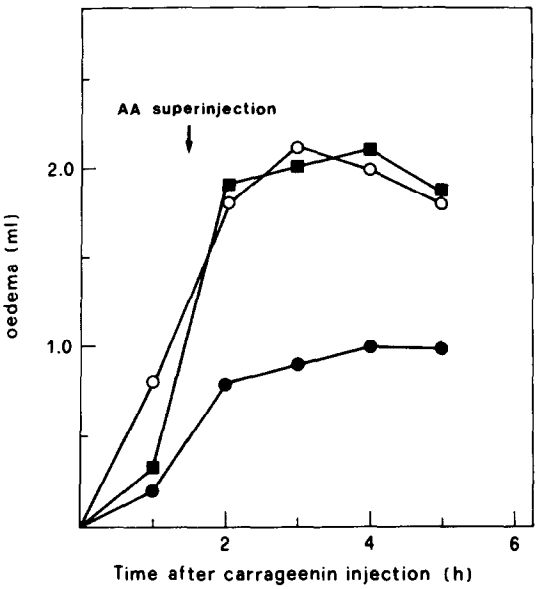

Fig. 2. Reversal of SV-IV inhibition on carrageenin oedema by arachidonic acid (AA) superinjection. Rats were injected with 0.1 ml of saline containing 1 mg of carrageenin alone (○——○) or plus SV-IV (1.5 mg/kg) (●——●). One group of animals was superinjected after 90 min with 0.1 ml of saline containing 0.1 mg of AA (■——■). Further experimental details are described in Materials and Methods.

SV-IV was injected into the animals in an attempt to inhibit a dextran-induced oedema (data not shown). A reversal of the inhibitory effect of SV-IV on carrageenin oedema was obtained by superinjection of arachidonic acid (Fig. 2).

The protein SV-IV has an inhibitory effect on PGE_2 synthesis and release from activated rat peritoneal leukocytes

The involvement of the arachidonic acid cascade in the anti-inflammatory mechanism of the protein SV-IV was investigated by determining the effect of SV-IV on the ability of the activated rat peritoneal leukocytes to synthesize and release PGE_2 in the surrounding medium. The data in Table 6 show that both dexamethasone (at a dose of 1 μg/ml) and SV-IV (at a dose of 130 μg/ml) possessed the ability to inhibit by 60% the synthesis and release of PGE_2.

Table 6. Inhibitory effect of SV-IV on PGE_2 release from rat peritoneal leukocytes phagocytosing killed bacteria and its reversal by arachidonic acid (AA)

Addition	μg/ml	ng $PGE_2/1 \times 10^6$ cells*	% of control
None (control)	—	14.7 ± 1.1 (10)	100
AA	1	14.9 ± 0.5 (3)	100
Dexamethasone	1	6.1 ± 0.6 (6)	42
SV-IV	0.5	13.8 ± 0.6 (3)	94
SV-IV	5	10.6 ± 0.2 (3)	72
SV-IV	30	8.3 ± 0.1 (3)	57
SV-IV	130	5.9 ± 0.3 (3)	40
SV-IV + AA	130 + 1	14.5 ± 0.4 (3)	100

* Values are expressed as means ± SEM; figures in parentheses represent the number of experiments. For further details see Materials and Methods.

Table 7. Effect of SV-IV on *in vitro* porcine pancreatic phospholipase A_2 activity*

Addition	PLA_2 activity ± SEM (nmoles of oleic acid released)	% of control
None	18.8 ± 0.2	100
SV-IV, 2 μM	18.9 ± 0.3	100
SV-IV, 10 μM	17.0 ± 0.2	90
SV-IV, 30 μM	15.5 ± 0.2	82
SV-IV, 40 μM	10.8 ± 0.2	57
SV-IV, 60 μM	8.3 ± 0.1	44
Bovine serum albumin, 15 μM	18.8 ± 0.2	100

* Experimental details are described in Materials and Methods.

The effect of SV-IV was, in addition, totally reverted by the simultaneous addition to the incubation medium of 1 μg/ml arachidonic acid.

