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Enhancement of granulocyte–endothelial cell adherence and granulocyte-induced cytotoxicity by platelet release products*

(serotonin/complement activation/neutrophil adherence/atherosclerosis)

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ABSTRACT **Complement-stimulated granulocytes adhere to and induce significant ^{51}Cr release from endothelial cells *in vitro*. Platelets were stimulated to undergo release, and these release products significantly enhanced granulocyte–endothelial cell adherence and granulocyte-induced ^{51}Cr release from endothelial cells. Platelet serotonin appeared to mediate these phenomena because serotonin antagonists blocked both the enhanced endothelial adherence and ^{51}Cr release. In addition, added serotonin mimicked the effect seen with the stimulated platelets upon granulocyte–endothelial cell adherence and cytotoxicity completely. This enhancement appeared to be due to serotonin effects upon both the granulocyte and endothelial cells. These data suggest that a released platelet constituent might modulate *in vivo* granulocyte–endothelial cell interactions in clinical disorders.**

Endothelial injury, followed by subendothelial cell proliferation, is thought to play a prominent role in virtually all models of vascular injury and atherogenesis (1). Speculation on the etiology of the proliferative response has centered upon the role of platelet-derived "growth factors" (2); however, the mechanisms for initiating endothelial cell damage have been given less attention. Recent studies from our laboratory have focused upon the damaging effects on the vascular endothelium of granulocytes (polymorphonuclear leukocytes) that have been stimulated by various immunologic stimuli, particularly activated complement components (3). These studies were fostered by our interest in the acute pulmonary dysfunction that occurs early in hemodialysis (4). Abnormalities in pulmonary dynamics that hemodialyzed patients manifest also may be reproduced in laboratory animals by reinfusing them with autologous plasma that had been allowed to contact hemodialyzer membranes (5, 6); pulmonary microvascular damage and associated pulmonary interstitial edema were evident in these animals (5, 6).

By visualizing the microvasculature of rat mesenteries through laser intravital microscopy, Hammerschmidt *et al.* (7) observed that infused complement, particularly C5a, first promoted granulocyte adhesion to endothelium, followed shortly thereafter by plasma leak into interstitial tissues (7). A potential mechanism for this vascular damage was suggested by *in vitro* studies of cultured endothelial cells (8). When exposed to granulocytes and a source of activated complement, labeled endothelial cells released chromium, and this release was largely inhibited by superoxide dismutase and catalase. It appeared likely, therefore, that the complement-stimulated production of reactive oxygen species by granulocytes underlies the endothelial cell alteration, a surmise validated by recent studies of others (9).

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Because platelets and their products have been suggested as important effectors in atherogenesis—possibly in proliferative response of injured vessels—we undertook the present *in vitro* studies to determine their possible role in complement-mediated vascular injury. This report indicates that platelets, although ineffectual alone, exacerbate the release of radioactive label from cultured endothelial cells exposed to complement-coated particles with granulocytes. One mediator of this amplification appears to be the platelet constituent, serotonin, which we find enhances the adhesion of complement-stimulated granulocytes to their target endothelial cells. The results, reported preliminarily elsewhere (10), suggest that platelets may play an endothelium-damaging role in addition to their previously suspected effects of inciting vascular wall proliferation.

METHODS AND MATERIALS

Preparation of Endothelial Cells. Endothelial cells were grown in culture in microtiter wells by our previously reported modification of established methods (8, 11, 12). Cells were cultured under 95% air/5% CO_2 at 37°C in medium 199 containing 20% calf serum (GIBCO); they were used ≈5 days after explanation, just prior to reaching confluence, and were identified as endothelium by their reaction with rabbit antisera to human Factor VIII antigen (13) (Boehringer Corporation, New York, NY). In selected studies, the cultured cells were incubated with sodium arachidonate (100 μM) or thrombin (1 unit/ml) for 5 min; the supernatant fluid was then aspirated and assayed for the stable prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ by using a radioimmunoassay (New England Nuclear).

