IN VITRO GRANULOCYTE ADHERENCE AND IN VIVO MARGINATION: TWO ASSOCIATED COMPLEMENT-DEPENDENT FUNCTIONS

Studies Based on the Acute Neutropenia of Filtration Leukophoresis*

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Granulocytes, to escape from the blood stream, must presumably adhere to the vascular wall before they enter the tissue by diapedesis and chemotaxis. Although such adherence seems essential to the early inflammatory process, the accumulated knowledge concerning the mechanisms of leukocyte emigration concentrates predominantly on motility and chemotaxis (1–3); the process of adherence of leukocytes to the endothelial surface has been less well defined and generally only at the level of light and electron microscopy (2, 4). Investigations utilizing transparent vessels in living animals indicate that leukocyte adhesion without emigration may be the only inflammatory reaction after minimal tissue damage – an observation clearly separating the process of adherence from chemotaxis. Moreover, the finding that temporary leukocyte attractions to undamaged endothelial walls occur regularly (4) suggests that adherence may also play an as yet undefined role in normal vascular physiology.

Since the development of methods of labeling blood granulocytes as with $DF^{32}P$, the existence of so-called marginated neutrophils (5), representing about one-half of all intravascular granulocytes, has become apparent. These cells exchange rapidly with freely circulating ones. An attractive hypothesis is that margination is a function of cell stickiness. In the present studies we have investigated this possibility by studying a situation in which profound neutropenia due to excessive margination occurs – the transient neutropenia which occurs in donors undergoing continuous flow filtration leukophoresis (CFFL) (6).[1] This neutropenia is virtually identical to that noted in uremic patients at the beginning of hemodialysis – another extracorporeal perfusion system (7, 8). In the latter situation, we have recently demonstrated that almost the total

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[1] *Abbreviations used in this paper:* AGG, aggregated gamma globulin; C, complement; CFFL, continuous flow filtration leukophoresis; CVF, cobra venom factor; EDTA, ethylenediaminetetraacetic acid; HBSS, Hank's balanced salt solution; MPO, myeloperoxidase; NF, nylon fibers; PBS, phosphate-buffered saline.

circulating granulocyte pool transiently sequesters in the lung (9), and does so in response to the infusion of complement (C) components activated by the dialysis coil cellophane (10). This has suggested to us that margination may somehow involve C-induced granulocyte adhesiveness. In the present studies we provide supportive evidence that the C system may play a central role in granulocyte adherence and, moreover, that granulocyte margination in vivo, and adhesiveness assayed in vitro, are closely related phenomena.

Materials and Methods

Human Studies. CFFL was performed as previously described (11). Blood cell counts were done on ethylenediaminetetraacetic acid (EDTA)-anti-coagulated samples drawn from the outline proximal to the filter system. Erythrocyte and leukocyte counts were done on a Coulter model S Counter (Coulter Electronics, Inc., Hialeah, Fla.) and differential counts were obtained from Wright-stained slides. Platelets were counted directly by phase contrast microscopy by using procaine HCl, 3.5 g per 100 ml, as a diluent.

Rabbit Studies. Pentothal-anesthesized male and female New Zealand White rabbits weighing 2.5–4.0 kg were prepared with sterile polyethylene cannulae in femoral or jugular veins for the infusion of plasma or other agents, and in femoral or carotid arteries for blood sampling. Fresh autologous cell-free plasma was obtained by collecting 35 ml blood into ice-cooled plastic syringes; after transfer into precooled plastic tubes and centrifugation at 4°C for 7 min at 20,000 *g*, the supernatant plasma was immediately decanted and anti-coagulated with 3 U/ml sodium heparin (Upjohn Co., Kalamazoo, Mich.). 20 ml of such plasma was incubated in a sterile polystyrene tube with 1 g of nylon fibers (NF) from a Fenwal filtration leukophoresis set system (Fenwal Laboratories, Inc., Morton Grove, Ill.) or with other specified agents; after removal of the perturbant, the activated plasma was reinfused over a 2-min period. Blood for leukocyte and differential counts was sampled at various intervals thereafter.

