Biochemical Pharmacology, Vol. 35, No. 15, pp. 2601–2606, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

ROLE OF POLYMORPHONUCLEAR LEUKOCYTES IN CONNECTIVE TISSUE BREAKDOWN DURING THE REVERSE PASSIVE ARTHUS REACTION

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(*Received* 9 *October* 1985; *accepted* 6 January 1986)

Abstract—The reverse passive Arthus (RPA) reaction performed in the skin of rats was modified to allow for the determination of polymorphonuclear leukocyte (PMN) infiltration and hemorrhage, as well as changes in vascular permeability. After initiation of the RPA reaction, PMN infiltration, monitored by measurement of tissue myeloperoxidase (MPO, EC 1.11.1.7) content, increased dramatically with time. Depending on the experimental conditions used, PMN accumulation reached a maximum 2–10 hr after increased vascular permeability (^{125}I-labeled albumin content) had peaked. Hemorrhage (^{59}Fe-labeled erythrocyte accumulation) began to occur only after significant levels of PMN were reached and continued to increase proportionately to the level of PMN infiltration attained. Indomethacin administered 30 min prior to initiating the RPA reaction had no effect on vascular permeability increase but suppressed both PMN accumulation and hemorrhage development about 50%. When indomethacin was given 2 hr after the RPA reaction was begun, no effect on any of the RPA variables was noted. Dexamethasone suppressed the increase in vascular permeability (53%), PMN accumulation (78%), and hemorrhage (90%) when given 30 min prior to initiation of the reaction. Dexamethasone given 2 hr after initiating the RPA suppressed the entire reaction, but to a lesser extent. Catalase, as well as trasylol, alpha-1-antiproteinase and soybean trypsin inhibitor, inhibited PMN accumulation as well as hemorrhage when given intravenously at plus 2 hr. These results indicate that the damage to blood vessels during a severe RPA reaction is a direct consequence of PMN activity.

The involvement of PMN in the reverse passive Arthus (RPA†) reaction has been well documented [1]. Crawford *et al.* [2, 3] have developed an inflammatory model in rabbits, based on the cutaneous RPA reaction, in which radiolabels are used to measure PMN, erythrocyte, platelet and plasma protein accumulations at the RPA site. These same techniques were used by Kopaniak *et al.* [4, 5] to measure inflammatory changes induced by *Escherichia coli* injected into rabbit skin. Their results added further confirmation that PMN are intimately involved in the tissue damage occurring during an inflammatory reaction such as the RPA.

The mechanism by which polymorphonuclear leukocytes damage tissue during inflammatory reactions has been postulated to involve enzymes and/or oxidative products derived from PMN [6–8]. Much of this evidence has been obtained from *in vitro* experiments examining release of degradative products from PMN in response to inflammatory stimuli [9, 10]. Oxygen metabolites are produced by PMN, such as superoxide anion, peroxides, and oxygen radicals [11], and are potentially damaging to tissue. Recently, *in vivo* experiments have produced added evidence for the direct involvement of PMN products in tissue damage. Hydroxyl radicals have been implicated in tissue injury occurring during the RPA [12]. Also, PMN lysosomal enzymes, capable of degrading connective tissue, may be released outside of these cells at the sites of inflammation [13]. Proteinase inhibitors, such as alpha-1-antiproteinase, present in the circulation and tissue fluids, are capable of neutralizing these enzymes. However, these antiproteinases are themselves susceptible to inactivation by PMN-derived oxygen species and myeloperoxidase-catalyzed reactions that utilize hydrogen peroxide and halide ion as cofactors [11, 14]. Revak *et al.* [15] have reported recently on a model of experimental pulmonary inflammation in monkeys in which tissue injury was related to the generation of both proteinases and oxidants. Thus, both enzymes and oxidative products from PMN appear to be intimately involved in the tissue damage occurring during acute inflammatory reactions.

In vivo experiments have mainly monitored vascular permeability changes and edema formation as the measure of tissue damage mediated by PMN. Although these changes also occur in tissues during inflammatory disease, the effects are transient and do not result in the irreversible damage to tissue seen in such diseases as rheumatoid arthritis. We have developed a model of tissue damage based on a severe RPA response in the skin of rats. Hemorrhage measurement (extravasation of ^{59}Fe-labeled erythrocytes [16], rather than measurement of the more transient increase in vascular permeability, was used as the indicator of vascular damage during the RPA response. Vascular permeability increase was measured as accumulation of ^{125}I-labeled albumin.

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† Abbreviations: RPA, reverse passive Arthus; PMN, polymorphonuclear leukocytes; and MPO, myeloperoxidase.