Blood Cell Preparation. Granulocytes were harvested from heparin-treated (6–8 unit/ml) volunteer peripheral blood by using standard techniques of dextran sedimentation, hypotonic lysis, and Ficoll/Hypaque sedimentation (8, 14). The enriched granulocytes were washed and resuspended (final concentration, 1×10^7 cells per ml) in Hanks' balanced salt solution (Microbiological Associates, Walkersville, MD) containing 0.5% human albumin. The purified granulocytes contained 5–10% mononuclear cells and $<1 \times 10^6$ platelets per ml. Platelets were harvested from citrated (0.36%) peripheral blood by centrifugation (400 g for 5 min) to yield platelet-rich plasma (PRP). "Purified platelets" were obtained by centrifugation of PRP (1,400 × *g* for 10 min), with resuspension in Ca/Mg-free Hanks'

Abbreviation: PRP, platelet-rich plasma.

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balanced salt solution (final concentration, 5×10^8 platelets per ml).

Determination of ^{51}Cr Release. Endothelial cells were labeled by addition of 5 μCi (1 Ci = 3.7×10^{10} becquerels) $Na_2{}^{51}CrO_4$ (New England Nuclear) to serum-free medium 16 hr before planned experiments (8, 11). In each experiment, controls for spontaneous ^{51}Cr release consisted of labeled cells from the same microtiter plate treated identically but without addition of immune reactants. Specific release refers to that obtained by subtracting the release from cultures containing unstimulated neutrophils from those that contained stimulated neutrophils. The reactants included, where appropriate: (*i*) 5×10^6 neutrophils; (*ii*) PRP or "purified platelets" (2×10^8); (*iii*) zymosan (Nutritional Biochemicals, Cleveland, OH) opsonized in fresh serum (30 min at 37°C) and resuspended to 5 mg/ml in Ca/Mg-free Hanks' balanced salt solution; (*iv*) various concentrations of serotonin (5-hydroxytryptamine; Sigma); (*v*) 100 μM methysergide maleate (a gift of Sandoz Pharmaceutical); or (*vi*) 1 μM imipramine hydrochloride (Sigma). The reaction wells, which contained $\approx 3 \times 10^5$ labeled endothelial cells and specific reactants in a final volume of 1.5 ml, were incubated at 37°C for 30 min and washed three times with Hanks' balanced salt solution containing 0.5% albumin. The washes were pooled for counting. The still-attached endothelial cells were mechanically removed from the wells and separately counted. For calculation of ^{51}Cr release, care was taken to sediment and recombine with the endothelial cell fraction any intact endothelial cells removed during washing; >90% of chromium was released from these cells during three freeze/thaw cycles. Our previous experiments (8, 11) had demonstrated that resting granulocytes or activated serum complement, when added separately, do not induce significant ^{51}Cr leak; this was confirmed in the present study.

The validity of using ^{51}Cr release as a marker of endothelial cell alteration was strengthened by using another cell label, [^{14}C]thymidine, which was preincorporated into endothelial cells for 72 hr prior to the experiment. In both situations, the released supernatant radioactivity (^{51}Cr or ^{14}C) was expressed as a percentage of the total cell plus supernatant fluid radioactivity recovered from each well.

Granulocyte Adherence Assay. Purified granulocytes (5×10^6) were placed atop either endothelial cells or "naked" plastic microtiter tissue culture wells (201 mm^2, Costar, Cambridge, MA) in the presence of Hanks' balanced salt solution with albumin (0.5%) or in this same buffer containing 15% (vol/vol) heated or zymosan-activated serum. After incubation for 30 min at 37°C, nonadherent cells were removed by vigorous aspiration and two additional washes. Adherent granulocytes were then removed with trypsin/EDTA; microscopic inspection revealed recovery of all granulocytes. The myeloperoxidase activity in both the pooled aspirates and adherent fractions was then determined by standard techniques (11, 15); the percentage adherence was calculated by dividing the myeloperoxidase activity of the adherent fraction by the total activity recoverable in each well. Recovery of myeloperoxidase activity was close to 100% of that predicted, and endothelial cells contained no demonstrable myeloperoxidase activity.