Complements Assays and Immunoreactants. Total complement (CH50) and C1 and C3 activities were assayed by standard hemolytic assays (12). Radial immunodiffusion, performed with commercial plates (Cordis Laboratories, Miami, Fla.), was also used to assay C3. Anticomplementary cobra venom factor (CVF) was isolated from lyophilized *Naja naja* cobra venom (Ross Allen's Reptile Institute, Inc., Silver Springs, Fla.) by diethylaminoethyl-cellulose column chromatography (13). Complement depletion in approximately 4-kg rabbits was produced by injecting intravenously 700 U of CVF 24 and again 12 h before experimental manipulation (13). Zymosan (Sigma Chemical Co., St. Louis, Mo.) in 0.9% saline was kept in a boiling waterbath for 30 min, washed twice, and resuspended at 50 mg/ml in phosphate-buffered saline (PBS) of pH 7.4 containing 1 mM magnesium. Plasma was activated by incubation for 30 min at 37°C with 5 mg zymosan/ml, followed by removal of zymosan by sedimentation at 20,000 *g* for 10 min. Inulin (Sigma Chemical Co.), at the same concentration, was sonicated instead of boiled and then used identically as zymosan. Aggregated gamma globulin (AGG) was prepared by diluting human gamma globulin (Swiss Red Cross, Berne, Switzerland) in PBS, pH 7.4, at a concentration of 50 mg/ml, heating at 63°C for 30 min, and washing the precipitate twice in PBS. The final concentration used to activate the C system in plasma was 5 mg/ml.

Hydrazine Treatment of Plasma. A 200-mM solution of hydrazine (Fisher Scientific Co., Fair Lawn, N. J.), freshly prepared in saline of pH corrected to 7.5 with 2 N HCl, was added to plasma to yield a final concentration of 10 mM hydrazine, and then incubated in a tumbling tube for 30 min at 37°C. Subsequently, the plasma was dialyzed at 4°C for 2 h against PBS, pH 7.4, and for 20 min against Hank's balanced salt solution (HBSS) before use.

Ultrafiltration of Activated Serum. Ultrafiltration membranes (Diaflo) and magnetically stirred cells from Amicon Corp., Scientific System Div., Lexington, Mass., were used. Serum, chosen to avoid precipitation of fibrinogen on the membranes, was prepared from cell-free plasma by immediate centrifugation of rabbit whole blood at 4°C for 6 min, followed by incubation at room temperature for 30 min and a further 45 min at 4°C. 20 ml serum incubated for 30 min with 1 g of NF at 37°C was subsequently ultrafiltrated with different membranes at 4°C. With membranes of mol wt cutoff limits lower than 50,000, repeated filtration and redilution with PBS, pH 7.4, was

found to be essential. The filtrates, after concentration by a UM 2 membrane (mol wt cutoff limit 1,000), were injected in a volume of 18–20 ml to mimic the experiments with neat plasma.

Granulocyte Adherence Assay. Leukocyte suspensions, prepared by our previously described technique (9, 10) were washed twice in HBSS (Microbiological Associates, Bethesda, Md.), and resuspended in HBSS at 4×10^6 granulocytes/ml. For quantitation of adherence to duplicate Petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.; tissue culture dish 3,001), 2.5 ml of the granulocyte suspension was pelleted and then resuspended in 2.5 ml of autologous plasma which had been treated in a variety of ways (e.g. heat-inactivated, fresh, inulin-activated etc.). After incubation for 5 min at 37°C, two 1-ml-samples were transferred into the duplicate Petri dishes which were incubated at 37°C for 30 min in a water-jacketed CO_2-incubator and subsequently washed in a sequence of six beakers with 0.9% saline and dried for 2 h in a refrigerator. The myeloperoxidase (MPO) content of adherent cells of each dish was extracted into 1 ml of 1.5 M NaCl (14) containing 0.5% Triton X-100 (Calbiochem, San Diego, Calif.) by freeze-thawing four times. The extraction fluid was assayed for MPO activity by the technique of Lundquist and Josefsson (15). This assay showed a linear relationship between the number of granulocytes assayed and the initial velocity of the enzyme activity between 1×10^4 and 30×10^4 cells. Thus, appropriate dilutions of the experimental samples were made to encompass this range. Pure plasma (90–100%) was chosen as suspension medium representing the most physiologic milieu, and because diluting the plasma from 80 to 25% resulted in a progressively decreasing adherence of cells, possibly revealing physiologic inhibitors, a finding which has been reported for chemotaxis (16). Below 25% plasma concentration, an unspecific protein-dependent increment in cell adherence was found. The sensitivity of the MPO method is in the range of 0.5 ng purified horse radish peroxidase and the activity of 50 ng of this peroxidase corresponded to the activity extracted from 1×10^5 granulocytes. By knowledge of the number of cells put on dishes and their initial MPO activity, the absolute number of adherent cells remaining after washing could be determined. The variability of the MPO assay was less than 3% and the variability between duplicate dishes less than 6%.

