ROLE OF THE CLOTTING SYSTEM IN CELL-MEDIATED HYPERSENSITIVITY

I. Fibrin Deposition in Delayed Skin Reactions in Man*

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(Received for publication 24 May 1973)

Indirect evidence has implicated the coagulation system in the pathogenesis of cell-mediated hypersensitivity. Thus, anticoagulants have been shown to inhibit the expression of a variety of delayed-type reactions in vivo in animals including classic tuberculin skin hypersensitivity (1–5) and allergic contact dermatitis (2), the ocular reaction to tuberculin (6), and the antigen-induced macrophage disappearance reaction (1, 7). Moreover, a variety of agents that interfere with different steps in the clotting sequence has each been effective in suppressing delayed reactivity (1, 2, 4, 5). In studies with one such agent, heparin, suppression occurred under conditions that did not measurably inhibit the complement system (1, 2), the competence of sensitized lymphocytes to transfer cell-mediated hypersensitivity passively (2), or the anamnestic antibody response (2). Despite these data there has been reluctance to accept a role for the coagulation system in the expression of delayed hypersensitivity, and, in fact, no direct evidence has been presented for activation of clotting in the evolution of these reactions.

Our interest in this problem stems from the recent observation that substantial amounts of a fibrin-like material accumulate in the dermis in lesions of allergic contact dermatitis (8). We have pursued this observation with fluorescent antibody methods and here demonstrate that fibrin deposition is a prominent and consistent feature of both allergic contact dermatitis and classic delayed hypersensitivity skin reactions in man.

Materials and Methods

Subjects, Immunizations, and Skin Tests.—A total of 43 adult volunteers participated in this study and were divided into three experimental groups:

* This work was supported by U.S. Public Health Service Grants AI-10,496, AI-09529, GM-02212, AM-05297, and by General Research Support Funds.

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(*a*) 21 male volunteers, ages 18–51 yr, were sensitized to dinitrochlorobenzene (DNCB)[1] by application of 0.1 ml of a 10% acetone solution to the volar surface of the forearm. 12–14 days later contact reactions were elicited by application of 25 μl of a 0.1 or 0.2% DNCB solution in acetone or methyl Cellosolve (Union Carbide Corp., New York) to the lateral aspect of the upper arms. As many as six separate test sites were applied to facilitate sequential biopsy of undisturbed lesions. Reactions were read and biopsied at various intervals from 4 h to 13 days. In addition, each subject received a skin test application at the time of sensitization. This reaction was invariably negative clinically and was biopsied at 48 h, well before the onset of active immunity, as a control.

(*b*) Naturally acquired contact reactivity to various environmental allergens was studied in 12 male and female outpatients, ages 17–56 yr, of Dr. Normand Olivier. These patients had symptomatic contact dermatitis to a variety of allergens but were otherwise healthy and on no known medication at the time of testing. Allergens included nickel, 4; formaldehyde, 2; 1,4-benzenediamine, 2; kerosine, 1; lanolin, 1; epoxy, 1; and rubber, 1. All were applied as patch tests in the standard concentrations recommended by Fisher (9). Patch tests were removed at 48 h and were read and biopsied at 72 h.

(*c*) 14 male volunteers, ages 20–49 yr, including four who also participated in the DNCB study, were tested intradermally with a battery of skin test antigens including old tuberculin (OT) (1:1000), *Candida albicans* (1:1000), streptokinase (5 U)/streptodornase (1.25 U) (SK/SD), and mumps (0.1 mg). Tests were read at 4, 24, and 48 h. Biopsies were taken at 48 h from 20 clinically positive reactions (>10 mm erythema and induration), from five negative intradermal test sites, from four control sites injected with isotonic saline, and from seven untreated control sites.