Granulocyte Function Studies. Granulocyte superoxide anion (O_2^-) was determined by the capacity of O_2^- to reduce ferricytochrome *c*, as previously described by Goldstein *et al.* (16). Release of H_2O_2 by purified granulocytes was assayed by using the decrease in fluorescence intensity of scopoletin due to its peroxidase-mediated oxidation by H_2O_2 (11, 17). cAMP levels were determined by radioimmunoassay (New England Nuclear) with purified granulocytes after their exposure to serotonin. After incubation at 37°C for various periods of time, the reactions were terminated by placing the tubes in boiling water (5 min), and extracting the cells by freezing and thawing in distilled water. Protein was measured by the method of Lowry *et al.* (18). Neutrophil hexose monophosphate-shunt activity was determined in granulocytes incubated with 1 μCi of D-[1-^{14}C]glucose (New England Nuclear) as described by DeChatelet *et al.* (19).

RESULTS

Platelets Amplify Granulocyte-Mediated Release of Endothelial Radiolabel. In confirmation of our earlier studies (8, 11), endothelial cells exposed to granulocytes that are stimulated by activated complement—either as zymosan-activated plasma (not shown) or as zymosan particles opsonized by serum complement components (shown in Table 1)—released significant amounts of $Na_2{}^{51}CrO_4$ (Table 1, line 2). To determine the possible role of circulating blood cells other than granulocytes, we exposed 1×10^7 cells from whole "buffy coat," rather than density-separated granulocytes, to opsonized zymosan and then added this stimulated, mixed cell population to the labeled endothelial cells; "buffy coat" cells induced roughly a 2-fold increase ($P < 0.01$) in specific ^{51}Cr release from the endothelial cells compared to that noted with more purified granulocytes (Table 1, line 3). The augmented specific ^{51}Cr release probably reflects the presence of platelets in the "buffy coat" because addition of PRP in quantities adjusted to the same approximate platelet radioactivity as that in "buffy coats" produced a similar amplification of endothelial cell ^{51}Cr release (Table 1, line 4).

Table 1. Platelets augment granulocyte-mediated ^{51}Cr release from endothelial cells

	^{51}Cr release		
	n	%	*P*
SAZ*	5	1.4 ± 0.6	—
PMN + SAZ	10	5.5 ± 0.8	—
"Buffy coat" cells + SAZ	3	9.7 ± 1.2	<0.05
PMN + platelets† + SAZ	8	10.0 ± 1.0	<0.01
Platelets† + SAZ	3	1.9 ± 1.6	—
PMN + platelet supernatant‡ fraction + SAZ	3	11.9 ± 0.8	<0.01
PMN + platelet pellet fraction‡ + SAZ	3	5.1 ± 1.1	—
PMN + aspirin-treated platelets§ + SAZ	3	4.6 ± 1.4	—

SAZ, serum-activated zymosan; PMN, granulocyte(s). Results are expressed as the mean percentage release ± SEM (30 min) of ^{51}Cr minus the spontaneous release from untreated cells in parallel wells. Each experiment utilized as an internal control the spontaneous ^{51}Cr release from cells grown from the same pool of umbilical cords. Mean spontaneous release was 4.4% per 30 min. Previous studies indicated that granulocytes alone cause no significant ^{51}Cr release (8, 11); individual studies were run in triplicate.

* +Zymosan (5.0 mg/ml) was added to fresh serum and incubated for 30 min; 0.15 ml was then added to the reaction wells.

† PRP (0.4 ml; 2×10^8 platelets) was added prior to addition of the zymosan particles. Release of labeled serotonin from platelets ranged between 35% and 57% with opsonized zymosan stimulation of platelet release.

‡ Platelet release supernatant fraction was prepared by incubating PRP with zymosan particles. After 30 min, the platelets and zymosan were sedimented (20,000 × *g* for 15 min), and the supernatant fraction or the pellet (resuspended in an equivalent volume of zymosan-activated serum) was then added to the endothelial cells.