Results

The Transient Leukopenia of CFFL. The sequence of leukocyte counts in four normal donors undergoing CFFL confirms recent observations by others (6), in that a parallel drop of neutrophils and monocytes occurs within 5–10 min after onset of the procedure (Fig. 1). Shortly thereafter, rebound granulocytosis with an increase in band forms occurs while monocytes return more sluggishly. Lymphocytes and thrombocyte counts do not significantly change, and the results correspond exactly with previously reported ones in patients undergoing hemodialysis (8–10).

Characterization of the Mediator of NF-Induced Neutropenia in Rabbits. By analogy to our studies in hemodialysis neutropenia (10), the possibility was analyzed that C activation by the NF of the filter system might be responsible for the profound changes in neutrophil kinetics. As shown in Table I, when 18–20 ml autologous plasma was incubated with NF at 37°C for 30 min and reinfused into rabbits over 2 min, a precipitous fall of circulating granulocytes to nearly zero was always observed; cells returned to circulation within 20–30 min, but only after the infusion was stopped. Continuing the infusion resulted in prolonged neutropenia, and repeated injections over a short time produced identical episodes of granulocytopenia. There was no difference in the reaction if untreated NF or those boiled and washed for prolonged periods were used. The temperature at which NF/plasma incubation was performed also did not seem critical, as 22°C incubations produced identical neutropenia to that noted at 37°C.

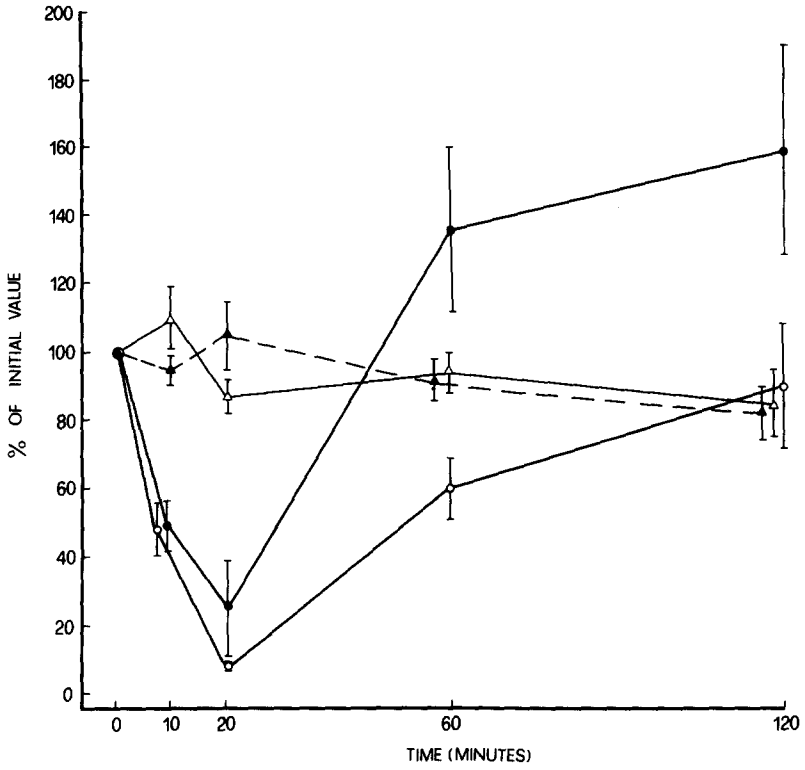

FIG. 1. The leukopenia in four donors during CFFL. Bars denote mean and range. ●—● neutrophils; ○—○ monocytes; △—△ lymphocytes; ▲---▲ thrombocytes.