Biopsy and Processing of Tissue for Microscopic Examination.—Tissue from a total of 111 (83 experimental and 28 control) biopsies was obtained with a 4 mm punch using Xylocaine field anesthesia (Astra Pharmaceutical Products, Inc., Worcester, Mass.). In this technique Xylocaine was injected intradermally around and subcutaneously below the site to be studied but never directly into the biopsied tissue. Hence, artifacts due to needle trauma were avoided. One half of each biopsy was fixed for 5 h in a mixture of paraformaldehyde-glutaraldehyde and was processed in Epon for the preparation of 1 μm, Giemsa-stained sections for light microscopy (8).

Immunofluorescence.—The other half of each biopsy was embedded in 7.5% gelatin and frozen in a dry ice-acetone slurry. Cryostat sections were washed 15 min in three changes of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.3 (PBS), and stained for 30 min with monospecific fluoresceinated goat or rabbit antisera to human fibrinogen/fibrin (Fib), IgG, IgM, IgA, IgE, β_{1C}/β_{1A} (C'3), and albumin (HSA) (Cappel Laboratories, Downingtown, Pa.) diluted 1:4 or 1:8 (10). After washing in PBS, sections were mounted in Elvanol (E. I. du Pont de Nemours and Co., Wilminton, Del.) (11) and examined in a Zeiss dark-field fluorescence microscope (Carl Zeiss, Inc., New York) equipped with a UG3 excitor filter and a 44 barrier filter to provide optimal color contrast between the green fluorescence of fluorescein and the blue autofluorescence of the dermal elastic fibers. The extent and overall intensity of Fib staining was graded 0 to 4+. Small but definite focal deposits were scored ±. Intermediate deposits were scored 1+ or 2+, and extensive, intense deposits 3+ or 4+. The activity and specificity of the fluoresceinated antisera were confirmed by double gel diffusion, immunoelectrophoresis, and inhibition by unlabeled specific antisera. Further, a washed fibrin clot, made from bovine thrombin-clotted human fibrinogen (Miles Laboratories, Inc., Kankakee, Ill.), completely absorbed the specific activity from the rabbit anti-Fib antiserum but did not diminish the staining capacity of anti-IgG.

Because antifibrinogen antibodies have specificity for fibrinogen, fibrin, and certain large fibrinogen fragments (12), the antigens detected with this antiserum will be collectively termed

[1] *Abbreviations used in this paper:* DNCB, dinitrochlorobenzene; Fib, fibrinogen/fibrin; HSA, human serum albumin; PBS, phosphate-buffered saline.

Fib, even though, as discussed below, fibrillar deposits in tissue that bind this antiserum certainly represent fibrin.

RESULTS

Gross and Light Microscopic Appearance.—The delayed reactions elicited with intradermal injections of protein antigens consisted of erythematous, indurated lesions that first became visible after a null period of 6–12 h and that achieved maximal intensity at 48 h. The reactions of allergic contact dermatitis exhibited less induration, were not maximal before 72 h, and were characterized by epidermal changes that included edema and vesiculation. In four of five instances, skin test with mumps virus elicited an early response of erythema and swelling maximal at 4 h that then receded by 8 h and that was followed subsequently by a typical delayed reaction. None of the other antigens studied produced this bimodal pattern of reactivity.

As studied in 1 μm light microscopic sections, all lesions were typical of delayed reactions and were characterized by perivascular accumulations of mononuclear cells that extended into the intervascular zones and infiltrated the epidermis as well (Fig. 1). The infiltrate of contact dermatitis was confined to the papillary and the superficial reticular dermis, in contrast to intradermal reactions to protein antigens in which the deeper layers of the dermis and sometimes the subcutis were involved as well. In addition, reactions of both types had prominent, loose, reticular deposits of fibrillar, fibrin-like material in the intervascular regions of the reticular dermis (Figs. 1 and 3). These were associated with collagen bundles and elastic fibers but not with infiltrating cells and spared both the perivascular cell accumulations and the vessels themselves.

