American Journal of Pathology, Vol. 137, No. 2, August 1990

C5a-induced Neutrophilia

A Primary Humoral Mechanism for Recruitment of Neutrophils

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The leukocytosis activity of C5a was studied in a rabbit model. Blood samples were drawn for cell counts through a catheter placed in an artery of one rabbit ear after injection of either porcine or human C5a into a vein in the opposite ear. These studies indicate the potential of C5a to mobilize bone marrow neutrophils following transient C5a-induced neutropenia, based on counts of nonsegmented neutrophils. The numbers of circulating neutrophils can be selectively elevated by 300% to 400%, within 1 to 2 hours, in rabbits given only microgram quantities (1 to 5 × 10^{-9} mol/l) of C5a or $C5a_{des\ Arg}$. These quantities of C5a are equivalent to 1% to 2% of complement C5 activation. The time-course of the induced neutrophilia is characteristic of C5a, increasing rapidly in the first 10 to 20 minutes after injection and attaining a maximum level at 2 to 5 hours, then decreasing slowly to normal levels over the next 4 to 6 hours. After boiling at 100°C for 10 minutes, C5a ($C5a_{des\ Arg}$) lost its leukocytosis activity, indicating that the cellular effect was not caused by endotoxin. Other known leukocytosis factors, such as epinephrine, dexamethasone, lipopolysaccharide, and the prostanoid 15(S)-15-methyl $PGF_{2\alpha}$, produced a distinctly different profile of leukocyte mobilization than that of C5a. The C5a-induced neutrophilia was not inhibited by pretreating these animals with indomethacin, suggesting that it is not a prostanoid-induced effect. One hypothesis is that no secondary cellular mediator system is involved in C5a-mediated leukocytosis, but rather that C5a alone is responsible for a rapid mobilization of neutrophils from bone marrow pools, and perhaps marginated pools, following neutropenia. Circulating neutrophils are activated by C5a and thereby become deformed and adherent, leading to a neutropenia, sequestration, and depletion of cells. After the neutropenic event an immediate neutrophilic response is required for replacement of this particular cell population and to re-establish homeostasis. Therefore the role of C5a may be just as important as other known leukocytosis factors (fragments from C3, for example) in promoting complement-dependent neutrophil mobilization in response to tissue injury, infections, or extracorporeal blood treatments. (Am J Pathol 1990, 137: 467–477)

Neutrophilic leukocytosis* is a common feature of many types of infections and a consequence of many inflammatory diseases. Mechanisms for controlling the number of neutrophils in circulation include the release of neutrophils from marginated and bone marrow storage pools. Many substances are known to induce neutrophilic leukocytosis, eg, glucocorticosteroid[1]; endotoxin[2,3]; interleukin-1 and/or tumor necrosis factor[4]; stable analogs of prostaglandin E_1, E_2, and $F_{2\alpha}$[5]; granulocyte/macrophage colony-stimulating factor (GM-CSF)[6,7]; and macrophage-derived neutrophil chemotactic factor (MDNCF).[8] Rother[9] demonstrated that a fragment of the complement component C3 exhibits leukocyte-mobilizing activity *in vitro* using a rat femur assay and that this C3 fragment induced leukocytosis in rabbits. Various C3-derived fragments (eg, C3e,[10] C3d-K,[11] and a synthetic nonpeptide of C3d-K[12]) have been isolated and they also have leukocytosis-inducing activities.

Complement activation induced during extracorporeal blood circulation, such as in hemodialysis[13] and coronary

Supported by National Institutes of Health grants HL16411 and HL25658.
Accepted for publication March 30, 1990.
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*Leukocytosis induced by C5a, as observed in the rabbit model, largely involves neutrophils and does not involve other leukocytic cell types. Hence the term neutrophilia will be used to describe the late cellular response to C5a.

bypass,[14] leads to acute leukopenia. The leukopenia presumably results from an interaction of C5a with polymorphonuclear leukocytes, which promotes adhesion of these cells, causing margination and eventual sequestration, an event observed prominently in the lungs of C5a-treated animals.[15] During hemodialysis in humans, a rebound in the number of circulating polymorphonuclear leukocytes occurs rapidly and incremental increases in immature polymorphonuclear leukocytes also was noted. This rebound leukocytosis effect could be attributed to the generation of bioactive C3 fragments, as mentioned above, and/or the C5 anaphylatoxin. The anaphylatoxin C5a has an important role in inflammatory reactions attributable to its chemotactic and spasmogenic functions and is well known to exhibit a marked neutropenic response.[15–17] However, relatively little attention has been given to the role of C5a as a major leukocytosis-inducing factor. Several investigators have shown that neutropenia induced by C5a is followed by significant neutrophilia in animal models of adult respiratory distress syndrome.[17,18] Accordingly, we have focused on characterizing the leukocytosis-inducing activity of C5a and have specifically examined the profile of the response, potency of the factor, and whether secondary mediators are involved in this phenomenon.

Materials and Methods

Animals

Male New Zealand White rabbits weighing between 2.0 and 3.0 kg were used for our experiments. Sometimes two experiments were performed in the same rabbit at intervals of more than 3 weeks. No influences from previous application of the factor or from previous bleeding were observed in these animals. However most animals were used only once.

Preparation of C5a, $C5a_{des\ Arg}$, and $C3a_{des\ Arg}$

Human C5a and $C5a_{des\ Arg}$ were prepared according to published methods.[19,20] Porcine $C5a_{des\ Arg}$ and $C3a_{des\ Arg}$ were prepared according to Gerard and Hugli[21] and Corbin and Hugli,[22] respectively. The purity of the C5a, $C5a_{des\ Arg}$, and $C3a_{des\ Arg}$ were confirmed by cellulose acetate (Microzone) electrophoresis at pH 8.7 and by amino acid analysis. Bioassays were performed using the guinea pig ileum for determining C5a activity. The purified anaphylatoxins were frozen in water at −20°C and diluted in 2 ml of 0.9% pyrogen-free saline (Travenol Labs, Deerfield, IL) just before administration.