TABLE I

Characterization of the Granulocytopenia-Inducing Factor in Rabbit

Treatment of or addition to plasma before NF incubation	*n*	Circulating granulocytes after injection of NF-incubated plasma (percent of initial level)					
		0 min	2.5 min	5 min	10 min	20 min	60 min
Untreated (37°C)	4	100	0.5 ± 0.5	3 ± 2	47 ± 13	83 ± 13	136 ± 17
Untreated (22°C)	2	100	0	4 ± 1	43 ± 5	150 ± 30	140 ± 20
56°C 30 min	4	100	89 ± 3	96 ± 7	104 ± 9	100 ± 4	103 ± 12
50°C 30 min	2	100	1 ± 0.5	0	16 ± 5	90 ± 15	150 ± 20
EDTA, 10 mM	3	100	88 ± 4	84 ± 6	94 ± 8	97 ± 10	120 ± 10

Results are means and ± SD, or means and range when less than three experiments were done. Duration of incubation of plasma with NF: 30 min 37°C, except where otherwise stated. Amount of plasma injected: 18-20 ml.

In ancillary studies (not shown) human plasma, incubated in vitro with NF at 37°C for 30 min, lost only 10% of total hemolytic complement, 25% of C1, and 15% of C3 (although C3 levels were not detectably altered when assayed by radial immunodiffusion). The same parameters monitored in human donors undergoing CFFL also were not unequivocally or consistently altered. Since these results did not conclusively demonstrate an association between C activation

and the transient leukocyte sequestration process, indirect ways were chosen to validate such an association. Thus, prevention of C activation by chelation of Ca^{++} and Mg^{++} with EDTA was found to block the neutropenic response (Table I). Moreover, heat (56°C) inactivation of rabbit plasma before NF exposure also prevented the neutropenia. In contrast, preheating plasma at 50°C, a procedure which blocks the alternative pathway of C activation by destroying C_3 proactivator, but which spares the enzyme activities of the classical pathway (17, 18), was not inhibitory, but even slightly prolonged the neutropenic response. Recombination experiments (19, 20) also supported the proposition that C activation underlies NF induction of neutropenia (Fig. 2). Thus, neither heat-inactivated plasma (56°C, 30 min), which lacks C1r, C2, and C_3 proactivator activity (21) nor hydrazine-treated plasma, which lacks C4 and C3 activity (18, 22) produced the neutropenic response after activation with NF. However if these plasmas were mixed in equal parts before NF incubation, which reconstitutes C3 activability, neutropenia-inducing potential was restored. That activated C components underlie CFFL neutropenia was further supported by studies in CVF-treated, C-depleted animals. That is, plasma of a depleted animal, when incubated with NF, does not cause neutropenia, although normal plasma induces profound neutropenia when infused into the C-depleted rabbit (not shown). Moreover, reinfusion of autologous rabbit plasma in which the classical pathway of C is activated in vitro by AGG (23) produces – after removal of the activator by high speed centrifugation – decrements of circulating neutrophils identical to that of CFFL; neutrophils remained below 10% of base line during the first 10 min, 43% at 20 min, and returned to 115% at 60 min. In addition, if C is activated in vivo in 2.5-kg rabbits by intravenous injection of 200 mg AGG (classical pathway activation) or by infusion of 700 U CVF (alternative pathway activation [24]), immediate neutropenia with circulating cells falling below 10% during the first 25 min is provoked.

An attempt to further characterize the neutropenia-inducing molecule(s) generated by the NF incubation was made by utilizing ultrafiltration through membranes of standardized pore size (Table II). Thus, the neutropenia factor was found to pass a membrane with a mol wt cutoff at 30,000, but was retained by a membrane with a cutoff at 10,000.

Granulocyte Adherence In Vitro and Its Dependence on Complement. As assayed by retained myeloperoxidase activity, granulocytes adhere in significant numbers to plastic Petri dishes when suspended in fresh plasma. When plasma is heat-inactivated at 56°C for 30 min, granulocyte adhesion is reduced significantly as shown in 10 such experiments. Thus, 7 ± 3% (SD) of surface-exposed cells adhere when suspended in heat-inactivated plasma, compared to 19 ± 4% when in fresh plasma ($P < 0.001$). Since these findings suggested the possible involvement of C components in in vitro granulocyte adherence, reconstitution techniques similar to those used for the characterization of the neutropenia-inducing factor of CFFL were undertaken (Fig. 3). Hydrazine treatment of plasma reduced the adherence-promoting capacity of fresh plasma to a similar extent to that of heat inactivation. However, adherence-promoting capacity was, at least partly, restored by mixing hydrazine and heated plasmas together. A similar restoration was obtained by adding purified C3 (Cordis Laboratories,