§ Aspirin-treated platelets were obtained from normal volunteers who had taken 600 mg of aspirin 12 and 24 hr prior to phlebotomy. These platelets did not undergo aggregation in the presence of opsonized zymosan (5.0 mg/ml). *P* values represent comparison with values in line 2 and were performed by utilizing a standard two-tailed *t* test.

However, complement-triggered platelets alone—that is, without added granulocytes but in the presence of activated complement—induced no significant ^{51}Cr release (Table 1, line 5). "Purified platelets" (PRP further enriched in platelets by recentrifugation) amplified endothelial ^{51}Cr release to a similar degree (results not shown). Furthermore, the concentration of added platelets was critical, with no significant amplification of ^{51}Cr release seen with $<1 \times 10^8$ platelets and only a slightly increased release (12.1 ± 1.3%) when the platelet concentration was increased 3-fold (6×10^8).

In ancillary studies, we noted that the platelet amplification phenomenon was only discernable if complement-opsonized particles, but not fluid-phase complement (e.g., plasma after removal of zymosan particles), were present in the system. Others have demonstrated that particulate- but not fluid-phase activated complement causes the release reaction in human platelets (20–22). We confirmed this observation; release of serotonin from labeled platelets engendered by particulate activated complement ranged between 35% and 57% in five separate experiments, whereas there was virtually no release when fluid-phase complement was used to stimulate platelets labeled with ^{14}C serotonin. Therefore, we postulated that a secreted platelet product was the amplifier of granulocyte-mediated, endothelial ^{51}Cr release. Indeed, centrifuged supernatants of platelets that had undergone release by exposure to opsonized zymosan particles potently amplified endothelial ^{51}Cr release (Table 1, line 6), whereas the "spent" platelet pellet was without effect (Table 1, line 7). Moreover, platelets either obtained from donors taking aspirin (Table 1, line 8) or from a patient with storage pool disease (not shown) were incapable of amplifying ^{51}Cr release from endothelial cells.

Identification of the Platelet-Derived Amplifier. Two separate lines of evidence indicate that serotonin (5-hydroxytryptamine) may be at least one platelet-derived amplifier of granulocyte-mediated ^{51}Cr release. When imipramine, an inhibitor of serotonin uptake, or methysergide, a serotonin competitive inhibitor, were added to platelet release products, these then failed to amplify granulocyte-induced ^{51}Cr release (Fig. 1 *Left*). Moreover, serotonin itself amplified granulocyte-mediated release from endothelial cells. This amplified release also could be blocked by including the same concentrations of imipramine or methysergide (Fig. 1 *Right*) in the mixture of serotonin and stimulated granulocytes. However, serotonin in the absence of stimulated granulocytes did not cause endothelial injury (<2% specific ^{51}Cr release, $n = 5$).

Serotonin amplified endothelial chromium release induced by activated granulocytes in a dose-dependent fashion; clearly detectable amplification was observed at 0.1 μM, and maximal effects were noted between 0.5 and 1 μM (Fig. 2). Confirmatory results were obtained with [^{14}C]thymidine as the endothelial cell label: unstimulated granulocytes provoked 1.6 ± 0.5% release of this label (as trichloroacetic acid-precipitable radioactivity), which increased to 6.5 ± 1.1% with addition of opsonized zymosan and further increased to 15.5 ± 1.6% in the presence of serotonin (1 μM).

Mechanism of Serotonin Amplification. Our previous studies showed that granulocyte-mediated damage to endothelial cells requires close apposition of effector and target cells—probably because the reactive oxygen species generated by complemented-stimulated granulocytes are rapidly dissipated (8, 11). Serotonin significantly enhanced adhesion of granulocytes (whether complement-stimulated or not) to endothelium but not to plastic (Table 2), providing one plausible mechanism for its augmentation of endothelial cytotoxicity. The augmentation of granulocyte adherence induced by serotonin was prevented by methysergide or imipramine (not shown). These inhibitors did not retard granulocyte adherence when serotonin was not present. In addition, a modest stimulation of granulocyte hexose monophosphate-shunt activity by serotonin (Table 2) might contribute also to its potentiating effects because increments in this metabolic pathway generally connote increased granulocyte production of reactive oxygen species. However, and somewhat surprisingly, we could not detect a serotonin-linked increment in superoxide anion or H_2O_2 generation in either unstimulated or complement-activated granulocytes (Table 2). Moreover, serotonin did not provoke detectable release of the granulocyte lysosomal enzymes, lysozyme, or myeloperoxidase (results not shown); other granulocyte lysosomal enzymes were not assayed.

