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Suppression of Cell-mediated Immune Reactivity by Peptides Cleaved from Human Fibrinogen

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ABSTRACT

Peptides derived from fibrinogen, known from earlier studies to inhibit the stimulation of lymphocytes in vitro and to suppress the humoral immune response in vivo, were investigated for their effect on cell-mediated immune reactivity in mice. An unfractioned mixture of peptides with molecular weights under 3,500 injected intraperitoneally at repeated intervals suppressed the contact hypersensitivity to oxazolone but did not influence the skin inflammatory reaction to croton oil. Local injections of peptides had a stronger effect on contact hypersensitivity. Four 200 µg local injections of peptides prior to sensitization abolished the increase in lymph node weight and the uptake of ^{125}I-iododeoxyuridine in the draining lymph node after sensitization. Three previously isolated peptides with vasoactive effects inhibited Con A-stimulated incorporation of ^{3}H-thymidine into spleen cells. The first, a pentapeptide (Ala-Arg-Pro-Ala-Lys), and the second, an undecapeptide (Ser-Glu-Leu-Gln-Lys-Val-Pro-Pro-Glu-Trp-Lys) both with an enhancing effect on microvascular permeability, were more potent than the third, a pentapeptide with slight vasoconstrictive properties (Thr-Ser-Glu-Val-Lys). Cell viability was not altered, as measured by trypan blue exclusion and the release of ^{86}Rb. Accumulating evidence indicates that peptides derived from fibrin may be of importance as modulators of cellular immunoreactivity in a number of clinical conditions.

INTRODUCTION

Plasmin, the main fibrinolytic enzyme of mammalian blood, degrades fibrinogen and fibrin to a number of macromolecular fragments and subsequently to a large number of peptides of molecular weights varying between 500 and 15,000 daltons (22,31). Three of those peptides have been isolated from a mixture of low molecular weight fibrinogen degradation products (LMW-FDP) on the bases of their vasoactivity in rat skin, and called peptides 6A, 6D and 6E. Their primary structures have been established as 6A: Ala-Arg-Pro-Ala-Lys, 6D: Ser-Glu-Leu-Glu-Lys-Val-Pro-Pro-Glu-Trp-Lys and 6E: Thr-Ser-Glu-Val-Lys (5, 4). The two former peptides enhance microvascular permeability, while peptide 6E has been found to be vasoconstrictive (14).

LMW-FDP, accounting for about one third of the total mass of fibrinogen after exhaustive proteolysis by plasmin (31) separated by dialysis or ultrafiltration from the whole digest of fibrinogen have recently been reported to suppress the humoral response *in vivo* and to inhibit the stimulation of lymphocytes cultured *in vitro* (16, 21, 13, 18).

Accumulation of FDP in the circulating blood has been detected in the same pathophysiological states as have been associated with diminished immunoreactivity and the presence of serum immune suppressive factors, *e.g.* malignancies (24, 17, 20, 9), glomerular kidney diseases (28, 1), and organ graft rejection (26, 12). Such accumulation has also been found in pregnancy (19, 6). Small peptides with immunosuppressive properties have been detected in sera (17) and ascites fluid (3) from patients with cancer. Hence a contribution of an LMW-FDP to immunosuppression has been suggestid (16). Information is lacking, however, about the influence of LMW-FDP on cell mediated immune responses under *in vivo* conditions. The purpose of the present work was therefore to investigate the effects of LMW-FDP administered *in vivo* on cell-mediated immune reactions, using delayed cutaneous hyper-sensitivity to oxazolone in mice as a model system. In addition, the three fibrin derived peptides, 6A, 6D and 6E, of which 6A and 6D were earlier found to suppress lymphocyte prolifiration *in vitro*, have been investigated for their ability to influence on Con A stimulation of spleen cells.