SV-IV Inhibits PLA_2 activity assayed in vitro

The possibility exists that PLA_2 is the main target of the SV-IV-mediated inhibitory effect on PGE_2 synthesis and, as a consequence, of the anti-inflammatory property of the protein. This hypothesis was tested by investigating the effect of SV-IV on the porcine pancreatic PLA_2 activity assayed *in vitro*. The data in Table 7 clearly indicate that at a concentration of 60 μM SV-IV inhibited *in vitro* 56% of PLA_2 activity. Bovine serum albumin, used as a control, did not influence the activity of the enzyme.

DISCUSSION

In this paper we report for the first time that one of the major proteins secreted from the rat SV epithelium, namely SV-IV, possesses non-species-specific immunosuppressive activity and a marked anti-inflammatory property. The immunosuppressive efficiency of SV-IV in the human PBL system treated with different mitogens was highest with Con A and progressively lower with OKT_3, SEB and PHA, the effect with LPS or A23187-treated PBL being practically undetectable. The human PBL used in this study were constituted by a polymorphic population of immunocytes, whose major components were T lymphocytes, with B lymphocytes and monocytes representing only a small percentage of the total cells. The finding that the immunosuppressive effect was not detectable when PBL were treated with LPS (a mitogen known to stimulate only B lymphocytes) suggests that the T lymphocytes were, probably the main target of SV-IV. In addition, when SV-IV was added to PBL stimulated by the calcium ionophore A23187, no immunosuppressive effect was detectable. This finding, in our opinion, merely indicates that the binding of SV-IV to the PBL surfaces did not interfere with the ability of the ionophore to increase the concentration of free Ca^{2+} in the cytosol of the immunocompetent cells.

The experimental data are not sufficient to explain the mechanism of the diverse immunosuppressive effect of SV-IV observed with Con A, OKT_3, SEB and PHA. The same human PBL batch was used in all of the experiments, thereby excluding differences in PBL stimulability. Therefore, the difference in effect of the various mitogens could be related to: (i) biochemical and/or functional features of the single mitogens; (ii) differences in the sensitivity to SV-IV by the specific transduction mechanisms of the various mitogenic signals used; (iii) diversity of interference of SV-IV on the interactions between

macrophages and specific lymphocyte subsets in the presence of the different types of mitogen.

Another interesting observation was that, under all the experimental conditions used, the SV-IV immunosuppressive effect was never complete. This was probably due to the selective binding of SV-IV to certain cell subsets present in the T lymphocyte population.

We observed that treatment of SV-IV with PBL at 37° before mitogen addition appeared to be of crucial importance for the occurrence of the immunosuppressive effect. The effectiveness of this treatment was detectable only when the treatment lasted at least 1 hr. What exactly occurred during the preincubation period is not clear, although we do have some evidence that this time period allows TGase to structurally modify the SV-IV such that a substantial amount of it can bind to PBL surfaces. Moreover, preincubating the protein with exogenous purified TGase and Ca^{2+}, produced larger amounts of TGase-modified SV-IV forms and, consequently, SV-IV binding to PBL was more evident. Extensive washings of the cells after the pretreatment completely abolished the immunosuppressive effect, probably as a consequence of the removal of the free forms of SV-IV and of the rapid endocytosis of the protein already bound.

The disappearance of the immunosuppressive effect when very high concentrations of SV-IV were used is difficult to explain. Still unclear is also the molecular mechanism of the SV-IV immunosuppressive effect. At the moment we cannot rule out interference of SV-IV binding to PBL both with the plasma membrane signal transduction machinery controlling cell growth and differentiation, and with macrophage biological activity, producing appropriate permissive signals for lymphocyte, blastogenesis. The first hypothesis seems to be supported by the finding that SV-IV is able to markedly inhibit *in vitro* PLA_2, one of the enzymes believed to play a role in the membrane signal transduction machineries [31, 32]. After binding to specific sites of PBL plasma membrane, the protein SV-IV could produce the immunosuppressive effect by interfering in the early phase of the proliferative response of T lymphocytes to mitogens with a mechanism leading to decreased expression of either IL2 or IL2 receptors, or both. This hypothesis is supported by our preliminary findings of lack of expression of IL2 receptors on the surface of T cells treated with SV-IV and activated by Con A.