Other Materials

Lipopolysaccharide (LPS) from salmonella abortus equi, and indomethacin were purchased from Sigma, St. Louis, MO. Lipopolysaccharide was dissolved in physiologic saline (1 μg/ml) and indomethacin was dissolved in 0.1 mol/l (molar) sodium carbonate (5 mg/ml). Epinephrine (USP) and dexamethasone sodium phosphate (USP) were obtained from Abbott Labs, North Chicago, IL and Elkinssin, Cherry Hill, NJ, respectively. The 15(S)-15-methyl $PGF_{2\alpha}$ was a gift from Upjohn Co. (Kalamazoo, MI) and was dissolved in 95% ethanol (10mg/ml), then diluted in physiologic saline.

Blood-collecting Procedure

Rabbits were restrained in a box for 4.5 hours without food or drink. A 24-gauge intravenous catheter (Johnson & Johnson Co., New Brunswick, NJ) was indwelt in a central artery of one ear for bleedings under local anesthesia (subcutaneous injection of lidocaine). Another catheter was placed in a marginal vein of the other ear for making injections. The catheters were connected with three-way stopcocks to a syringe with heparinized saline (1 U/ml saline) to prevent coagulation. The catheters were rinsed continuously with heparinized saline (5 to 8 ml/hours) by an infusion pump (Harvard Apparatus, Model 975, South Natick, MA). Blood samples (300 μl) were collected in tubes containing 5 μl of 10% EDTA (Sigma) at designated times. After a rabbit was sedated, three initial bleeds were made at intervals of 5 minutes to establish a zero time baseline level. Epinephrine and 15(S)-15-methyl $PGF_{2\alpha}$ were infused over 4 minutes; all other agents, including C5a, were infused over 30 seconds. All experiments were begun between 9 and 10 A.M. for consistency. In some extended experiments, rabbits were transferred to a cage 4.5 hours after the experiment had begun and further blood samples were then obtained by needle puncture of an ear artery.

Leukocyte Count

Total leukocyte counts were made with a Coulter counter (Coulter Electronics, Model ZBI, Hileah, FL). Absolute values for each type of leukocyte were calculated from the manual differential counts obtained for 100 to 200 cells in blood smears and the percentage of each cell was normalized to total leukocyte count. These smears were stained with Wright and Giemsa stain. Nonsegmented neutrophils were defined as any neutrophil in which nuclear lobes were not clearly separated by a filament.

Table 1. *Effect of Human $C5a_{DES\,ARG}$ or Porcine $C5a_{DES\,ARG}$ on Circulating White Blood Cell Subsets in Rabbits*

Time	Total WBC	Neutrophils	Nonsegmented neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Human $C5a_{des\,Arg}$							
0 time	6372 ± 688	3024 ± 751	322 ± 98	3022 ± 866	254 ± 89	85 ± 29	114 ± 34
2 min.	2645 ± 873*	0 ± 0*	0 ± 0*	2645 ± 873	0 ± 0*	5 ± 7*	0 ± 0*
30 min.	7990 ± 2102	5168 ± 2099	1615 ± 620	2230 ± 690	205 ± 31	80 ± 21	98 ± 13
1 hr.	8517 ± 1967	5690 ± 2067	1845 ± 623†	2576 ± 798	199 ± 59	85 ± 20	105 ± 14
4 hrs.	11940 ± 2697†	8716 ± 2541†	2945 ± 859†	2728 ± 491	254 ± 44	74 ± 53	116 ± 21
Porcine $C5a_{des\,Arg}$							
0 time	6778 ± 325	2692 ± 586	251 ± 78	3424 ± 423	285 ± 120	132 ± 59	65 ± 51
2 min.	1726 ± 305*	0 ± 0*	0 ± 0*	1725 ± 306*	4 ± 6*	0 ± 0*	7 ± 9*
30 min.	10452 ± 1787†	5890 ± 1417†	2315 ± 717†	4052 ± 805	295 ± 157	262 ± 182	62 ± 44
1 hr.	12882 ± 1641†	7678 ± 1660†	2694 ± 960†	4755 ± 624	229 ± 157	142 ± 123	37 ± 52
4 hrs.	15519 ± 2560†	9827 ± 2580†	2364 ± 1319	3960 ± 637	290 ± 80	219 ± 114	92 ± 65
8 hrs.	9399 ± 1241†	5289 ± 1595	746 ± 286	3732 ± 190	189 ± 139	125 ± 44	94 ± 13

Mean value ± SE, n = 3.
* Significant ($P < 0.05$) for leukopenia compared to control 0 time values.
† Significant ($P < 0.05$) for granulocytosis compared to control 0 time values.

Visual identification indicated primarily bands and virtually no promyelocytes or myelocytes. Postinfusion leukocyte counts were expressed as a percentage of the average of three preinfusion counts.

Statistics

Data in Table 1 and the figures are given as mean ± standard error (SE). For calculation of statistical significance, Student's paired *t*-test (Table 1) or unpaired *t*-test (Figs. 1 to 6) was used, and a P value < 0.05 was considered significant.