MATERIALS AND METHODS

LMW-FDP were prepared as described previously from fibrinogen isolated from human citrated plasma (29) and were not contaminated with plasminogen or factor VIII (21). Two per cent fibrinogen was digested by plasmin overnight at room temperature at an enzyme to substrate protein ration of 1 to 100. Plasmin was obtained by activation of human plasminogen with streptokinase (both from AB Kabi, Stockholm, Sweden) at 37° C for 30 min., using 250 units of streptokinase per mg of plasminogen. The fibrinogen digest was dialysed against 10 volumes of distilled water and the dialysate was concentrated under vacuum to one-tenth of its initial volume. The concentration of LMW-FDP was calculated on the assumption that the peptides contained 16.5 % nitrogen. The concentrations of the final LMW-FDP preparations ranged from 20 to 35 mg/ml. LMW-FDP obtained in this manner were found to contain peptides with molecular weights below 3,500 judged from an analytical gel filtration on BioGel P-10 (29). Phosphate-buffered saline (PBS) or culture medium was used for adjusting LMW-FDP to the required concentrations.

Peptides 6A, 6D and 6E were isolated as described by Belew et al (5).

Mice: Eight- to 12-week-old Balb/c mice, weighing 19-21 g obtained from the State Institute of Hygiene, Warsaw, were used for all experiments. They were housed in plastic cages under standardized conditions (room temperature 22° C, relative humidity 55 %) and were kept on a standard diet and allowed water ad libitum.

Delayed cutaneous hypersensitivity to oxazolone was achieved according to the method of Asherson and Ptak (2). Mice (5 animals per group) were immunized with 0.1 ml of a 3 % solution of oxazolone in ethanol (2-ethoxymethylene-4-phenyloxazolone, BDH Chemicals Ltd., Poole, England) by painting this on the shaved abdominal skin. The thickness of each ear was measured with an Oditest micrometer (H.C. Kröplin GmbH, Göttingen, Germany) five days after sensitization and both sides of the ears were then smeared with 20 µl of oxazolone solution. The thickness of the ears was measured again 24 h after challenge, and the results are expressed as the mean increment in ear thickness ($\pm$SD) in units

of 10^{-3} cm. Control mice received the challenge alone.

Skin inflammatory reaction provoked by croton oil. This was evaluated in the same way as above 24 h after both sides of the ears had been smeared with a 10 % croton oil solution in acetone (Eastman Kodak Ltd., Rochester, USA).

Incorporation of ^{125}I UdR into lymph nodes draining the site of sensitization with oxazolone. Fluorodeoxyuridine (UdR) (Calbiochem Ltd., Hereford, England), 8 x 10^{-8} mol in 0.2 ml of PBS, was injected intraperitoneally (i.p.) followed in 10 min by an i.p. injection of ^{125}I-UdR (^{125}I-iodo-2-deoxyuridine, 1-6 mCi/mg, Radiochemical Centre, Amersham, Buckinghamshire, England), 1 µCi in 0.2 ml of PBS (23). The mice were killed by cervical dislocation 2 h later. Inguinal lymph nodes on the side of the body used for sensitization were excised, weighed and fixed in neutral formalin. Non-incorporated ^{125}I-UdR was washed out with several changes of ethanol, and radioactivity incorporated into lymph nodes was counted in a scintillation counter (Corumat 2700, ICN, Belgium). The radioactivity of ^{125}I-UdR used for each experiment was determined and compared with a standard of 10^6 cpm. The lymph node radioactivity was converted to this standard and is expressed as the mean cpm $\pm$ SD per lymph node from results obtained in five mice.

Incorporation of ^{3}H-TdR into cultures of mice spleen cells. Immediately after killing the spleens were removed and dissected with preparative needles at room temperature. Spleen cells were forced through a steel sieve, treated with 0.84 % ammonium chloride at room temperature an then washed and suspended in RPMI 1640 medium (Gibco Biocult Ltd., Scotland) supplemented with 10 % foetal calf serum (Gibco), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cell suspensions, 4 x 10^5 cells, were cultivated in 0.2 ml in a microculture system in an atmosphere of 5 % CO_2 in air for 72 h. Concanavalin A (Con A, Calbiochem Ltd., San Diego, Calif., USA), 1 µg per culture, was added as a stimulant. Eighteen hours before harvesting by a Dynatech automatic cell harvester, 1 µCi of ^{3}H-TdR (25 mCi/nmol, UVVR, Prague, Czechoslovakia) in 10 µl of medium was added. Incorporation of ^{3}H-TdR into acid-insoluble material was determined in a liquid scintillation spectrometer, using a Corumat 2700 counter. The

cell viability was assessed by a trypan blue exclusion test before cultivation, and suspensions containing over 85 % of viable cells were used for experiments. The viability was recheched before cell harvesting. Results are expressed as mean cpm $\pm$ SD for four to five cultures.