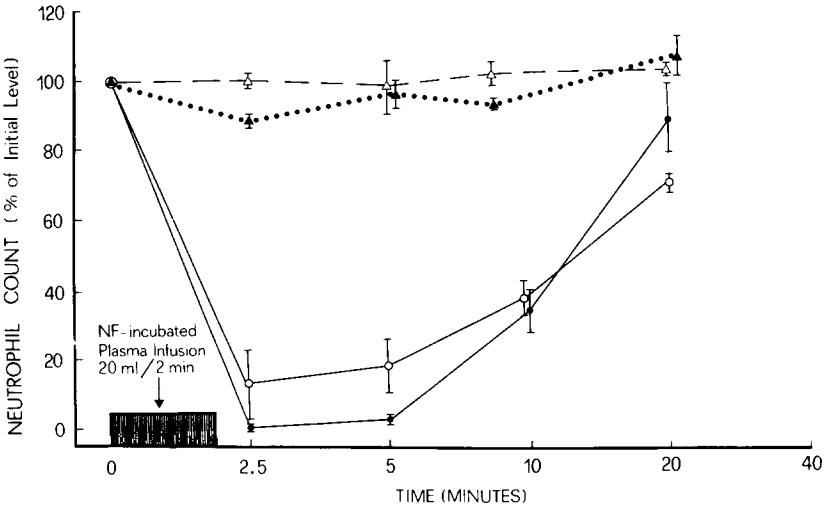

FIG. 2. Complement dependence of NF-induced neutropenia: recombination experiments in rabbits. ●—● fresh plasma; ▲· · · · ▲ heated plasma (56°C); △- - -△ hydrazine-treated plasma; ○—○ equal amounts of heated and hydrazine-treated plasmas. Means and range of triplicate experiments.

TABLE II

Partial Separation of Neutropenia-Inducing Substance by Ultrafiltration of Rabbit NF-Activated Serum

Ultrafiltration membrane* (mol wt cutoff limit)	Circulating granulocytes after injection of ultrafiltrate (percent of initial level)			
	0 min	1.5 min	4 min	10 min
	%	%	%	%
XM 300 (300, 000)	100	2	10	52
XM 100 (100, 000)	100	27	26	61
PM 30‡ (30, 000)	100	1.3	10	61
PM 10‡ (10, 000)	100	90	78	77

Results are means of two experiments; the range did not exceed 6%.

* Diaflo, Amicon Corp.

‡ Repeated redilution and filtration with five times the original serum volume.

Miami, Fla.), 200 U/ml, to hydrazine-treated plasma; in two experiments this component doubled the amount of cells from 23–24% (range of two experiments) in hydrazine-treated plasma to 48–56% in the same plasma to which was added purified C3 (not shown). Moreover, granulocyte adherence was strikingly increased when cells were suspended in plasmas in which the C system was intentionally activated. Thus, activation of the alternative pathway of C by inulin and zymosan (Fig. 4, right part) or CVF (not shown) roughly doubled cell adherence.

To determine whether the effect of the C-derived granulocyte adherence-promoting factor was reversible, cells were exposed for 5 min at 37°C to plasma activated with zymosan (top, Fig. 4) or inulin (bottom, Fig. 4) and after the removal of the activated plasma by centrifugation at 4°C, they were resuspended