Results

Control Study with Saline

As shown in Figure 1A, no significant changes in leukocyte counts were observed during the course of the experiment after 2 ml of saline was injected into the animal. The small increase in leukocytes after 4 to 5 hours could be caused by the stress of multiple bleedings or by exercise, feeding, and/or drinking by the unrestrained animals once they were returned to their cages at 4 hours into the experiment. A modest decrease in the hematocrit was noted between 4 and 6 hours (Figure 1B). The decrease in hematocrit was less than 7% throughout these experi-

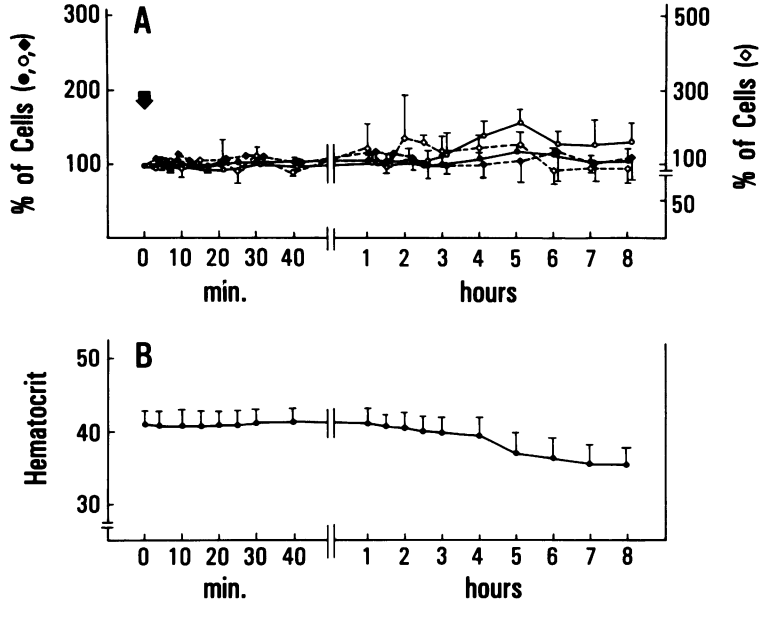

Figure 1. A *shows the changes in total leukocytes (●), neutrophils (○), nonsegmented neutrophils (◇), and lymphocytes (◆) for a control animal study using saline. Two milliliters of saline were injected intravenously into adult rabbits (▼). The rabbits were removed from a restraining box after 4 hours. Postinjection leukocyte cell counts are expressed as a percentage of an average of three preinjection cell counts. All values are given as the mean ± SE for three animals.* B *shows the average change in hematocrit during 8 hours from a total of given as the mean ± SE for 10 animals. Significant decreases in hematocrit and slight increase in neutrophils between 4 and 8 hours could be due to either multiple bleedings or to exercise, eating, and drinking.*

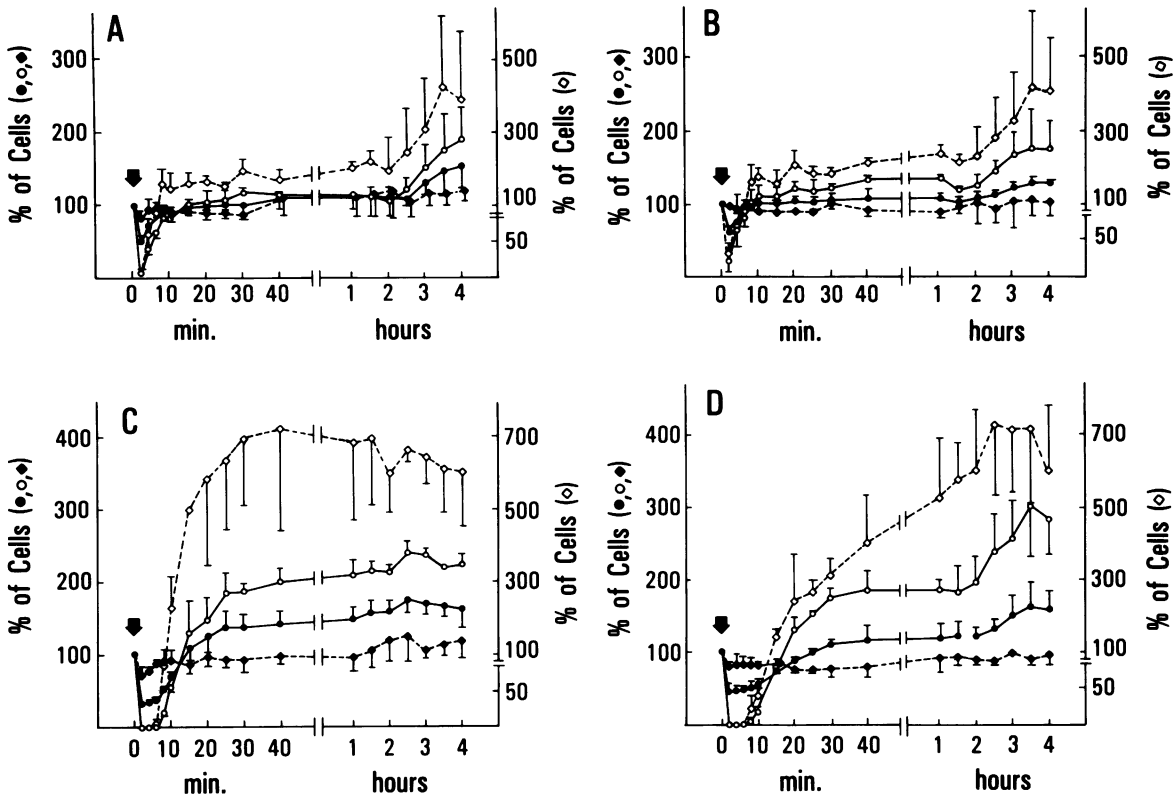

Figure 2. A *and* B *show the changes in circulating of total leukocytes (●), neutrophils (○), nonsegmented neutrophils (◇), and lymphocytes (◆) induced by 1 μg/kg of human C5a (A) or human* $C5a_{des\ Arg}$ *(B). Each C5a preparation was injected along with 100 μg of porcine* $C3a_{des\ Arg}$ *used as a carrier protein. This quantity of porcine* $C3a_{des\ Arg}$ *alone produces no significant change in the leukocyte count. A short, but significant, neutropenia was observed immediately after administration of the C5a (*$C5a_{des\ Arg}$*); however neutrophilia was not pronounced in either study when using 1 μg/kg of material.* C *and* D *show the larger changes of leukocyte counts induced by injection of 5 μg/kg of human C5a* (C) *or human* $C5a_{des\ Arg}$ (D)*, also mixed with 100 μg of porcine* $C3a_{des\ Arg}$*. More profound neutropenia was seen at these levels of C5a and significant neutrophilia is observed after 20 to 25 minutes accompanied by rather remarkable increases in nonsegmented neutrophils in each study. Significant lymphopenia was not observed. Values are given as the mean ± SE of three experiments.*

ments and so the influence of anemia is considered minor.