The myeloperoxidase content of the skin site was taken to be indicative of the amount of PMN infiltration [17]. This system was characterized using the standard anti-inflammatory compounds, indomethacin and dexamethasone. In addition, various enzyme inhibitors were tested in an attempt to demonstrate a direct role for PMN enzymes in tissue degradation, resulting in the hemorrhage. The results indicated that a correlation existed between the amount of PMN accumulating in the RPA skin sites and the degree of vascular damage that occurred. The effect of enzyme inhibitors in reducing the hemorrhage associated with the skin reaction could not be attributed solely to direct inhibition of PMN degradative enzymes, since all of these agents decreased the amount of PMN infiltration.

MATERIALS AND METHODS

RPA reaction. Rats whose erythrocytes had been labeled previously with ^{59}Fe were used. Metofane inhalant anesthesia was used at each injection step. Each rat was shaved along the backside and injected intradermally at four sites with 50 μl of rabbit anti-ovalbumin serum containing 200 μg antibody protein (antiserum was reconstituted from lyophilized whole serum, Cappel Laboratories, West Chester, PA) followed immediately by an intravenous injection of 2 mg ovalbumin (Calbiochem, La Jolla, CA) and 1 μCi ^{125}I-labeled human serum albumin (Mallinckrodt, St. Louis, MO) in 1 ml saline. Controls received no ovalbumin in the intravenous injection. At specific times, 1 ml of blood was removed by cardiac puncture. The animals were then killed using carbon dioxide asphyxiation, and the skin areas containing the RPA sites were removed. Each reaction site was punched from the skin using a 16-mm metal punch. The isotope contents of the blood and skin samples were determined by counting in an Autogamma 800 (Packard Instrument Co., Downers Grove, IL), and the counts were corrected for isotope crossover between channels. The skin sites were then homogenized in 10 ml of 0.5% hexadecyltrimethylammonium bromide using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After centrifugation, the supernatant fractions were assayed for MPO activity as described below.

In vivo *erythrocyte labeling.* Male CDF rats (Charles River, Kingston, NY) (200 g) were injected intravenously with 0.5 ml saline containing 10 μCi [^{59}Fe] ferrous citrate (1 μCi/μl, New England Nuclear, Boston, MA) immediately after withdrawal of 2.5 ml blood by cardiac puncture to stimulate erythropoiesis. Rats were used for RPA experiments 3–4 days later when analyses of blood samples showed that all (>99%) of the radioisotope was bound within the erythrocytes [16].

Myeloperoxidase assay. Samples were assayed for MPO content using a modification of the method described by Bradley *et al.* [17]. Samples (10 μl) were mixed with 200 μl of 50 mM phosphate buffer, pH 6.0, containing 1 mM *O*-dianisidine dihydrochloride and 0.001% hydrogen peroxide in microtiter plate (Costar, Cambridge, MA) wells. Absorbance at 450 nm was determined using a Multiscan MC microtiter plate scanner (Flow Laboratories Inc., McClean, VA) after color development had been stopped by the addition of 10 μl of 0.2% sodium azide.

Compound testing. Compounds were administered as solutions in 1 ml PBS by intravenous or intraperitoneal injections at the times and doses stated. Control and reaction groups received an equal volume of saline using the same protocol as test animals.

Calculation of results. The accumulation of erythrocytes in the RPA skin site (hemorrhage) was expressed in terms of μl equivalents of blood. This value was obtained for each rat by dividing the average ^{59}Fe/cpm of the skin sites by the ^{59}Fe/cpm of 1 μl of whole blood. The amount of accumulated plasma protein (vascular permeability) was expressed as μl equivalents of plasma by a similar calculation using the ^{125}I/cpm of the skin sites and blood plasma. The spectrophotometric reading (A_{450nm}) of the myeloperoxidase assay was used directly as an expression of the amount of PMN accumulation in the RPA sites. For all three RPA variables, the values reported have control values subtracted.

RESULTS

Cutaneous RPA model. Following initiation of the RPA reaction, vascular permeability, PMN infiltration and hemorrhage increased in a time-dependent manner. The time course for development of each of the three RPA variables measured under the conditions used for compound testing is shown in Fig. 1. Accumulation of plasma proteins peaked much earlier than PMN accumulation or hemorrhage development, reaching about 75% of maximum during the first hour. PMN accumulation, as measured by myeloperoxidase activity, typically reached a maximum between 4 and 6 hr, the increase beginning as vascular permeability reached maximum. Erythrocyte accumulation increased in parallel with PMN accumulation but did not begin until a certain level of PMN infiltration occurred. These time relationships among the three RPA variables remained the same, regardless of the severity of the RPA reaction or changes in its overall time course achieved by varying the conditions (severity of the RPA was found to increase in proportion to the amount of antiserum used and the reaction was found to develop more quickly in older rats—data not shown). An example of this is shown in Fig. 2, where the use of younger rats resulted in a slower development of the total RPA reaction. Regardless, the vascular permeability increase developed far more rapidly than PMN infiltration, and hemorrhage occurred in proportion to the amount of PMN accumulated at the skin sites.