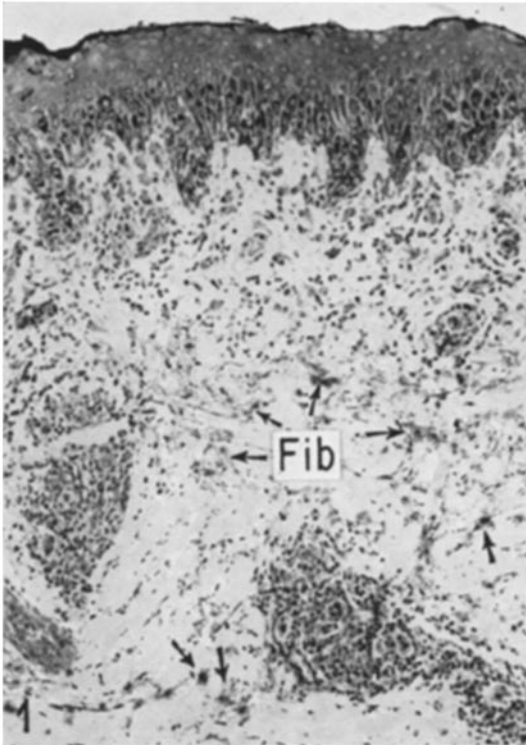
Photomicrographs of light microscopic and fluorescent antibody preparations of allergic contact dermatitis reactions to DNCB.

FIG. 1. Low-power field from a 72 h reaction. Mononuclear cells form cuffs about small veins and venules and are scattered in the intervascular zones. Fib deposits (*arrows*) are located primarily in the intervascular portion of the upper reticular dermis, but focal patches are seen deeper in the reticular dermis as well. × 110.

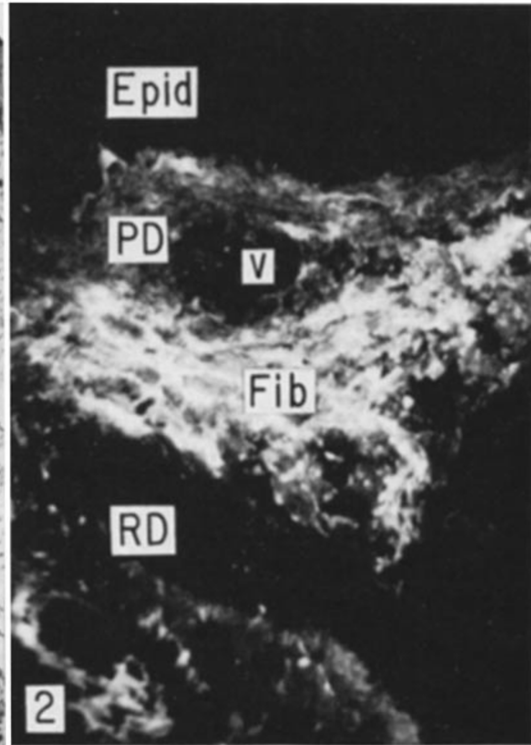
FIG. 2. Fib deposits as demonstrated by fluorescein-conjugated, rabbit antihuman fibrinogen/fibrin in a 24 h reaction to DNCB photographed at comparable magnification to Fig. 1. *Epid*, epidermis; *PD*, papillary dermis; *v*, negatively staining vessel with mononuclear cell cuff at junction between papillary and reticular dermis; *Fib*, fibrinogen/fibrin in upper reticular dermis; *RD*, midreticular dermis. Fib is deposited primarily in the upper reticular dermis but focal deposits are detected in the papillary dermis as well as deeper in the reticular dermis.

FIG. 3. High magnification photomicrograph illustrating morphology of Fib deposits in the upper reticular dermis of a 24 h reaction to DNCB. Fib is arranged as a loose meshwork interlacing between collagen bundles and forms striking associations with elastic fibers (*E*) that in some instances are coated with Fib. Scattered mononuclear cells are present but do not exhibit close associations with the Fib deposits. × 1,000.

FIG. 4. High magnification photomicrograph of a 72 h DNCB reaction stained with fluorescein-conjugated antihuman fibrinogen/fibrin for comparison with Fig. 3. Note fibrillar appearance of Fib meshwork. Two elastic fibers (*E*) exhibited blue autofluorescence but no specific staining except for small portions that are coated with Fib.