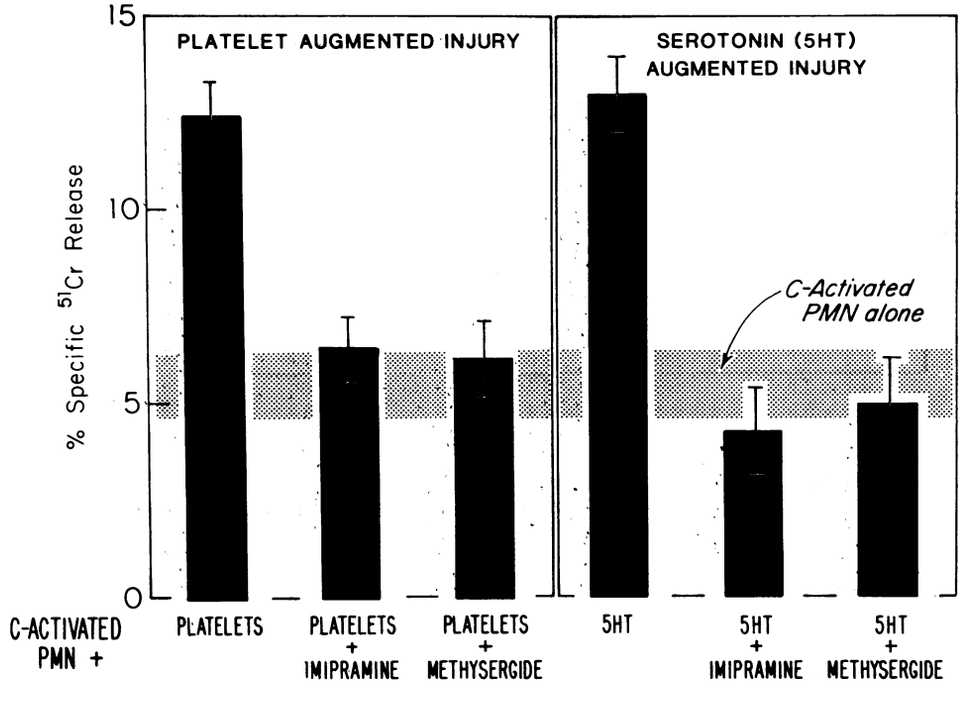

FIG. 1. Specific ^{51}Cr release from labeled endothelial cells incubated in the presence of 5×10^6 granulocytes (PMN) and opsonized zymosan. (*Left*) PRP (0.4 ml) enhances endothelial ^{51}Cr leak but not when the serotonin antagonists methysergide or imipramine were included with the granulocytes and opsonized zymosan. (*Right*) Serotonin (5-hydroxytryptamine; 5 HT) reproduces the platelet-augmenting effect. The stippled bar represents the mean and SEM for ^{51}Cr release induced by granulocytes and opsonized zymosan alone. The PRP or serotonin (1 μM) was incubated with imipramine (1 μM) or methysergide (100 μM) for 15 min in the indicated experiments, and then the granulocytes were added. This mixture (PMN, platelet release products, or 5HT, and inhibitors) was then added to the endothelial cells.