Responses to Human C5a and $C5a_{des\ Arg}$

Human C5a or $C5a_{des\ Arg}$ was administered to the rabbits as shown in Figure 2. Human C5a or $C5a_{des\ Arg}$ was mixed with 100 μg of porcine $C3a_{des\ Arg}$, a physically similar molecule, to minimize losses of the C5a from binding to the surfaces of tubes or syringes during infusion. We avoided using albumin or rabbit serum as carriers to assure that complement was not activated in the experimental animals. This quantity of porcine $C3a_{des\ Arg}$ did not produce significant changes in the leukocyte count when injected into one animal (data not shown). As shown in Figure 2A and B, 1 μg/kg of human C5a or $C5a_{des\ Arg}$ caused a short (ie, less than 10 minutes) but significant neutropenia immediately after administration of the factor. The neutropenia was followed by a late and statistically insignificant neutrophilia compared to the results of the control study. Neutropenia induced by 5 μg/kg of human C5a or $C5a_{des\ Arg}$ were of significantly longer duration (more than 10 minutes) than that induced by 1 μg of material and were not accompanied by significant lymphopenia, as shown in Figure 2C and D. Neutropenia was immediately followed by neutrophilia, and the pronounced leukocytosis effect was accompanied by a remarkable increase in nonsegmented neutrophils. The neutrophilia observed after 25 minutes was statistically significant compared to that of the control study in Figure 1 and was not accompanied by either lymphopenia or lymphocytosis. In these studies there are no significant differences between the results obtained by human C5a or $C5a_{des\ Arg}$, suggesting that serum carboxypeptidase may have converted C5a efficiently to $C5a_{des\ Arg}$.

Response to Porcine $C5a_{des\ Arg}$

A 5-μg/kg dose of porcine $C5a_{des\ Arg}$ was administered to rabbits and observations of the cellular response were

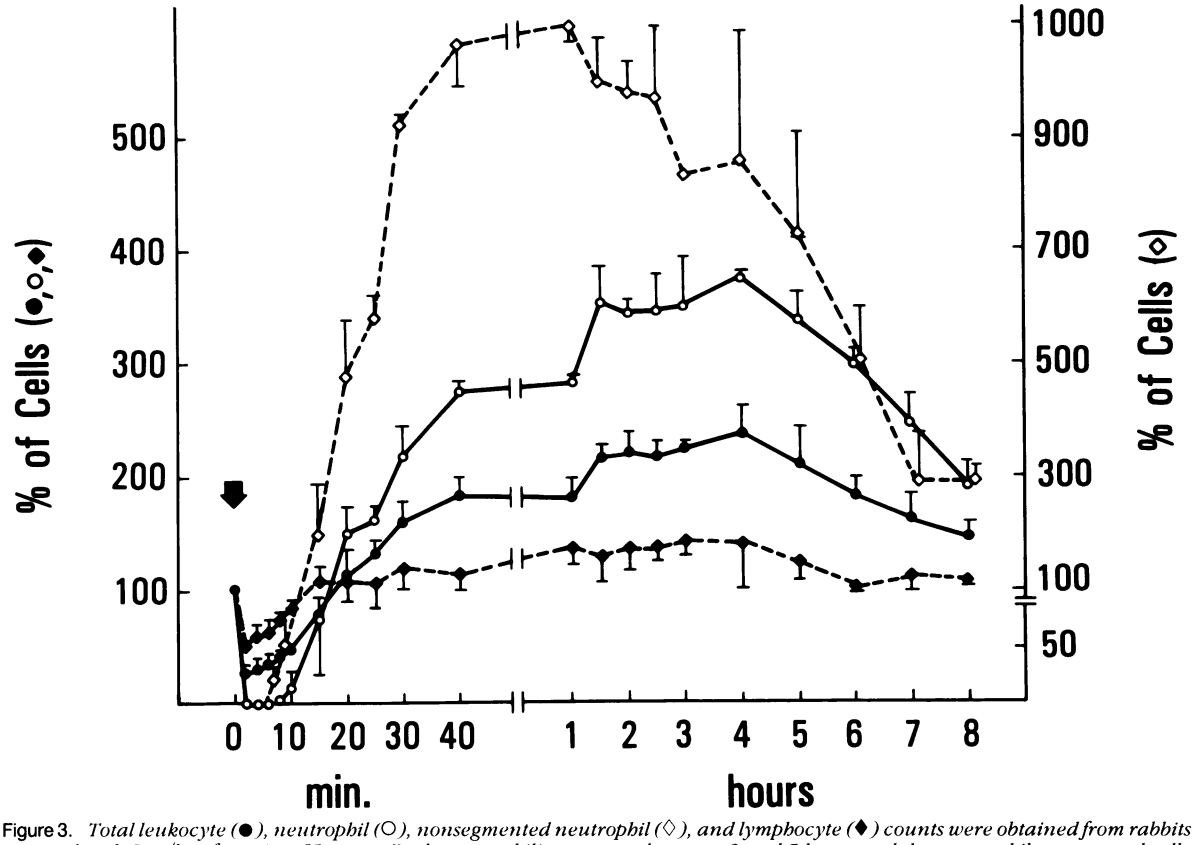

Figure 3. *Total leukocyte (●), neutrophil (○), nonsegmented neutrophil (◇), and lymphocyte (◆) counts were obtained from rabbits injected with 5 μg/kg of porcine $C5a_{des\ Arg}$. Peak neutrophilia was seen between 2 and 5 hours and the neutrophil counts gradually decreased after 4 to 5 hours. The neutrophilic response to porcine $C5a_{des\ Arg}$ appears to be greater than that to human $C5a_{des\ Arg}$. Total nonsegmented neutrophils increased markedly and the ratio of segmented to nonsegmented neutrophils was similar to that for animals exposed to human $C5a_{des\ Arg}$. Lymphocyte counts decreased in parallel with neutropenia but no lymphocytosis was noted during neutrophilia. Values are given as the mean ± SE for three separate experiments.*