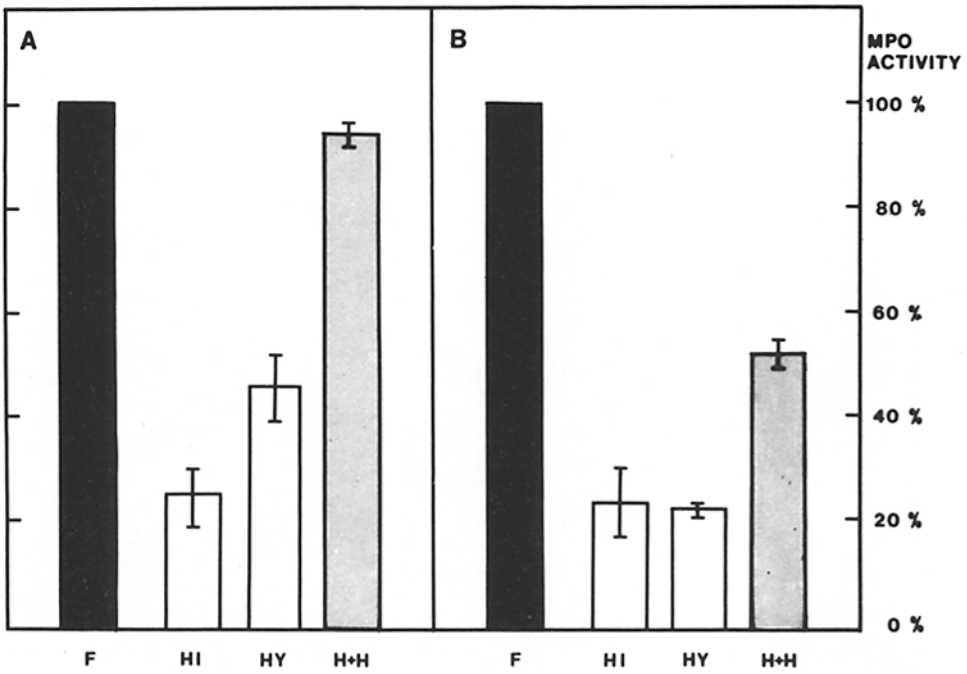

FIG. 3. Complement dependence of granulocyte adherence: adherence-promoting capacity of fresh plasma is taken as 100%. Bars denote mean and range of twicely repeated experiments in two different individuals (A + B). F, fresh plasma; HI, heat-inactivated plasma (56°C, 30 min); HY, hydrazine-treated plasma; H + H, recombination of equal amounts of HI and HY plasmas.

in prewarmed untreated plasma. At various times thereafter a 1-ml sample of this suspension was removed to duplicate Petri dishes and granulocyte adherence assayed. When resuspended in fresh plasma at 37°C, cells progressively lost their increased stickiness between 15 and 20 min and then maintained a constant adhesiveness (at the level of fresh plasma adherence) over the next 45–60 min (Fig. 4, solid symbols). In contrast, when resuspended in heat-inactivated (56°C, 30 min) plasma, no significant decrease in the intense cell adhesiveness was noted (bottom, Fig. 4, open symbols).

Discussion

To study mechanisms regulating the distribution of intravascular granulocytes between circulating and marginated pools, we took advantage of a human model with extreme transient margination, the neutropenia of CFFL (6). The early profound transient neutropenia, which has been noted by others during the procedure, was confirmed, and additionally we documented that monocytes decreased as well. Moreover, we showed that simple exposure of plasma to NF produces, upon reinfusion, the neutropenic response. In analyzing C involvement in this transient sequestration process, it became evident that routine laboratory assays which measure C consumption were not sensitive enough to document a role for C. In contrast, by utilizing our infused rabbit model, we could demonstrate that a complement-derived granulocytopenia-inducing factor