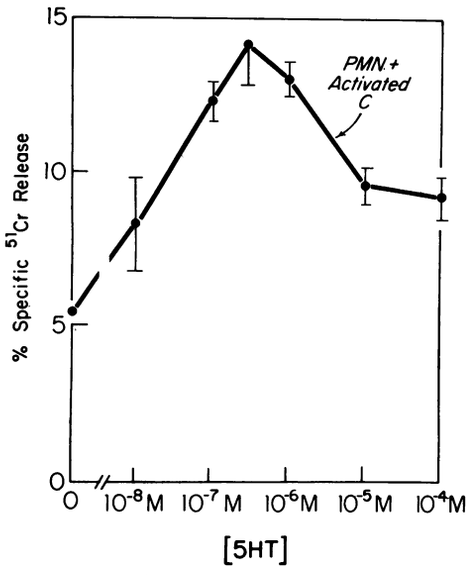

FIG. 2. Dose-dependent augmentation of granulocyte (PMN)-mediated specific ^{51}Cr release from labeled endothelial cells. PMN (5×10^6), opsonized zymosan, and various concentrations of serotonin (5 HT) were incubated with endothelial cells. Each point represents the mean and standard error of at least three separate triplicate determinations.

DISCUSSION

We have shown that complement-activated granulocytes adhere to and damage endothelial cells *in vitro* (8, 11) and microvasculature *in vivo* as well (3, 5–7). Other circulating blood cells also may affect granulocyte–endothelial cell interactions, but the present *in vitro* studies suggest that platelets augment this toxicity through release of their granule constituents, such as serotonin or one of its metabolites. That other platelet constituents also may interact with granulocytes and render them more cytotoxic has not been excluded and may be important. For instance, platelets amplify granulocyte adherence to nylon fibers (23), and others have shown platelet Factor IV can increase the activity of granulocyte proteases such as elastase (24). Moreover, evidence has been provided that thrombin-stimulated but not resting platelets (in the presence of endotoxin) enhance granulocyte adherence to nylon fibers (25), further implicating constituents of the platelet-release reaction in granulocyte function. Nevertheless, our ability to completely abrogate the platelet-augmenting effect on granulocyte-mediated endothelial radiolabel release with two serotonin antagonists, imipramine and methysergide (Fig. 1), implies that serotonin may well be a particularly important platelet constituent effecting granulocyte–endothelial interactions.

The mechanism by which added serotonin acts in this regard is not obvious. Platelets stimulated with opsonized zymosan released at least 40% of their serotonin for a final concentration of between 0.05 and 0.2 μM. Amplified ^{51}Cr release clearly was achieved at 0.1 μM, and while most of our studies were performed at a higher concentration, the dose–response curve plateaus between 1 and 0.1 μM. Serotonin itself is not toxic to endothelium; it does not alter either spontaneous endothelial leak of label or that provoked by the oxygen species generated with xanthine and xanthine oxidase (results not shown): therefore, its effects on granulocytes seem more relevant. In this regard, its capacity to increase the hexose monophosphate-shunt metabolism of granulocytes and, by implication, their production of potentially toxic oxidant compounds is probably of some importance. We find more compelling, however, the ability of serotonin to increase granulocyte adherence to target endothelial cells because, in our previous studies (3, 8, 11), diverse agents that diminish such adherence, such as cytochalasin B, lidocaine, and corticosteroids, decreased PMN-mediated endothelial cytotoxicity. How serotonin promotes granulocyte stickiness is not well defined, but a role for cAMP is not supported by data so far collected. Although cAMP of granulocytes has been found to affect their adhesion to glass and nylon fibers (26, 27), we have no evidence that altered granulocyte cAMP underlies serotonin's promotion of granulocyte–endothelial cell adhesion; that is, no detectable change in granulocyte cAMP levels accompanies serotonin exposure (results not shown). Conversely, we have gathered preliminary data that implicate endothelial cell-derived prostacyclin. As shown in Table 2, serotonin enhances granulocyte adhesion to endothelial cells but not to plastic, implying that endothelial cell function might be more than passively involved in the cell–cell interaction. Boxer and his co-workers have reported that prostacyclin decreases granulocyte adhesion to endothelium (28); therefore, any reduction in this endothelial cell-derived prostaglandin might be expected to promote granulocyte adhesion. In fact, serotonin inhibits by 34% the release of the prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ from thrombin-stimulated endothelial cells (16.7 ± 3.7 vs. 25.3 ± 1.2 μg/μg of protein) and by 15% from arachidonate-treated endothelial cells (128.1 ± 9.6 vs. 151.2 ± 7.5 μg/μg of protein). These studies support a postulated sequence: serotonin released from complement-stimulated platelets retards endothelial prostacyclin production