performed during an 8-hour period, as shown in Figure 3. No $C3a_{des\ Arg}$ carrier was added to the porcine $C5a_{des\ Arg}$. After a protracted 10-minute neutropenia, a statistically significant neutrophilia was observed 25 minutes after C5a injection. Peak neutrophilia occurred between 2 and 5 hours and then neutrophil counts decreased steadily during the next few hours. The maximum neutrophil count approaches four times (average percentage change is 357% ± 32%) as many cells as in the initial counts. The neutrophilic response to porcine $C5a_{des\ Arg}$ after 30 minutes appears to be greater than compared to that elicited by human $C5a_{des\ Arg}$ at the same concentration. The total number of segmented and nonsegmented neutrophils increased markedly, but the ratio of segmented to nonsegmented neutrophils was not significantly different from that using human C5a/$C5a_{des\ Arg}$. Lymphocyte counts decreased simultaneously with leukopenia and, unlike the response to human C5a, the lymphopenia was significant (see also Figure 4). The reason for this difference may be that porcine C5a is biologically more potent than human C5a in this system. Neither lymphopenia nor lymphocytosis was seen during the neutrophilia phase.

The numbers of various types of leukocytes stimulated by 5 μg/kg of human $C5a_{des\ Arg}$ or porcine $C5a_{des\ Arg}$ are shown in Table 1. Neutrophils, monocytes, eosinophils, and basophils each virtually disappeared immediately after administration of the factors. However, no significant incremental rise in any leukocyte, other than the neutrophils, was observed 1 hour to 4 hours after injection, indicating a selectivity in the response.

Responses to Other Leukocytosis Factors

Dexamethasone and epinephrine were administered to the rabbits to investigate the potential contribution of factors derived from the adrenal gland on the C5a effect. Epinephrine is known to shift leukocytes from the marginated granulocyte pool to the circulating granulocyte pool.[23] As shown in Figure 5A, when 20 μg of epinephrine were infused in rabbit for 4 minutes, both neutrophil and lymphocyte counts began to increase as soon as epinephrine was infused, and levels returned to baseline in approximately 10 minutes after the end of infusion. The

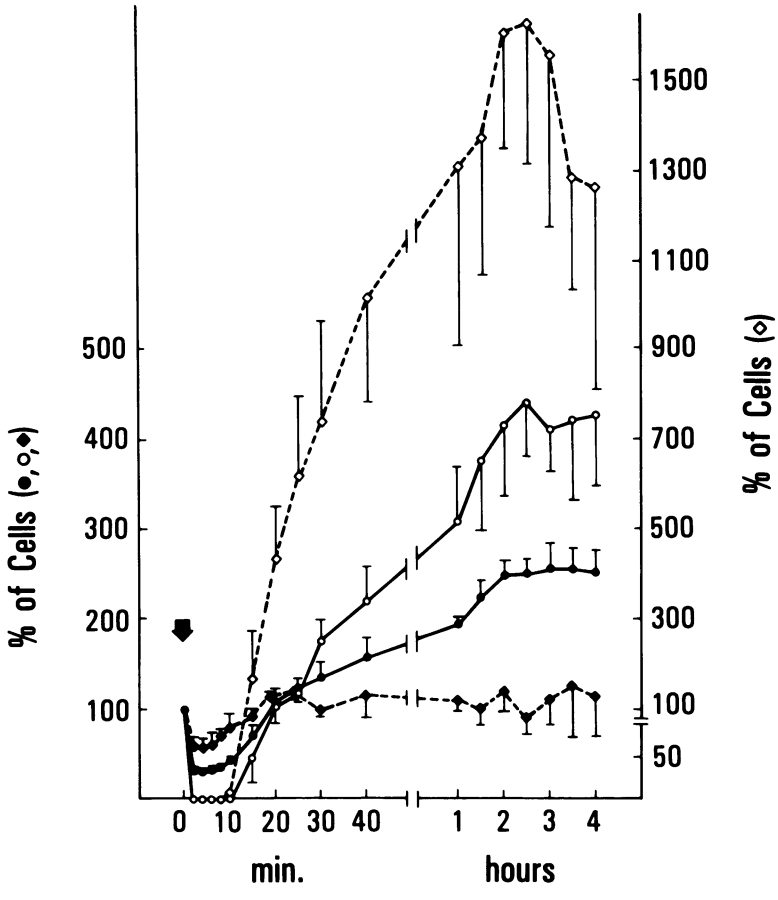

Figure 4. *The effect of indomethacin (5 mg/kg) pretreatment on total leukocyte (●), neutrophil (○), nonsegmented (◇), and lymphocyte (◆) counts induced by 5 μg/kg of porcine $C5a_{des\ Arg}$ was measured. No significant differences are observed between the counts obtained from injection of porcine $C5a_{des\ Arg}$ with or without indomethacin present. Each leukocyte count is expressed relative to the value obtained after injection of indomethacin from three samples taken before C5a injection. Values are given as the mean ± SE of three animal experiments.*

ratio of segmented and nonsegmented neutrophils was unchanged when compared to the ratio before infusion. This pattern of leukocytosis is similar to the response observed in humans,[23] except it is less pronounced in the animal model.