When SV-IV was added to the culture medium 24 or 72 hr after activation of PBL by mitogens, no immunosuppressive effect was detected. This result indicates that SV-IV is not able to interfere with the proliferation of T lymphocytes previously activated by the mitogens. We also exclude the possibility that SV-IV exerts its immunosuppressive effect by entering the cells via endocytosis. Blocking the enzymes involved in DNA synthesis should be ruled out because both the native protein and its TGase-modified forms are absolutely unable to interfere *in vitro* with the activity of the α-DNA polymerase/DNA primase complex, the major enzyme system involved in DNA replication. The inhibitory effect of SV-IV on the cell proliferation seems to be limited to mitogen-treated immunocompetent cells, since no antiproliferative effect was observed when the protein was added to other normal or transformed cells actively proliferating in culture.

Proteins immunorelated to SV-IV have been found in normal human seminal fluid both as a free form in seminal plasma and as a surface-associated form in the ejaculated sperm cells [15, 16]. In human seminal plasma we have recently shown also the presence of a variable but significant TGase activity [33]. In a preliminary study carried out on seminal fluids of men with idiopathic infertility, we have observed (unpublished observations): (1) decreased immunosuppressive activity of seminal plasma; (2) decreased levels of SV-IV-related antigens and TGase; (3) a marked increase in the number of immunocompetent cells; (4) oligospermia and/or asthenospermia; (5) a cytotoxic effect *in vitro* of the seminal lymphocytes on ejaculated spermatozoa obtained from healthy fertile donors. These observations point out the importance of the role played by immunosuppressive substances occurring in the seminal fluid for the structural and functional integrity of the ejaculated spermatozoa. These agents (such as SV-IV, UG, TGase, prostaglandins, endorphins, etc.) should keep the immunosurveillance system, present both in the male and female genital tracts, in a physiological condition that can guarantee the survival of the male gametes in these potentially dangerous anatomical regions.

The protein SV-IV also has marked anti-inflammatory activity. The mechanism of action underlying this effect involves the inhibition of PLA_2, which seems to be the main target of SV-IV anti-inflammatory activity. The inhibitory effect of SV-IV on PLA_2 produces an effective block of the whole arachidonic acid cascade, as demonstrated by the ability of the protein to produce a marked decrease in the synthesis of PGE_2, one of the more efficient mediator of the late phase of the inflammatory response. Similar inhibitory activity is shown by UG [34]. The hypothesis that PLA_2 was the main target of the anti-inflammatory effect of SV-IV is supported also by the observation that an appropriate administration of arachidonic acid completely reverted the inhibitory effect of SV-IV both on the inflammatory response to carrageenin *in vivo* and on the ability of activated peritoneal leukocytes to produce PGE_2 *in vitro*. Histamine release, one of the first steps in an acute inflammatory process, is not involved in the anti-inflammatory effect of SV-IV because this protein does not seem to inhibit a dextran-induced inflammatory reaction. Therefore, taking into account its pharmacological properties and the control of its synthesis by steroid hormones, the protein SV-IV can be considered a possible candidate for membership in the family of proteins named "lipocortins" [35].

By immunological techniques we have found SV-IV-related antigens in many tissues, secretions and cell culture media [6, 15, 16, 18 and unpublished observations]. UG-like immunoreactivity has also been found in many tissues and body fluids [31]. These two proteins (SV-IV and UG), in fact, share several biological and biochemical properties. Their widespread distribution suggests that these macro-

molecules can play a specific role as modulators of the tissue reactivity against the possible invasion of the organism by potentially harmful alien elements or macromolecules. Their biological activities and their concentrations, particularly high in male and female genital tracts, probably serve (i) to protect these regions against the aggression of dangerous nonself intruders, (ii) to avoid autoimmune responses in male and alloimmune responses in women against sperm auto- and allo-antigens, and (iii) to avoid massive release of inflammation mediators (in particular superoxide anions and free hydroxyl radicals) highly toxic and, therefore, harmful to the survival of the germ line. The possibility that alterations of finely balanced control mechanisms of these functions can give rise to various pathologies (such as infertility, chronic inflammation or tumours) in both the female and male genital tract is, in our opinion, something more than an interesting hypothesis.