Release of ^{86}Rb from prelabelled spleen cells. Spleen cells prepared as above were washed three times in PBS at room temperature and incubated in PBS with the addition of ^{86}RbCl (135 mCi/g, IBJ, Poland), 5 µCi/ml, at 37° C for 1 h. After three washes with large volumes of PBS they were then resuspended in Parker's medium supplemented with 10 % foetal calf serum. To 0.1 ml of the labelled cell suspension (5 x 10^6 cells), 0.1 ml of peptide 6A, 6D or 6E in PBS was added. After 30 min incubation at 37° C, samples were centrifuged at 400 g at room temperature and the radioactivity of cell pellets and of supernatants was counted separately in a gamma scintillation counter. Four to five samples were examined in parallel. Radioactivity in the supernatant was calculated as per cent of the total ^{86}Rb incorporated, i.e. of the sum of ^{86}Rb radioactivity recovered in the cell pellet and supernatant.

RESULTS

Suppression of delayed cutaneous hypersensitivity to oxazolone by LMW-FDP

Fig. 1 shows scheme of the experiments in which the effects of i.p. or subcutaneous administration of LMW-FDP on hypersensitivity to oxazolone were investigated.

As seen in Table I, repeated i.p. injections of LMW-FDP in doses of 200 µg per mouse , a dose that enhances the progression of L-1 sarcoma in mice (27), prior to and after sensitization suppressed the contact hypersensitivity to oxazolone moderately but significantly. In contract, similar treatment with LMW-FDP did not influence the skin inflammatory reaction provoked by croton oil. The inhibitory effect of LMW-FDP on the cutaneous hypersensitivity to oxazolone was much more pronounced when peptides were injected in the area drained by the lymph node involved

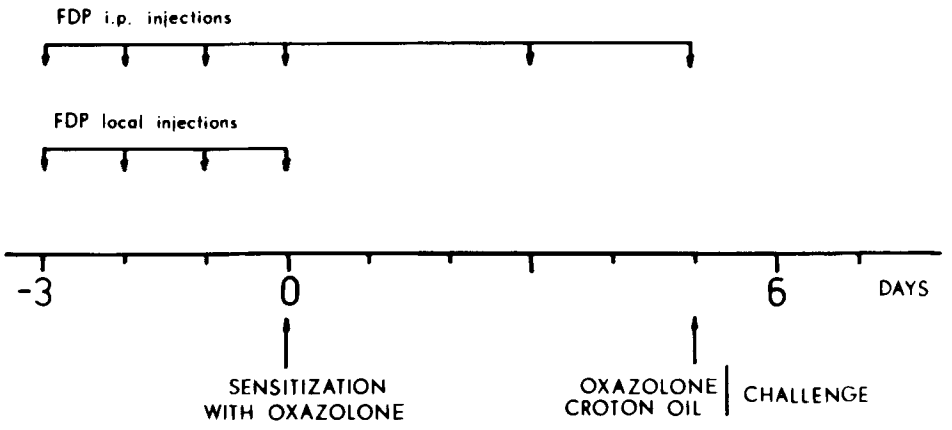

Fig. 1 Experimental design for the studies on suppression of delayed cutaneous hypersensitivity.

FDP i.p.: intraperitoneal injection of 200 µg of LMW-FDP in 0.1 ml of PBS.

FDP local: subcutaneous injection of 200 µg of LMW-FDP in 0.1 ml of PBS at the interior aspect of the thigh below the groin.

Table I. Effect of LMW-FDP on delayed cutaneous hypersensitivity to oxazolone and on the skin inflammatory reaction to croton oil. For dosage regimens, see Fig. 1 Mean values ($\pm$ S.D.) of the increment in ear thickness in units of 10^{-3} cm are given.

	Animals injected with		
Treatment	PBS i.p.	LMW-FDP i.p.	LMW-FDP locally
none	1.2 ± 1.1		
oxazolone	21.4 ± 2.8^{a}	15.5 ± 2.1^{x}	7.6 ± 1.4^{xx}
croton oil	19.9 ± 2.8	20.4 ± 2.3	

a: the response to oxazolone in controls treated s.c. with PBS was almost identical, i.e. 21.7 ± 2.4

x: $p < 0.05$

xx: $p < 0.001$

in the central phase of the response to the sensitizing agent. In animals treated in this manner with four daily doses of 200 µg of LMW-FDP, the contact hypersensitivity to oxazolone was reduced to 35 % of the value obtained in the control group injected with PBS.