Adrenocortical steroids induce granulocytosis by decreasing the rate of egress of cells from the total blood granulocyte pool as well as increase an influx of cells from the bone marrow.[1] Dexamethasone (5 mg) induced neutrophilia and was accompanied by an incremental rise in nonsegmented neutrophils, as shown in Figure 5B. No response was observed until 1 hour after injection and the maximum response occurred between 4 and 8 hours. This peak response is delayed 2 or 3 hours compared with the C5a response; however, the extent of neutrophilia and the ratio of segmented to nonsegmented neutrophils are similar to the response induced by C5a. Significant decreases in lymphocyte and eosinophil counts were also observed with dexamethasone correlating with the response observed in humans.[24] These results suggest that C5a-induced neutrophilia is not mediated by adrenocorticoid.

When 0.25 to 1 μg of lipopolysaccharide from salmonella abortus equi (LPS) was administered, a late-phase leukocytosis response was observed, as shown in Figure 5C. We examined LPS to negate the possibility that endotoxin contamination in the C5a preparations were responsible for the effect. Direct measurement of endotoxin in the porcine C5a preparation (Sigma E-Toxate Kit, Sigma Chemical Co., St. Louis, Mo.) indicated less than 0.5 ng of LPS in 5 μg of the active factor and less than 8 ng of LPS in the 100-μg sample of porcine $C3a_{des\ Arg}$ used as a carrier.

Although significant neutrophilia was observed 3 or 4 hours after exposure of the animals to LPS, accompanied by an increase of nonsegmented cells, the neutropenia was delayed compared to that induced by C5a. Significant long-term lymphopenia was induced by LPS concurrent with the late-phase neutrophilia, unlike C5a, which induces lymphopenia immediately after injection of the factor. When porcine $C5a_{des\ Arg}$ was boiled at 100°C for 10 minutes, this preparation no longer demonstrated measurable effects on the circulating leukocyte count (data not shown), indicating that the C5a effect was authentic and not induced by LPS.

We showed that 15(S)-15-methyl $PGF_{2\alpha}$ could cause neutrophilia, as reported by Ulich et al.[5] The 15(S)-15-methyl $PGF_{2\alpha}$ was dissolved in 20% ethyl alcohol and it

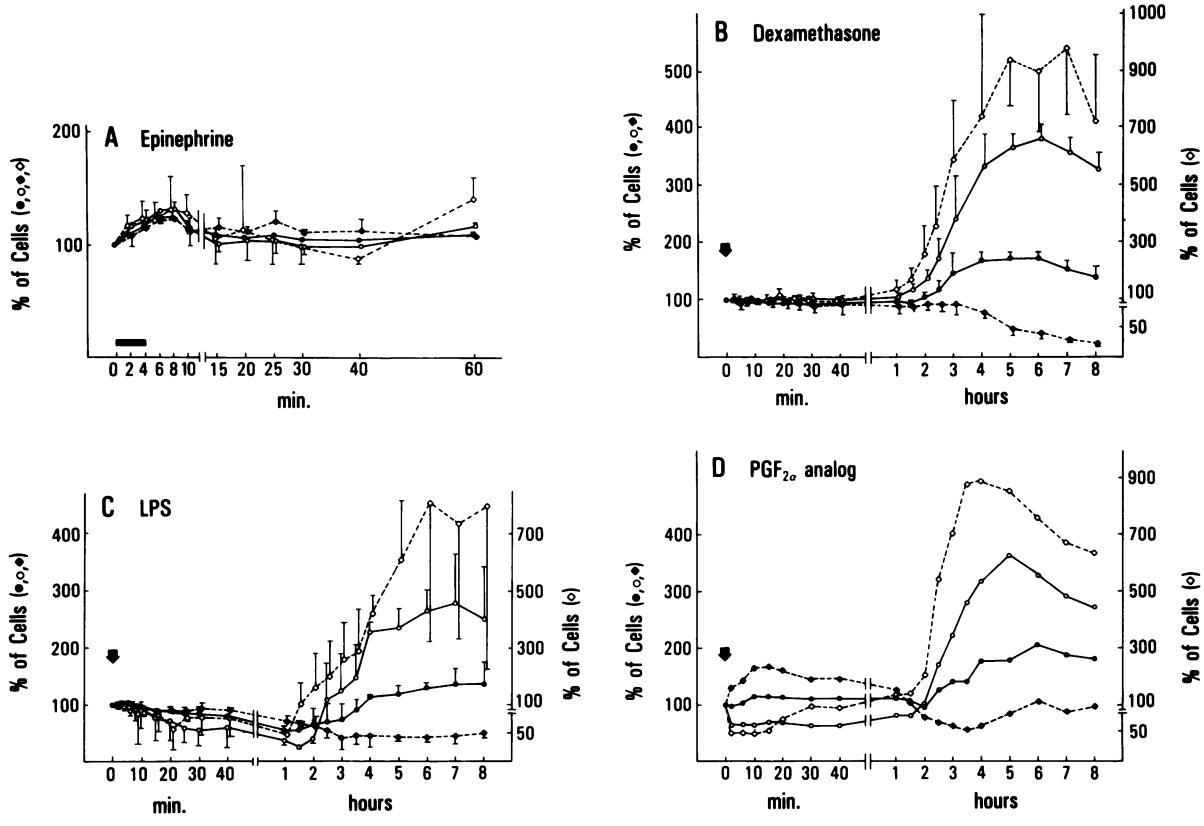

Figure 5. *Changes in total leukocyte (●), neutrophil (○), non-segmented neutrophil (◇), and lymphocyte (◆) counts induced by 20 μg of epinephrine* (A), *5 mg of dexamethasone* (B), *0.25–1 μg of lipopolysaccharide* (C) *and 0.25 mg of 15(S)-15-methyl* $PGF_{2\alpha}$. *Epinephrine and 15(S)-15-methyl* $PGF_{2\alpha}$ *were infused during a period of 4 minutes and the 0 time indicates the beginning of an intravenous infusion of the agent. No significant increase in neutrophils were seen until after 1 hour* (B, C *and* D) *and maximal responses were observed between 4 and 8 hours. The ratios of segmented to nonsegmented neutrophils were similar to those obtained for C5a. Significant decreases in lymphocytes were also noted during neutrophilia in* B, C *and* D. *Values in* A, B *and* C *are given as mean ± SE for three experiments. Each point in* D *indicates the average of only two experiments.*