Table 2. Effect of serotonin on human granulocytes

PMN medium	Assays of PMN function: Superoxide*	H_2O_2†	Ratio hexose monophosphate-shunt activity‡	PMN adherence, %: Plastic	Endothelium
HBSS/albumin buffer	3.1 ± 0.7	<0.8	1.0 —	53.4 ± 9.3	7.4 ± 4.2
5HT (1 μM) + buffer	4.2 ± 1.6	<0.8	3.1 ± 0.4	47.1 ± 7.6	17.6 ± 5.8
Activated serum	27.9 ± 0.9	4.9 ± 1.2	6.4 ± 0.6	39.8 ± 6.2	45.0 ± 5.8
Activated serum + 5HT (1 μM)	25.8 ± 0.4	5.2 ± 1.6	7.0 ± 0.5	41.5 ± 7.0	55.2 ± 8.3

PMN, granulocytes; 5HT, serotonin; HBSS, Hanks' balanced salt solution. The results represent the mean and SEM from three experiments, each in triplicate. The adherence assay results represent the mean ± SD for at least five experiments, each in triplicate. Enhanced adherence in the presence of serotonin was significant ($P = 0.01$) in Student t test.

* Expressed as nanomoles of cytochrome c reduced per 5×10^6 PMN per 15 min.

† Expressed as nanomoles per 1×10^6 PMN per 10 min.

‡ Hexose monophosphate-shunt activities, measured as $^{14}CO_2$ release, are expressed as the cpm in stimulated cells divided by the cpm obtained with the same number of resting neutrophils in parallel flasks. Serum-activated zymosan particles were prepared as described in Table 1 and were used in metabolic studies; for adherence studies, zymosan-activated serum (15%, vol/vol) was used instead of opsonized zymosan particles.

or release; as a result, potentially toxic granulocytes may more readily adhere to and damage the endothelium.

The temptation to extrapolate these results to situations in which *in vivo* vascular injury occurs is tempered by the artificiality of our *in vitro* system. The actual meaning of endothelial ^{51}Cr (or [^{14}C]thymidine) release is not clear. Our previous studies (8, 11), which used a confirmatory assay of cytotoxicity (that of fluorochromasia), did support the suggestion that radiolabel release, at least in part, represents true cytotoxicity. However, other possibilities such as enhanced endothelial membrane turnover or nonlethal cellular "leak" are not completely excluded. In addition, others using similar, but not identical, endothelial cell culture systems have suggested that released granulocyte proteases may cause endothelial cell detachment from culture dishes rather than cell death (29). We believe this formulation unlikely in our studies because our assay methods recapture detached, intact cells by centrifugation of culture supernates and include their radioactivity with that of the cell contents. The assay medium contains serum or plasma that inhibits endothelial-detaching proteases released by complement-stimulated granulocytes (29). In fact, only 2–10% of the cell-associated radioactivity usually detached during the assay. There was no relationship between cellular detachment and soluble ^{51}Cr release in the presence or absence of serotonin in the assay mixture (not shown).

With this caveat, our studies suggest that platelets that become adherent to sites of *in vivo* endothelial injury might by their release of serotonin (and other constituents) promote adhesion of granulocytes to the same or neighboring sites. If these granulocytes are or subsequently become stimulated, as by exposure to activated complement, further endothelial injury might supervene. Although this formulation—particularly the role of serotonin in vascular injury—will need to be validated by further experiments, it has not escaped our attention that hyperserotoninemic individuals with the carcinoid syndrome develop pulmonary arteriosclerosis in addition to right-sided endocardial damage. (30, 31).

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