Effect of LMW-FDP on the changes elicited in the central phase of the response in the lymph node draining the site of sensitization with oxazolone. Incorporation of i.p. injected ^{125}I-UdR into the lymph node draining the site of sensitization increased progressively after treatment of the skin with oxazolone (Fig. 2). The maximal value was observed on the third day and was 20 times as high as the corresponding value in non-sensitized animals. There was a parallel increase in lymph node weight, attaining a maximum on the 3rd and 4th day after sensitization, and the values exceeded control values in non-sensitized mice about five-fold. Four 200 µg doses of LMW-FDP injected locally, prior to sensitization, into the area drained by the same lymph nodes almost completely abolished the oxazolone-provoked incorporation of ^{125}I-UdR (Fig. 2) and increase in lymph node weight (not shown).

Effect of peptides 6A, 6D and 6E on stimulation of spleen cells by Con A. Increasing amounts of peptides 6A, 6D and 6E were added to spleen cell suspensions stimulated with Con A at the beginning of cultivation. All three peptides were found to inhibit Con A-stimulated incorporation of ^{3}H-UdR into spleen cells (Fig. 3). This inhibitory effect was dose-dependent and distinct at a concentration of 100 nmol of peptides 6A or 6D per ml of culture medium. A similar degree of inhibition was observed at a concentration of 400 nmol per ml for peptide 6E. In contrast, the cell viability evaluated by the trypan blue exclusion test was not changed by the continuous presence of peptide 6A, 6D or 6E in concentrations of up to 400 nmol per ml during the entire cultivation time. Neither did peptides in these concentrations induce any significant release of ^{86}Rb from prelabelled cell suspensions during a 30-min incubation period.

DISCUSSION

Low molecular weight degradation products from human fibrinogen

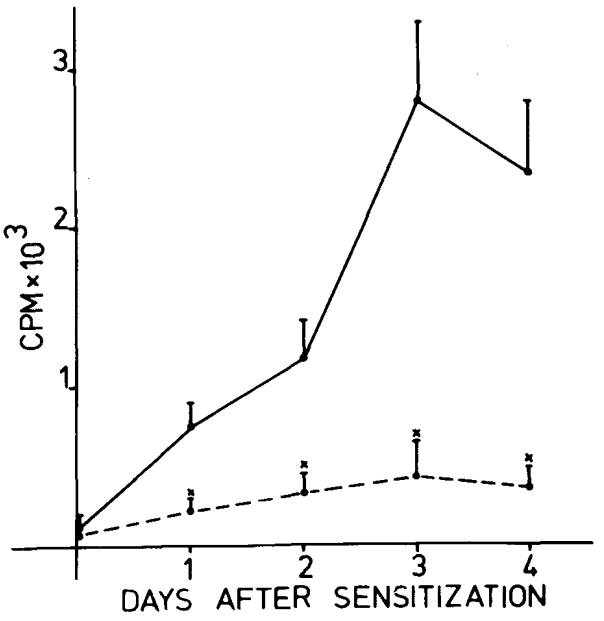

Fig. 2 Uptake of ^{125}I-UdR in the draining lymph node at various intervals after sensitization with oxazolone in mice treated with local injection of PBS (——) and of LMW-FDP (---). Results are expressed as means ± SD of cpm per lymph node from five mice. For dosage regimen of LMW-FDP, see Fig. 1.

x = $p < 0.05$.

have been reported to inhibit the humoral response in vivo to sheep red blood cells (SRBC) in mice (16,13). A suppression of cell-mediated immune reactions by LMW-FDP has been assumed. This is based on results which have shown their inhibitory effect in vitro on proliferation of lymphocytes stimulated by phytohaemagglutinin (16,21,13,18), Con A (18, 15), or allogenic leucocytes (13).

The suppressive effect appears to include as well human, mouse and rat cells (21, 15) and was not associated with cytotoxicity to cells in culture (16,21,13,15).