induced an effect on the circulating leukocyte count similar to that of dexamethasone and lipopolysaccharide. When 2 ml of 20% ethyl alcohol were injected as a control, it did not influence the leukocyte counts. Consequently, the neutrophilia induced by the $PGF_{2\alpha}$ analog does not appear to compare with that induced by C5a based on differences in the time course of the neutrophilia.

Effect of Indomethacin Pretreatment on C5a-induced Leukocytosis

It is possible that the neutrophilia induced by C5a is mediated by prostanoids other than $PGF_{2\alpha}$.[5] To confirm or dismiss this possibility, rabbits were pretreated with 5 mg/kg of indomethacin 20 minutes before injection of porcine $C5a_{des\ Arg}$. As demonstrated in Figure 4, these animals were unaffected by pretreatment with the indomethacin in terms of the C5a-induced leukocyte response.

Discussion

Complement activation is well known for inducing a transient neutropenia in animal models.[25] Earlier studies have implicated C5a generation as being responsible for the neutropenia observed in humans during hemodialysis and cardiobypass.[13,14] Although C5a was presumed to be largely responsible for the complement-dependent cellular response in humans, the purified factor was initially used in rabbit experiments by Webster et al.[15] These investigators monitored circulating neutrophil levels after injection of purified C5a. They observed an immediate and transient neutropenia, lasting only minutes, followed by a significant neutrophilia persisting during a 4-hour period. A later study in guinea pigs indicated that purified C5a produced not only a biphasic response of neutropenia followed by neutrophilia but also that the platelet counts initially fell and then recovered to normal levels.[17] In the guinea pig model, platelet involvement was expected because these cells contain C5a receptors.[26] The

neutrophilia in guinea pigs lasted for more than 6 hours. In both animal models, neutrophils were sequestered in the lung microvasculature and remained in the lungs at elevated levels for more than 1 hour after injection of the C5a. It appears that significant neutrophil depletion occurs and that a sizable pool of noncirculating neutrophils are mobilized by the C5a immediately after the neutropenic phase. Previous studies have also demonstrated that several fragments of component C3 are leukocytotic factors, and therefore complement activation is apparently responsible for generating several potent factors capable of mobilizing leukocytes *in vivo*.

In this study we have considered the cell types recruited, the magnitude of the neutrophil response, its duration, evidence for bone marrow-derived cells, and the mechanism that is responsible for C5a-induced neutrophilia. We considered the leukocyte response to be either a direct effect of the complement-dependent mediator C5a on receptor-bearing cells, such as the neutrophils, or a result of secondary mediator(s) release by C5a. In addition, we examined whether the C5a-induced neutrophils came directly from bone marrow maturation pools or were mainly marginated cells. In previous studies, using radiolabeled white blood cells, most of the neutrophils that sequester in marginated pools after C5a exposure returned rapidly to the circulation.[17] However neutrophils that sequestered in the microvasculature were observed histologically to be in place for more than 1 hour after C5a administration.[15,17] Therefore the leukocytes in marginated pools appear to contribute to the neutrophilia induced by C5a, but presumably cannot account for the many circulating neutrophils present within minutes of the neutropenic event. Evidence presented here suggests that a significant portion of the neutrophils recruited by C5a originate in the bone marrow pool based on the high levels of nonsegmented (ie, band form) neutrophils observed.

The profile of the leukocytosis response to C5a is characterized by an early and rapid rise in circulating segmented and nonsegmented neutrophils. The elevated levels are almost entirely neutrophilic leukocytes and these high levels persist for 6 to 8 hours. Although porcine C5a proved to be slightly more active than human C5a in the rabbit model, a response of 300% to 400% elevation in circulating neutrophils is usually produced by levels of the factor equivalent to only 1% to 2% conversion of C5 (eg, 5μg/kg of C5a is approximately 50 to 75 ng/ml of C5a in a 3-kg animal compared to a potential 3000 to 4000 ng/ml of C5a for total C5 conversion). Therefore 5×10^{-9} mol/l C5a can produce a leukocytosis response equal to or greater than that produced by comparable levels of the C3 fragments C3e[10] or C3dK.[11] Because the concentration of C3 in blood is approximately 20 times higher than C5, the potential activity for C3 fragments to induce neutrophilia appears to be greater than that of C5a. However, the C3 contribution depends on the rate that C3b, which is not leukocytotic, is converted to degradation fragments exhibiting this cellular activity.

Certain cytokines (GM-CSF and MDNCF) are known leukocytosis-inducing factors and could be involved in C5a-induced neutrophilia. Mediators released from neutrophils or macrophages are the most likely candidates for C5a-mediated responses. We conclude that monokines like MDNCF, TNF, and IL-1 are not likely candidates for mediating C5a-induced neutrophilia because unstimulated or unprimed monocytes have low levels of C5a receptors[27] and respond weakly to physiologic levels of C5a.[28,29] The neutrophilic response presumably occurs too uniformly and rapidly for MDNCF or other monokines to be generated and return to the circulation, even if tissue macrophages are stimulated by the C5a. However it must still be confirmed whether cytokines participate actively in the C5a neutrophilic response.