Acknowledgements—This work has been supported by a CNR grant from Progetto Finalizzato "Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie", a CNR grant from Progetto Finalizzato "Oncologia", and a grant from the Italian Ministry of Education (40%). We are very grateful to Prof. H. G. Williams-Ashman for the critical reading of the manuscript.

REFERENCES

1. Hogart PJ, *Immunological Aspects of Mammalian Reproduction*. Blackie, Glasgow, 1982.
2. James K and Hargreave TB, Immunosuppression by seminal plasma and its possible clinical significance. *Immunology Today* **5**: 357–363, 1984.
3. Alexander NJ and Anderson DJ, Immunology of semen. *Fert Ster* **47**: 192–205, 1987.
4. Mukherjee DJ, Agrawal AK, Manjunath R and Mukherjee AB, Suppression of epididymal sperm antigenicity in the rabbit by uteroglobin and transglutaminase *in vitro*. *Science* **219**: 989–991, 1983.
5. Manjunath R, Chung SI and Mukherjee AB, Cross-linking of uteroglobin by transglutaminase. *Biochem Biophys Res Commun* **121**: 400–407, 1984.
6. Metafora S, Facchiano F, Facchiano A, Esposito C, Peluso G and Porta R, Homology between rabbit uteroglobin and the rat seminal vesicle sperm-binding protein: prediction of structural features of glutamine substrates for transglutaminase. *J Prot Chem* **6**: 353–359, 1987.
7. Ostrowski MC, Kistler MK and Kistler WS, Purification and cell-free synthesis of a major protein from rat seminal vesicle secretion. A potential marker for androgen action. *J Biol Chem* **254**: 383–390, 1979.
8. Pan Y-CE and S-L.Li S, Structure of secretory protein IV from rat seminal vesicles. *Int J Pept Prot Res* **20**: 177–187, 1982.
9. Abrescia P, Corbo L and Metafora S, Maturation in different translational systems of the protein RSV-IV secreted from the rat seminal vesicle epithelium. *Bull Mol Biol Med* **11**: 19–33, 1986.
10. Metafora S, Guardiola J, Paonessa G and Abrescia P, The 11s rat seminal vesicle mRNA directs the *in vitro* synthesis of two precursors of the major secretory protein IV. *Nucleic Acid Res* **12**: 1901–1910, 1984.
11. Mansson PE, Sugino A and Harris SE, Use of a cloned double stranded cDNA for a major androgen dependent protein in rat seminal vesicle secretion: the effect of testosterone in gene expression. *Nucleic Acid Res* **9**: 935–946, 1981.
12. McDonald C, Williams L, McTurck P, Fuller F, McIntosh E and Higgins S, Isolation and characterization of genes for androgen responsive secretory proteins of rat seminal vesicles. *Nucleic Acid Res* **11**: 917–930, 1983.
13. Kandala C, Kistler MK, Lawther RP and Kistler WS, Characterization of a genomic clone for rat seminal vesicle secretory protein IV. *Nucleic Acid Res* **11**: 3169–3186, 1983.
14. Harris SE, Mansson P-E, Tully DB and Burkhart B, Seminal vesicle secretion IV gene: allelic difference due to a series of 20 base-pair direct tandem repeats within an intron. *Proc Natl Acad Sci USA* **80**: 6460–6464, 1983.
15. Abrescia P, Lombardi G, De Rosa M, Quagliozzi L, Guardiola J and Metafora S, Identification and preliminary characterization of a sperm binding protein in normal human semen. *J Reprod Fertil* **73**: 71–77, 1985.
16. Metafora S, Lombardi G, De Rosa M, Quagliozzi L, Ravagnan G, Peluso G and Abrescia P, A protein family immunorelated to a sperm-binding protein and its regulation in human semen. *Gamete Res* **16**: 229–241, 1987.
17. Porta R, Esposito C, Persico P, Peluso G and Metafora S, Transglutaminase-catalyzed crosslinking of an immunosuppressive and anti-inflammatory protein secreted from the rat seminal vesicles. In *Advances in Post-translational Modification of Proteins and Aging* (Eds. Zappia V, Galetti P, Porta R and Wold F), pp. 153–160. Plenum Press, New York, 1988.
18. Metafora S, Peluso G, Ravagnan G, Gentile V, Fusco A and Porta R, Factors modulating immunocompatibility of spermatozoa: role of transglutaminase and SV-IV, one of the major proteins secreted from the rat seminal vesicle epithelium. In: *Morphological Basis of Human Reproductive Function* (Eds. Spera G and de Kretser DM) pp. 187–196. Plenum Press, New York, 1987.
19. Porta R, Peluso G, Esposito C, Fusco A, Gentile V and Metafora S, Suppression of the rat epididymal sperm antigenicity *in vitro* by transglutaminase and one of the major protein secreted from the rat seminal vesicle epithelium (SV-IV). *Ital J Biochem* **36**: 35A–37A, 1987.
20. Witkin SS, Suppressor T lymphocytes and cross-reactive sperm antigens in human semen. *AIDS Res* **1**: 339–346, 1984.
21. Zagury D, Bernard J, Leibowitch J, Safal B, Groupman JE, Feldman M, Sarngadharan MG and Gallo RC, HTLV-III in cells cultured from semen of two patients with AIDS. *Science* **22**: 449–451, 1984.
22. Nieto A, Postingl H and Beato M, Purification and quaternary structure of the hormonally induced protein uteroglobin. *Arch Biochem Biophys* **180**: 82–92, 1977.
23. Lowry OH, Rosebrough NY, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
24. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond)* **309**: 116–117, 1970.
25. Bonner WM and Laskey RA, A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur J Biochem* **46**: 83–88, 1974.
26. Grippo P, Locorotondo G and Caruso A, Characterization of the two major DNA polymerase activities in oocytes and eggs of Xenopus laevis. *FEBS Lett* **51**: 137–142, 1975.
27. Parente L, Ammendola G, Persico P and Di Rosa M, Glucocorticoids, prostaglandins and the inflammatory process. *Pol J Pharmacol Pharm* **30**: 141–155, 1978.
28. Blackwell GJ, Carnuccio A, Di Rosa M, Flower RJ, Parente L and Persico P, Macrocortin: a polypeptide causing the antiphospholipase effect of glucocorticoids.