The results of this work indicate that human LMW-FDP suppress also a cell-mediated immune reaction in vivo. Products of fibrinogen proteolysis can affect immune reactions in vivo by a variety of mechanisms. They have been claimed to possess properties by which the non-specific inflammatory components of the immune reaction can be modulated (8, 7, 25). In the present study, the

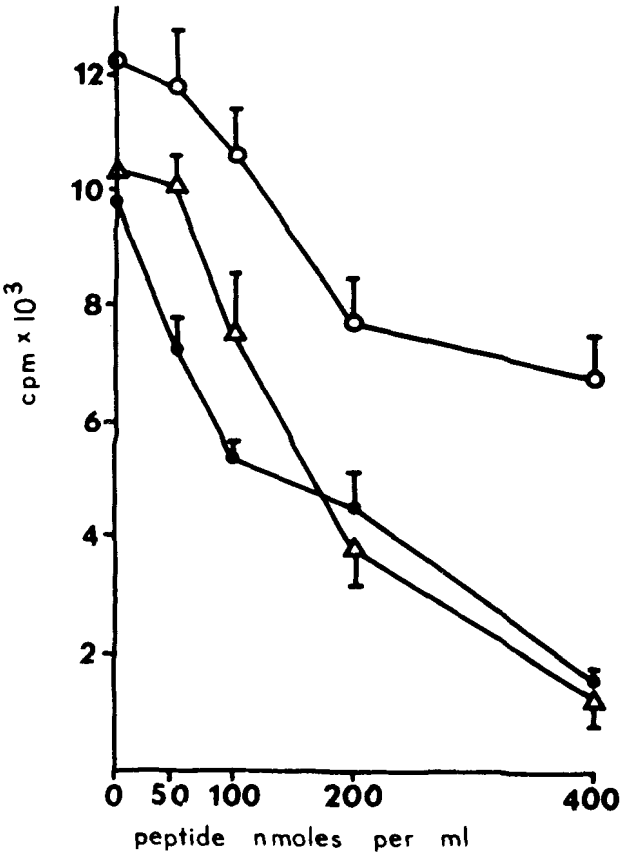

Fig. 3 Effects of peptides 6A (Δ), 6D (©) and 6E (o) on incorporation of ^{3}H-TdR into spleen cells after stimulation with Con A. Results are mean values ± SD obtained from four to five cultures.

inflammatory reaction provoked by croton oil appeared to be unaffected by LMW-FDP. They rather seem to impair the immune response in a more specific way judged from the inhibition in ^{125}I-UdR incorporation in the draining lymph nodes after sensitization of oxazolone. It could therefore be concluded that LMW-FDP inhibit the central proliferative phase of the response to the sensitizing agent. Local treatment of mice with LMW-FDP prior to sensitization was found to suppress the cutaneous hypersensitivity with greater potency than i.p. administration which might be explained by differences in the concentrations of active peptides reaching the lymphatic tissue involved in the immune response.

In most studies on the biological effects of LMW-FDP, unfrac-

tioned mixtures of peptides have been used, containing a variety of molecular peptides species, some perhaps with opposite biological effects. In the present study we compared the effects of three peptides of established primary structure isolated from the mixture of LMW-FDP. All of them inhibited the ^{3}H-TdR incorporation into stimulated mouse spleen cells in culture in a concentration-dependent manner. As shown in our previous investigation (25) we found that a significant inhibitory effect was exerted by peptides 6A and 6D in a concentration of 100 nmol per ml. Higher doses of peptide 6E (400 nmol/ml) were required for a similar effect. LMW-FDP at concentrations below 100 µg/ml suppress the stimulation of blood and spleen lymphocytes (21, 13, 18, 15). The minimal inhibitory concentrations of peptides 6A and 6D (100 nmol/ml) are about 50 and 100 µg/ml respectively. As those peptides constitute only a small precentage of LMW-FDP the contribution of these peptides to the immunosuppressive effect of the whole mixture of LMW-FDP in in vitro conditions is probably minute. However, this assumption is not necessarily true if the effects of peptides are synergistic and not additive. Limited availability of isolated peptides prevented us from investigating this alternative.