Fib
1



Epid
PD
V
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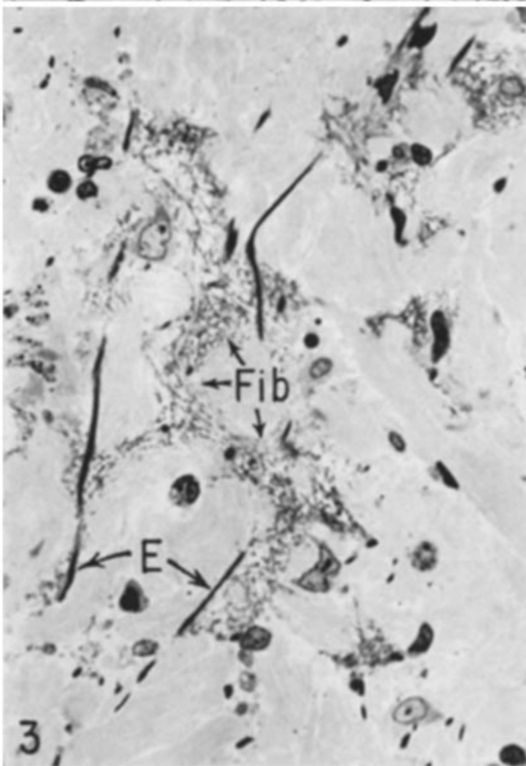
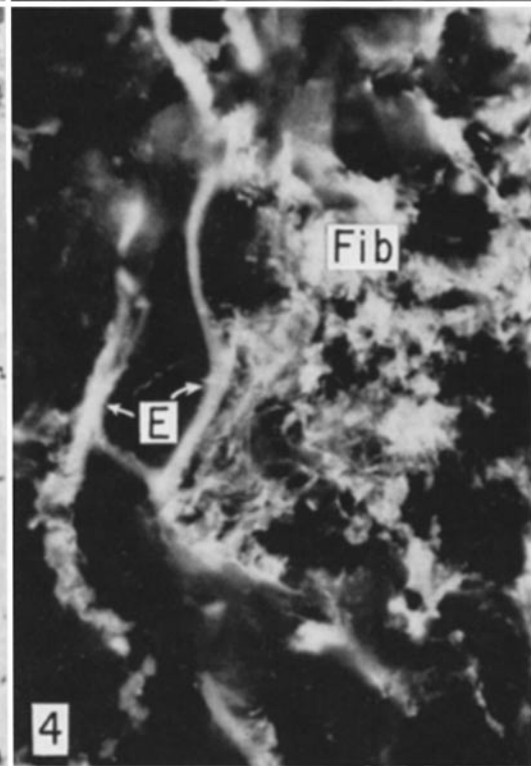
2



Fib
E
3



Fib
E
4

Similar deposits were sometimes observed in the papillary dermis of severe contact reactions but were not seen in this location in skin tests with protein antigens. Features of Arthus reactivity, such as vascular thrombosis, vasculitis, gross hemorrhage, or extensive neutrophil exudation, were not observed in any of these reactions.

Immunofluorescence: Fibrinogen/Fibrin (Fib).—Fully developed delayed reactions elicited with various contact allergens and protein antigens were characterized by extensive dermal deposits of Fib (Tables I and II, Figs. 2 and 4). These intensely staining deposits often appeared amorphous under low magnifications. However, under higher power they were clearly seen to consist of a delicate fibrillar meshwork interspersed between collagen bundles and coating elastic fibers (Fig. 4). Fib was most abundant in the intervascular portions of the superficial reticular dermis but characteristically spared the perivascular regions, which contained the highest density of infiltrating cells (Fig. 2). With rare exceptions (see below), Fib was not seen in blood vessel walls.