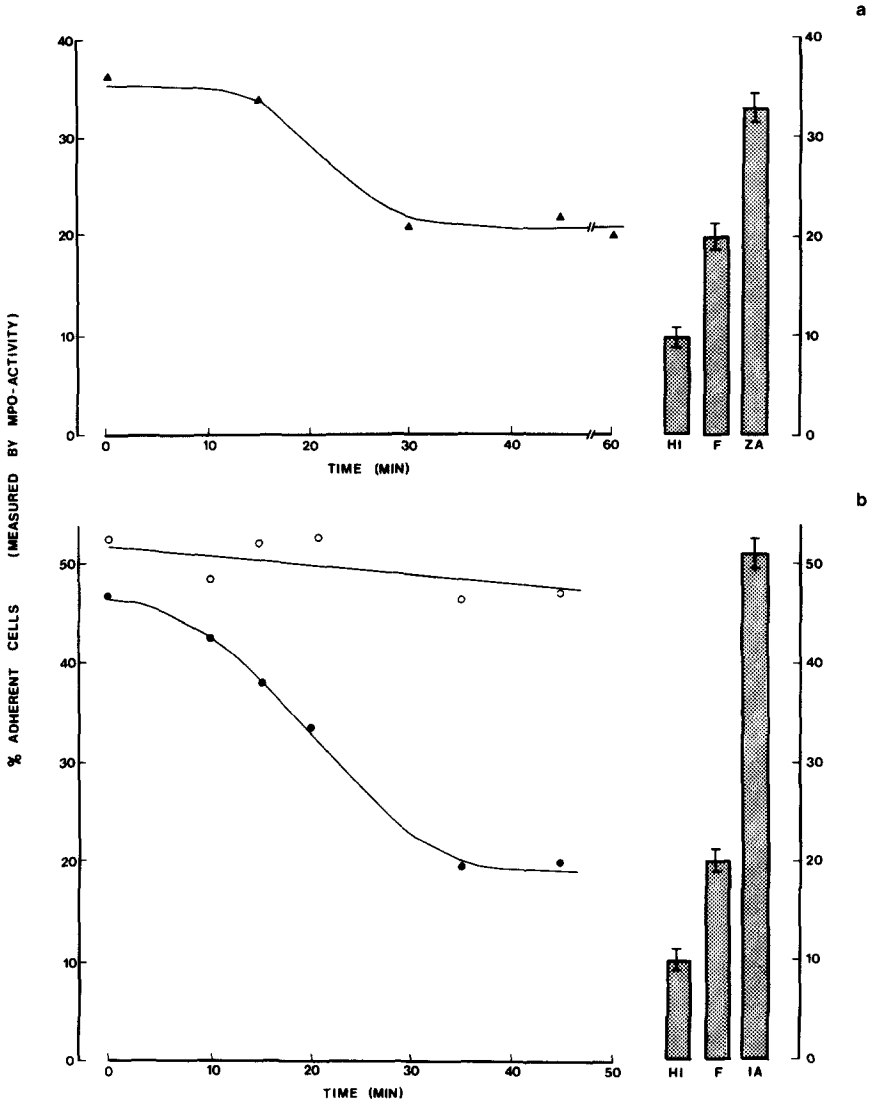

FIG. 4. Reversible effect of the complement-derived adherence-promoting factor on granulocytes in fresh plasma and sustained effect in heat-inactivated plasma. ▲—▲ cells briefly exposed to zymosan-activated plasma and kept in fresh plasma; ●—● cells briefly exposed to inulin-activated plasma and kept in fresh plasma; ○—○ cells briefly exposed to inulin-activated plasma and kept in heat-inactivated (56°C, 30 min) plasma. Bars on the right denote cell adherence in heat-inactivated (HI), fresh (F), and activated (ZA, zymosan, IA, inulin) plasma, respectively, without preexposure to C-activated plasma (mean ± SD; n for HI, F, and ZA = 10; n for IA = 3). Differences between HI and F, F and ZA, F and IA are statistically significant ($P < 0.001$, Student's t test). Each point represents the mean value of duplicate Petri dishes, and the experiments were repeated twice with similar results.

is generated by exposing plasma to NF. Whether NF activate predominantly the classical or the alternative pathway of C cannot be stated with certainty. If C3PA of rabbits has the same exquisite heat lability at 50°C as documented for human and guinea pig C3PA (17, 18, 25), the continued induction of neutropenia

by NF-treated plasma which had been preheated at 50°C (Table I) indicates that activation of the classical pathway, at least partially, underlies CFFL neutropenia. Nevertheless, C induction of neutropenia is evidently not solely restricted to activation through the classical pathway, since activators both of the classical pathway, for instance AGG (23, 26), as well as the alternative pathway, as CVF (24), produced neutropenia identical to that provoked by incubation with NF. In any case, our recombination experiments with hydrazine-treated and heat-inactivated plasmas, combined with the demonstrated abolishing effect of C depletion in animals with CVF, strongly suggest an absolute requirement for C3 in the NF induction of neutropenia-generating activity, since C3 is the common convergence point for these experimental systems. The mechanism by which NF activates C3 and possibly later components remains to be determined. The role for Hageman factor, a known contact-activated component of the coagulation and kinin systems (27, 28) is suspected and is currently under investigation.