It is possible that all chemotactic factors induce neutropenia *via* mechanisms of enhanced surface adherence and increased cellular rigidity associated with morphologic shape changes in the cell. Worthen et al.[30] report that cytoskeletal actin assembly results in a mechanical retention of the neutrophils in microvessels because of the inability of the cell to deform. Visible morphologic changes in the stimulated neutrophil are slowly reversible[31] and the time course is consistent with the duration of sequestered cells in the lungs.[31] A transient adhesion of neutrophils to the vascular endothelium, involving the leukocyte integrins, a group of leukocyte receptors referred to as the CD11/CD18 complex, has been demonstrated *in vitro*.[32] Reversal of this process appears to be responsible for the rapid reappearance of the adherent neutrophils to the circulation. A similar series of mechanisms could explain the recruitment of marginated and bone marrow stores of neutrophils by chemotactic factors. The morphologic changes in marginated neutrophils stimulated by chemotactic factor may help to dislodge these cells, as well as a dynamic reorganization of the CD11/CD18 complex of surface adherence molecules, thus producing the secondary phase of neutrophilia. Such events resulting from direct activation of the neutrophil by C5a would preclude the need for secondary mediator involvement.

Several chemotactic factors are known to induce neutropenia.[16,33] These same chemotactic factors might also stimulate neutrophilia if their influence on bone marrow and marginated cells leads to a mobilization by altering the adherent properties of this pool of receptor-bearing cells. In the case of f-Met-Leu-Phe, neutrophilia was not observed in rabbits receiving 0.2 μg of the peptide per

kilogram.[33] In our study, a prominent leukocytosis was induced in rabbits by f-Met-Leu-Phe at 2 μg/kg; however the cellular response was somewhat delayed in its onset (eg, 1 to 2 hours) compared to that induced by C5a (data not shown). Therefore at least two different chemotactic factors can promote a rebound leukocytosis response after neutropenia.

Lipid-mediator substances, including certain prostanoids and perhaps LTB_4, possess leukocytosis-inducing activity and could mediate the C5a effect. C5a induces a reversible systemic hypotension in rabbits when injected intravenously. This effect is found to be dependent on and parallel to the release of cyclooxygenase products. Consequently indomethacin effectively inhibited C5a-induced hypotension.[16] On the other hand, because hypotension is itself stressful and induces release of steroids, leukocytosis could, in this case, be induced by hypotension.[34] In our experiments, pretreatment with indomethacin did not prevent the elevation in leukocyte counts induced by C5a and consequently prostanoids or prostanoid-derived stress are apparently excluded as a cause of the neutrophilia promoted by C5a.

In Table 1, monocytes, eosinophils, and basophils, as well as neutrophils, disappeared from circulation immediately after the administration of C5a. This leukopenic response is indicative that adherent cell types[35] having C5a receptors on their surface disappear from circulation in the early phase of the response. However it is only the neutrophils that return to circulation in excess of their original circulating levels. The selective influence of C5a on neutrophils indicates that a specific mechanism exists for recruiting this cell type.

An unidentified leukocytosis-inducing factor is reportedly generated in blood whenever the leukocytes are removed from the circulation by endotoxin[36] or vinblastine.[37] Hemodialysis[38] and leukophoresis[39] are well-characterized blood treatments that lead to leukocytosis. Rother[9] suggested that leukocyte-mobilizing factor (LMF) may be derived from the complement component C3 and that it was a novel humoral factor capable of mobilizing bone marrow cells and causing leukocytosis. The profiles of leukocytosis that are produced by hemodialysis[38] or leukophoresis[39] are very similar to that induced by either C5a or the C3 fragments. Therefore leukocytosis induced by extracorporeal treatment of blood appears to result from the generation of one or both of these active complement fragments. When one considers the duration and magnitude of the late neutrophil response to C5a, it is apparent that this factor may play an important role in the systemic recruitment of neutrophils. The homeostatic mechanisms for replacement of locally sequestered cells or of depleted neutrophil stores, particularly from inflammatory responses, may involve the same factor that initiates depletion *via* its chemotactic and permeability functions. This concept is illustrated in Figure 6, in which the dynamic action of C5a, in stimulating neutrophils and other cell types to become adherent over a relatively short period of time, is overcompensated by the ability of this same factor to induce mobilization of bone marrow and marginated cells. The late-phase recruitment of segmented and nonsegmented neutrophils by C5a is a dynamic means of replenishing a depleted circulating pool. Although other mediators, including C3-derived complement factors, participate in the same process of recruitment to establish neutrophil homeostasis, the role of C5a must be considered whenever complement is involved.

Humoral Regulation of Circulating Neutrophils: A Proposed Scheme for Homeostasis

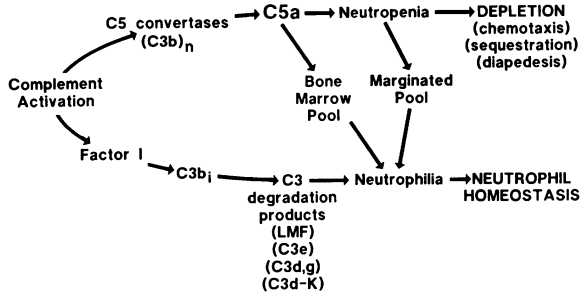

Figure 6. *Complement activation leads to an enzymatic conversion of the third component C3 to C3a and C3b. Factor I cleaves C3b to C3bi and further degradation leads to various bioactive C3 fragments known to promote neutrophilia. Late component activation results in the cleavage of C5 to C5a and C5b. The fragment C5a is a potent chemotactic factor and activator of granulocytes. The ability to promote an immediate and transient neutropenia followed by a prolonged neutrophilia indicates a potentially significant role for C5a in leukocyte homeostatic mechanisms associated with infections and inflammation.*

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Acknowledgment

The authors thank Ellye Lukaschewsky for her secretarial assistance.