Peptide 6D is one of three small peptides released from the highly exposed middle region of the fibrinogen α-chain during the initial attack of plasmin (30). Peptide 6A is split upon limited proteolysis of fibrinogen from a region close to the N-terminal of the β-chain at a high rate (4). Under in vivo conditions degradation of fibrin(ogen) is limited by proteolytic enzyme inhibitors in plasma and body fluids. Thus, concentrations of peptides released at the initial or early stages of fibrinolysis can probably attain higher levels than those of products of far advanced proteolysis. For this reason it cannot be excluded that the contribution of peptides 6A and 6D to the mass and biological effect of LMW-FDP cleaved in vivo may differ from that evaluated on the basis of our experiments in vitro.

It is worth mentioning that a negative relationship has been demonstrated between the suppressive effects of LMW-FDP and the degree of lymphocyte stimulation in vitro. Specific antigenic stimulation of lymphocytes in vivo is usually much lower than that produced by non-specific mitogens in cell cultures. It is

therefore possible that the effects of peptides in vivo may be stronger than those observed in vitro.

Deposition of abundant amounts of fibrinogen-related material has been demonstrated in delayed cutaneous hypersensitivity (11) in kidney grafts during rejection (10) and many types of experimental and human malignant tumours (for review see 32). It has also been suggested that the fibrin deposited at the periphery of the tumour may facilitate tumour progression by forming a lattice which supports cell proliferation, provides nutrients and/or stimulants of growth, and protects the tumour from the defence mechanisms of the host (24, 32).

Cell mediated immune reactions play a major role in graft rejection and contribute to antitumour defence mechanisms. Previously published data and the results of the present report suggest that proteolysis of fibrinogen with liberation of immunosuppressive peptides can modulate both humoral and cell mediated immune reactions in vivo. Local generation of LMW-FDP, inhibiting the proliferative response to sensitization, may constitute a feed-back mechanism limiting progression of an immune reaction.

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REFERENCES

1. Ambrus, J.L., Balian, T., Ambrus, C.M., Mink, I.B. & Murphy, G.P.: Fibrin-fibrinogen degradation products in children with renal disease. N.Y. State J. Med. 74:1396-1402, 1974.
2. Asherson, G.L. & Ptak, W.: Contact skin hypersensitivity in the mouse. I. Active sensitization and passive transfer. Immunology 15:405-416, 1968.
3. Badger, A.M., Buehler, R.J. & Cooperband, S.R.: Immunosuppressive activity and tissue polypeptide antigen content of human ascitic fluids. Cancer Res. 38:3365-3370, 1978.

4. Belew, M., Gerdin, B., Lindeberg, G., Porath, J., Saldeen, T. & Wallin, R.: Structure-activity relationships of vasoactive peptides derived form fibrin or fibrinogen degraded by plasmin. Biochem. Biophys. Acta 621:169-178, 1980.

5. Belew, M., Gerdin, B., Porath, J, & Saldeen, T.: Isolation of vasoactive peptides from human fibrin and fibrinogen degraded by plasmin. Thrombos. Res. 13:983-994, 1978.

6. Bissenden, J.G., Lind, N.R. & Mackintosh, P.: Suppression of mixed lymphocyte reactions by pregnancy serum. Clin. Immunol. 39:195-202, 1980.

7. Buluk, K. & Malofiejew, M. The pharmacological properties of fibrinogen degradation products. Br. J. Pharmac. 35:76-89, 1969.

8. Buxzko, W.: Effect of fibrinogen degradation products on the rat blood vessels. Acta Physiol. Pol. 28:153-159, 1977.

9. Carlsson, S.: Fibrinogen degradation products in serum from patients with cancer. Acta Chir, Scand. 139:499-502, 1973.

10. Claes, G. & Svalander, C.: Studies on platelets and fibrin during rejection of canine renal allografts. Scand. J. Urol. Neprol. Suppl 10. 1972.

11. Colvin, R.B., & Dvorak, H.F.: Role of the clotting system in cell-mediated hypersensitivity. II. Kinetics of fibrinogen/fibrin accumulation and vascular permeability changes in tuberculin and cutaneous basophil hypersensitivity reaction. J. Immunol. 114:377-387, 1975.

12. Dobbelstein, H.: Immunmechanismen bei Urämie. Klin. Wschr. 53:463-466, 1975.

13. Edgington, T.S., Curtis, L.K. & Plow, E.G.: The immunosuppressive activity of plasmic degradation products of human fibrinogen. Thrombos. haemostas. 38:170, 1977.