Although both allergic contact dermatitis and delayed reactions to protein antigens were characterized by prominent accumulations of Fib in the upper

TABLE I

Fib Distribution in Allergic Contact Dermatitis Reactions to DNCB and Environmental Contact Allergens at Various Times after Skin Test as Detected by Immunofluorescence

Skin test*	No. of subjects	Mean clinical score‡	Upper reticular dermis§				Papillary dermis§			
			0	±	1+/2+	3+/4+	0	±	1+/2+	3+/4+
I. DNCB										
(A) Sensitized										
4 h	5	0	2	3	—	—	5	—	—	—
8 h	7	0, 3 e/ei, 4	3	3	1	—	4	3	—	—
24 h	13	ei/eiv	1	2	3	7	2	1	6	4
48 h	5	eiv	—	—	—	5	—	—	3	2
72 h	8	eiv	—	—	2	6	1	1	6	—
6 days	5	eiv	—	—	3	2	3	—	1	1
11–13 days	8	e/ei	2	1	5	—	2	4	2	—
(B) Unsensitized										
8 h	3	0	3	—	—	—	3	—	—	—
48 h	9	0	9	—	—	—	9	—	—	—
II. Environmental allergens (combined)										
72 h	12	ei/eiv	2	3	4	3	3	5	3	1

* Conditions of skin test are recorded in Materials and Methods.

‡ Reactions were scored grossly as follows: 0, no reaction; e, erythema; i, induration; v, vesiculation.

§ Extent of staining after reaction with fluoresceinated rabbit antihuman fibrinogen/fibrin. 0, negative; ±, small, focal deposits only; 1+/2+, deposits of intermediate extent; 3+/4+, extensive intense deposits.

TABLE II

Fib Distribution in 48-h Delayed Hypersensitivity Reactions after Intradermal Challenge with Various Antigens as Detected by Immunofluorescence

Skin test*	No. of subjects	Mean clinical score‡	Reticular dermis§				Papillary dermis§			
			0	±	1+/2+	3+/4+	0	±	1+/2+	3+/4+
OT	5	18^{++}	—	—	2	3	2	2	1	—
Candida	7	17^{++}	—	—	4	3	6	1	—	—
SK/SD	3	22^{++}	—	—	1	2	1	1	1	—
Mumps	5	16^{++}	—	—	3	2	4	1	—	—
Negative tests‖	5	$6^{+/++}$	3	—	2	—	5	—	—	—
Saline	4	0	4	—	—	—	4	—	—	—
Normal skin	7	0	7	—	—	—	7	—	—	—

* Conditions of skin test are recorded in Materials and Methods.

‡ Reactions were scored as diameter of erythema (millimeters) and degree of induration (0 to +++).

§ Extent of staining after reaction with fluoresceinated rabbit antihuman fibrinogen/fibrin. Scoring same as in Table I.

‖ Negative 48-h skin reactions (<10 mm erythema and induration) to OT (2), SK/SD (1) and mumps (2).

reticular dermis, certain differences were noted with regard to the distribution of Fib elsewhere in these two types of reactions. In contact dermatitis Fib was frequently present in the papillary dermis (42 of 63 samples), but in only 1 of 63 biopsies were Fib deposits more extensive in the papillary than in the reticular dermis. In addition, focal fibrillar deposits of Fib were noted in epidermal vesicles. By contrast, in delayed reactions elicited with intradermal injections of protein antigens, Fib was less commonly present in the papillary dermis (focal deposits were found in 7 of 20 samples); however, patchy deposits of Fib were present deeper in the reticular dermis and these extended into the subcutis as well. Thus, in both types of lesion Fib was deposited in relation to areas of cellular infiltration but at the same time exhibited no close association with the infiltrating cells themselves (Fig. 5).