Nature (Lond) **287**: 147–149, 1980.

29. Blackwell GJ, Carnuccio R, Di Rosa M. Flower RJ, Langham CSJ, Parente L, Persico P, Russell-Smith NC and Stone D, Glucocorticoids induce the formation and release of anti-inflammatory and anti-phospholipase proteins into the peritoneal cavity of the rat. *Br J Pharmac* **76**: 185–194, 1982.
30. Metafora S, Peluso G, Ravagnan G, Fusco A, Gentile V and Porta R, Implication of transglutaminase in mitogen-induced human lymphocyte blast transformation. In: *Advances in Post-translational Modification of Proteins and Aging* (Eds. Zappia V, Galletti P, Porta R and Wold F), pp. 175–184. Plenum Press, New York, 1988.
31. Miele L, Cordella-Miele E and Mukherjee AB, Uteroglobin: structure, molecular biology, and new perspectives on its function as a phospholipase A_2 inhibitor. *Endocrine Rev* **8**: 474–490, 1987.
32. Burgoyne RD, Cheek TR and O'Sullivan AJ, Receptor-activation of phospholipase A_2 cellular signalling. *Trends Biochem Sci* **12**: 332–333, 1987.
33. Porta R, Esposito C, De Santis A, Fusco A, Iannone M and Metafora S, Sperm maturation in human semen: role of transglutaminase-mediated reactions. *Biol Reprod* **35**: 965–970, 1986.
34. Levin SW, Butler J DeB, Schumaker UK, Wightman PD and Mukherjee AB, Uteroglobin inhibits phospholipase A_2 activity. *Life Sci* **38**: 1813–1819, 1986.
35. Di Rosa M, Flower RJ, Hirata F, Parente L and Russo-Marie F, Anti-phospholipase proteins. *Prostaglandins* **28**: 441–442, 1984.