14. Gerdin, B. & Saldeen, T.: Effect of fibrin degradation products on microvascular permeability. Thrombos. Res. 13: 995-1006, 1978.

15. Gerdin, B., Saldeen, T., Roszkowski, W., Szmigielski, S., Stachurska, J. & Kopéc, M.: Immunosuppressive effect of vasoactive peptides derived from human fibrinogen. Thrombos. Res. 18:461-468, 1980.

16. Girman, G., Pees, H., Schwarze, G. & Scheurlen, P.G.: Immunosuppression by micromolecular fibrinogen degradation products in cancer. Nature 259:399-401, 1976.

17. Glasgow, A.H., Nimberg, R.B., Menzoian, J.O. Saporoschetz, L. & Cooperband, S.R.: Association of anergy with an immunosuppressive peptide fraction in the serum of patients with cancer. New Engl. J. Med. 291:1263-1267, 1974.

18. Harvey, H.A., Albright, C. & Lipton, A.: The effect of fibrinogen degradation products on in vitro lymphocyte function. Thrombos. Haemostas. 38:226, 1977.

19. Hedner, U. & Nilsson, I.M.: Clinical experience with determination of fibrinogen degradation products. Acta Med. Scand. 189:471-477, 1971.

20. Izumi, T., Nagai, S. & Suginoshita, T.: Serum immunosuppres-

sion as a new tool for immunodiagnosis of lung cancer. Cancer Res. 40:444-447, 1980.

21. Krzystyniak, K., Stachurska, J., Ryzewski, J., Bykowska, K. & Kopeć, M.: Suppressive effect of low molecular weight fibrinogen degradation products on human and rat lymphocytes. Thrombos. Res. 12:523-530, 1978.

22. Marder, V.J. & Budzynzki, A.Z.: Data for defining fibrinogen and degradation products. Thrombos. Diathes. Haemorh. 33:199 207, 1975.

23. Pritchard, H. & Micklem, H.S.: Immune responses in congenitally thymus-less mice. I. Absence of response to oxazolone. Clin. Exp. Immunol. 10:151-161, 1972.

24. Rasche, H. & Dietrich, M.: Hemostatic abnormalities associatid with malignant diseases. Eur. J. Cancer 13:1053-1064, 1977.

25. Richardson, D.L., Pepper, D.S. & Kay, A.B.: Chemotoxis for human monocytes by fibrinogen-derived peptides. Brit. J. Haemat. 32:507-513, 1976.

26. Riggio, R.R., Schwartz, G.H., Bull, F.G., Stenzel, K.H. & Rubin, A.L.; α_2-globulins in renal graft rejection. Effects on in vitro lymphocyte function. Transplantation 8:689-694, 1969.

27. Roszkowski, W., Stachurska, J., Gerdin, B., Saldeen, T. & Kopeć, M.: Peptides cleaved from fibrinogen by plasmin enhance the progression of L-1 Sarcoma in BALB/c Mice. Eur. J. Cancer Clin. Oncol. 17:889-892, 1981.

28. Scheurlen, P.G., Baaka, M., Frey, N., Moers, P. & Sieberth, G.: Über Immunoinsuffizienz bei chronischen uremischen Nierenkrankungen. Dtsch Med. Wschr. 94:17-20, 1969.

29. Stachurska, J., Lopaciuk, S., Gerdin, B., Saldeen, T., Korościk, A. & Kopeć, M. Effects of proteolytic degradation products of human fibrinogen and of human Factor VIII on platelet aggregation and vascular permeability. Thrombos. Res. 15:663-672, 1979.

30. Takagi, T. & Doolittle, R.F. Amino acid sequence studies on the α-chain of human fibrinogen. Biochemistry 14:5149-5145, 1975.

31. Triantaphyllopoulos, E. & Triantaphyllopoulos, D.C. Fibrinolysis: the micromolecular derivatives. Brit. J. Haemat. 15: 337-343, 1968.

32. Zacharski, L.R., Henderson, W.G., Richles, F.R., Forman, W.B. Cornell, Jr. C.J. et al.: Rational and experimental design for the VA Cooperative Study of Anticoagulation (Warfarin) in the Treatment of Cancer. Cancer 44:632-741, 1979.

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