Fib deposits were a consistent feature of delayed reactions in man, and no significant differences in the amount of deposition could be related to the particular allergen or antigen employed (Tables I and II). Thus, Fib was found in the reticular dermis in 18, and in the papillary dermis in 16, of 20 reactions of allergic contact dermatitis studied at 72 h. In this group, Fib was absent from the reticular dermis only in weak reactions elicited with formalin and in the single case of kerosine allergy studied; in the latter instance, however, Fib was found in the papillary dermis. With regard to delayed reactions elicited by intradermal injection of protein antigens, Fib deposits were detected in the reticular dermis in all of 20 positive lesions studied at 48 h (5 OT; 7 *Candida*; 3 SK/SD; and 5 mumps).

The time-course of Fib accumulation was studied in serial biopsies taken

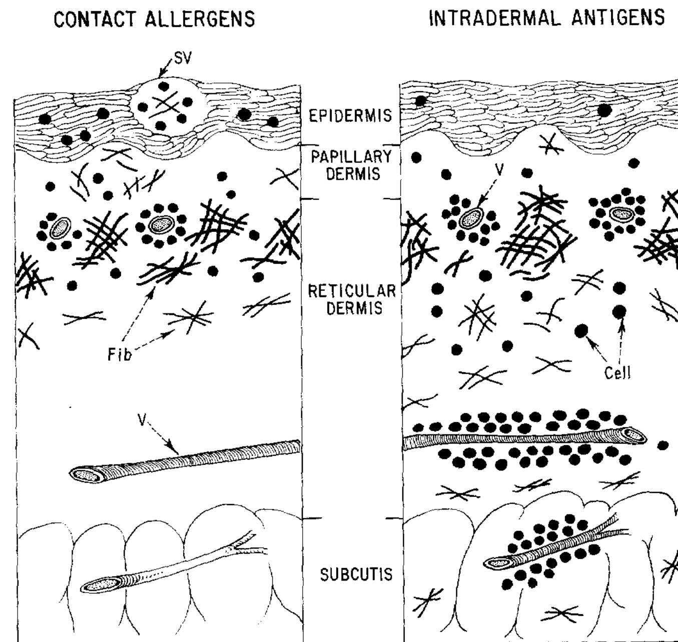


FIG. 5. Schematic diagram illustrating the distribution of Fib deposits in the lesions of allergic contact dermatitis and delayed hypersensitivity to intradermal antigens as detected both by immunofluorescence and by the 1 μm Epon techniques.

from volunteers sensitized and tested with DNCB (Table I). In 8 of 12 biopsies taken at 4 and 8 h after application of DNCB, isolated, focal deposits of Fib were seen, particularly around dermal appendages and occasionally in the papillary dermis. By 24 h Fib deposits had increased markedly and often extended throughout the upper reticular dermis. Maximal amounts of Fib were observed at 24–72 h, but even at 6 and 11 days after testing Fib was generally still detectable in the reticular dermis.

A striking feature of these reactions was the absence of detectable Fib in blood vessel walls. Such vascular wall deposition is characteristic of antibody-mediated reactions of the Arthus type (13–16). In only two instances out of 94 biopsies studied was significant vascular staining (1+) present in addition to intervascular Fib accumulation (one 24 h DNCB and one 72 h 1,4-benzenediamine contact reaction). In the latter instance, vessels positive for Fib were also stained by antisera with specificity for IgM, IgA, and C′3, and Epon sections revealed intravascular accumulations of neutrophils, suggesting an Arthus component. In an additional six biopsies faint Fib deposits were found in rare vessel walls, but the significance of these is uncertain.

Immunofluorescence: Immunoglobulins, C′3, and HSA.—No specific deposits of IgG, IgM, IgA, IgE, C′3, or HSA could be detected by immunofluorescence

in the skin test sites, aside from the single exception noted above. Nonspecific staining of the cytoplasm of occasional epidermal cells and the lining of vesicles in contact lesions and variable staining of granulocytes (17) occurred with all fluoresceinated antisera used including antirabbit IgG. Occasional dead or damaged epidermal cells were observed in 1 μm Epon sections in these reactions, and it is likely that the nonspecific epidermal staining was related to cell injury (18).