Our ultrafiltration studies indicate that the NF-provoked neutropenia-inducing factor has a mol wt in the range of 10,000–30,000. The prime candidates of known C split products with such small molecular weights, but high biologic activity (27, 29), are C3a and C5a (30) with their mol wt of 9,000 (31) and 17,500 (32), respectively. Taking into account that protein admixture raises the cutoff limit of these ultrafiltration membranes (33), it can be assumed that both C3a and C5a are retained by the PM 10 membrane. In this regard, it has recently been proposed that the acute disappearance of granulocytes after intravascular activation of the C system may be the consequence of neutrophil and monocyte margination at sites of increased vascular permeability, caused by the anaphylatoxin activity of C3a and C5a (34). However, we doubt this interpretation has validity in our studies since the neutropenia-inducing factor generated by NF in vitro maintains its activity in neat plasma for hours while anaphylatoxin activity is rapidly destroyed by an anaphylatoxin inactivator (35). Moreover, in our previous studies (9), injection of C-activated plasma into neutropenic sheep produced no alteration of intravascular blood pressures and no indication of increased vascular permeability, suggesting that such plasma is devoid of anaphylatoxin activity and that it is similar to the more stable chemotactic activities of C3a and C5a (35, 36).

Possible mechanisms by which activated C components might produce neutropenia have been considered; we favor the hypothesis that neutropenia results from C-induced, increased cell adhesiveness to endothelial surfaces. To our knowledge, the influence of C on granulocyte adherence has not been studied systematically. A few indirect indications that C activation might play a role in leukocytes adhesiveness had been noted. Thus: (*a*) in transparent ear vessels of rabbits sensitized to horse serum, leukocytes adhere to vessel walls during antigenic serum infusion (37); and (*b*) decomplemented rabbits maintain considerably higher circulating granulocyte counts than normal ones (38) which might be a consequence of demargination.

The high degree of association between in vivo margination and Petri dish adhesiveness demonstrated in the present studies suggests the two processes in fact reflect a common membrane-perturbing effect of C on granulocytes. In this regard it has recently been shown that exposure of granulocytes to C5a decreases their net negative surface charge (39), engendering the suggestion that

adhesion occurs when powerful electrostatic repulsive forces between cells and substrate (endothelium or plastic dishes) are overcome, thereby permitting short range forces (Van der Waals and hydrophobic) to act (40, 41). Finally our results demonstrating partial reversibility of C-derived adherence in vitro (Fig. 4) coupled with the reversibility of neutropenia in vivo permits the postulate that a heat-labile C-inactivating system in the circulation is responsible for the fact that granulocytes demarginate so quickly after the neutropenia-inducing stimulus has been withdrawn. This postulate is currently under active investigation.

Summary

To study mechanisms and mediators regulating the distribution of intravascular granulocytes between circulating and marginated pools, a human model with extreme transient margination, the neutropenia of continuous flow filtration leukophoresis, was analyzed. Studies in animals demonstrated the existence of a complement (C)-derived granulocytopenia-inducing factor. Thus, autologous plasma, exposed to nylon fibers (NF) of the filtration system, produced an acute selective decrement of circulating granulocytes and monocytes. This phenomenon was blocked by decomplementing plasma, by pretreatment of plasma with EDTA or hydrazine, and by preheating at 56°C, but did occur after recombination of heat-inactivated and hydrazine-treated plasma before NF exposure. Preheating plasma at 50°C did not inhibit the neutropenic response, suggesting involvement of the classical pathway of C activation. Ultrafiltration studies indicated that the NF-provoked neutropenia-inducing factor has a mol wt in the range of 10,000–30,000, and is heat stable (56°C). To analyze the hypothesis that C-induced neutrophil margination might be consequent to increased cell adhesiveness to endothelial surfaces, the role of C in promoting granulocyte adherence was evaluated in vitro. Measured with a plastic Petri dish assay, granulocyte adherence was significantly reduced in heat-inactivated (56°C) and hydrazine-treated plasma, but adherence promoting capacity was restored by mixing the two plasmas, or by adding purified C3 to hydrazine-treated plasma. After exposure to activated C, neutrophils showed significantly increased adhesiveness which was maintained when cells were resuspended in heat-inactivated plasma, but progressively lost when resuspended in fresh plasma. On the basis of these results we conclude that granulocyte adhesiveness in vitro and margination in vivo are closely associated, C-dependent phenomena.

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