Controls.—12 biopsies of 48-h DNCB skin test sites in unsensitized individuals, 5 biopsies of negative skin reactions to protein antigens, 4 biopsies of 48-h saline test sites, and 7 biopsies of normal skin were studied (Tables I and II). Control DNCB test sites lacked erythema and Fib deposits but regularly exhibited a mild perivascular mononuclear cell infiltrate in Epon sections. Four of five "negative" intradermal test sites exhibited erythema (up to 7 mm diameter) and mild induration and all exhibited a mononuclear infiltrate; Fib deposits were found in two of these biopsies. Saline skin test sites and normal skin were negative by gross, microscopic, and fluorescent antibody criteria.

DISCUSSION

The data presented here indicate that extensive extravascular dermal deposits of fibrin are a distinctive and consistent feature of delayed hypersensitivity skin reactions in man and identify the fibrillar material observed in Epon-embedded, light microscopic sections of these reactions as fibrin. That the Fib detected was not unpolymerized fibrinogen is indicated by its fibrillar appearance and by its characteristic periodicity (19) in the electron microscope (A. M. Dvorak, unpublished data). The distribution of fibrin deposition—principally in the intervascular portions of the reticular dermis with sparing of vessels and the immediate perivascular space—is quite different from that described in antibody-mediated lesions in animals or man (13–16). These findings provide convincing direct evidence for activation of the coagulation system in the course of delayed reactions and complement earlier studies that demonstrated inhibition of cellular hypersensitivity by anticoagulants (1–7).

Previous limited immunofluorescence studies of delayed reactions have presented a confusing picture. Paronetto et al., in a preliminary investigation of guinea pig (14) and human (13) tuberculin reactions, reported fibrin deposits in and around blood vessel walls, in a similar distribution to that observed in Arthus reactions (14) and in vasculitis (13–16). In contrast, our own data on a large series of patients indicate that vascular and perivascular localization of fibrin is extremely rare (2 of 94 biopsies), whereas intervascular fibrin deposits, removed from vessels and their surrounding cuffs of lymphocytes, were identified in almost every reaction studied at the peak of its intensity (55 of 58 biopsies). In one of the two exceptional cases exhibiting significant fibrin deposits in blood vessel walls we also found deposits of immunoglobulin and C′3

in a similar distribution. Aside from this single case, and in general agreement with most other workers, immunoglobulin and C′3 deposits were not found in these delayed reactions (13, 14, 20). We conclude that vessel-associated deposits of fibrin are not an intrinsic part of cellular hypersensitivity but rather reflect activation of the clotting system secondary either to nonspecific vascular damage or to the presence of a complicating Arthus component.

The pathogenesis of fibrin deposition in delayed hypersensitivity reactions is not yet clear. Fibrin is ultimately derived from circulating fibrinogen, and its accumulation in delayed skin reactions provides evidence for locally increased vascular permeability (21), particularly involving the vessels of the upper reticular dermis where Fib deposition is most prominent. In fact, fibrinogen may provide an unusually sensitive measure of altered vascular permeability since, unlike other plasma proteins that contribute to the extracellular fluid, it may polymerize under appropriate circumstances to form an insoluble product that serves as an enduring permeability indicator. The characteristic pattern of fibrin deposition in the intervascular portions of the upper reticular dermis is unusual and, to our knowledge, has not yet been described in other skin disorders (16, 22–25).

Polymerization of extravascular fibrinogen in delayed hypersensitivity reactions could be initiated in several ways that might involve either the intrinsic or extrinsic coagulation pathways. That fibrin deposition is not simply a consequence of tissue injury (such as trauma from intradermal skin testing, biopsy artifact, or severe reactions with associated necrosis) is indicated 1— by its deposition in allergic contact dermatitis after epicutaneous application of allergen; 2— by its appearance as early as 4–8 h after testing with DNCB in a majority of sensitized subjects (Table I); 3— by its absence from a variety of control test sites (Tables I and II). The distinctive anatomic associations between fibrin and elastic fibers and collagen suggest that these may have a role in initiating the clotting sequence, and such a role has in fact been claimed for collagen (26, 27). It is possible, therefore, that fibrin deposition in delayed hypersensitivity is merely a necessary consequence of enhanced vascular permeability that allows increased leakage of fibrinogen into the interstitial space where connective tissue elements initiate clotting. Alternatively, coagulation could be triggered by a product of sensitized lymphocytes (28), or these cells could exert their effect less directly, as by interfering with the fibrinolytic mechanism. Relevant to these hypotheses is the demonstration that fibrinolytic activity is concentrated about small cutaneous blood vessels (29, 30); that a number of inflammatory reactions of the skin are characterized by a measurable reduction in vessel-associated fibrinolytic activity (20, 31); and that fibrin deposits spare those portions of the dermis (the perivascular zones) where lymphocytes, the fibrinolytic mechanism, and presumably fibrinogen are present in highest concentration. The possibility of an antibody-mediated activation of the clotting system is difficult to exclude with certainty, although

the absence of detectable immunoglobulins and the observed pattern of fibrin distribution argue against it. Moreover, similar fibrin deposits have been found in delayed reactions in guinea pigs sensitized so as to avoid antibody production (R. B. Colvin and H. F. Dvorak, unpublished data).

The significance of fibrin deposition in delayed-type hypersensitivity is uncertain, but fibrin could contribute to the pathogenesis of these reactions in at least two ways. On the one hand, initiation of clotting by thrombin involves the splitting off of two small bioactive peptides from fibrinogen monomer (31) and subsequent limited proteolysis of fibrinogen or fibrin by plasmin yields other biologically potent fragments (31). The phaımacologic properties of these fibrinogen/fibrin derivatives are as yet poorly characterized, particularly in man, but include potentiation of bradykinin-induced smooth muscle contraction (32), increased vascular permeability (33, 34), and chemotaxis of granulocytes (35, 36). Finally, insertion of a loose meshwork of long chain, insoluble fibrin polymer into the connective tissue matrix could lead to an expansion of the extravascular space with accumulation of water and plasma proteins. This, in turn, could explain the "swollen" appearance of the dermal collagen in delayed reactions studied with conventional histologic methods in which delicate fibrin strands may be difficult to visualize against a background of dense collagen fibers (37). Such accumulations of fluid might contribute significantly to the induration that is a characteristic feature of delayed hypersensitivity reactions.

SUMMARY

The expression of delayed-type hypersensitivity in animals has been inhibited by a variety of anticoagulants, but direct evidence for activation of clotting in the evolution of these reactions has been lacking. Using the fluorescent antibody technique we here demonstrate that fibrin deposition is a prominent and consistent feature of both allergic contact dermatitis and classic delayed hypersensitivity skin reactions in man. Fib was detected in 55 of 58 delayed reactions studied at the peak of their intensity. The characteristic distribution of Fib—principally in the intervascular portions of the reticular dermis with sparing of vessels and their associated cuffs of mononuclear cells—is unusual and quite different from that described in antibody-mediated lesions in animals or man. Fib was found in vessel walls in only 2 of 94 biopsies studied. With a single exception, deposition of immunoglobulins and complement was not observed.

The pathogensis and significance of Fib deposition in these reactions are not yet clear. Fib is ultimately derived from circulating fibrinogen, and its accumulation provides additional evidence for locally increased vascular permeability in delayed hypersensitivity. Polymerization of extravascular fibrinogen could be triggered nonspecifically by dermal elements (e.g., collagen) or by a product of sensitized lymphocytes. The appearance of Fib early in the development of these reactions (4–8 h after epicutaneous test with DNCB) and inhibition

studies with anticoagulants together suggest that clotting may have a role in their pathogenesis, possibly by the release of bioactive peptides from fibrinogen/fibrin or by contributing to the induration characteristic of delayed hypersensitivity.

The authors gratefully acknowledge the expert technical assistance of Eleanor J. Manseau.

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