A direct correlation (correlation coefficient = 0.75) existed between PMN accumulation (as measured by myeloperoxidase levels) and hemorrhage development (Fig. 3). Likewise, a correlation (correlation coefficient = 0.89) between total vascular permeability increase and maximum PMN accumulation was found in a series of RPA reactions of various intensities (Fig. 4).

Although histological examination of the RPA reaction sites showed extravasation of erythrocytes, vasodilation that resulted in an increased total blood

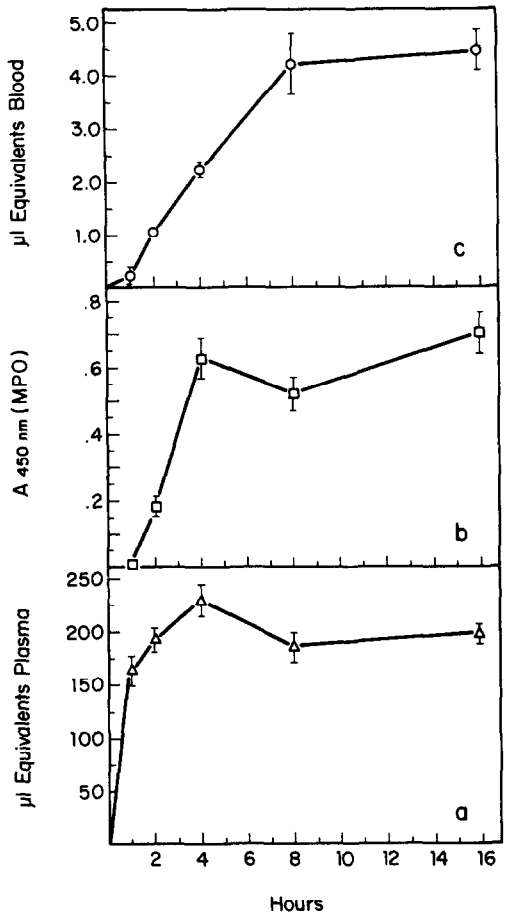

Fig. 1. Time course of the cutaneous RPA reaction in the rat. Rats (200 g) were injected intradermally with antiserum to ovalbumin (50 μl containing 200 μg of antibody protein) followed immediately by an intravenous injection of 2 mg of ovalbumin. Animals were killed at various times after initiating the RPA, and vascular permeability (a), PMN accumulation (b) and hemorrhage (c) were measured as described in the text. Results are expressed as the mean ±S.E.M. (N = 4).

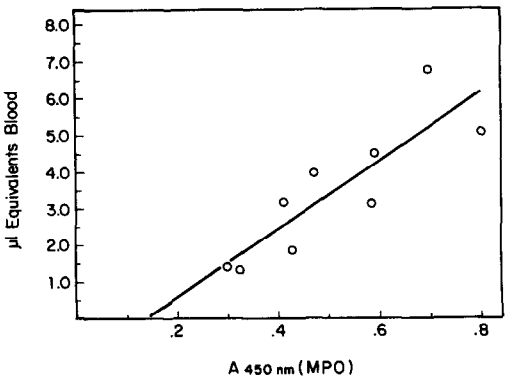

Fig. 3. Linear regression plot of hemorrhage vs PMN accumulation in response to development of the RPA in the skin of rats. Data points represent 8-hr RPA development using different conditions to evoke reactions of various intensities (various rat sizes and antibody concentrations). Below a certain level of PMN accumulation, hemorrhage did not develop. These points were omitted from the linear regression plot (correlation coefficient = 0.744).

flow might account for the increased ^{59}Fe content of these sites. To show that this was not the case, ^{51}Cr-labeled erythrocytes were injected intravenously into the rats and allowed to disperse throughout the circulation for 5 min before killing the animals and measuring the RPA variables. ^{51}Cr content was then used to calculate the amount of blood in each skin site in the same manner as used with ^{59}Fe. ^{51}Cr-labeled erythrocyte content (blood volume) of the RPA sites was the same as that of the control sites, indicating that increased erythrocyte accumulation, as measured by ^{59}Fe content, was not merely due to increased vascular volume (Fig. 5).

Effect of anti-inflammatory drugs. The effects of various agents on the RPA reaction were determined by using single or multiple injections at the times

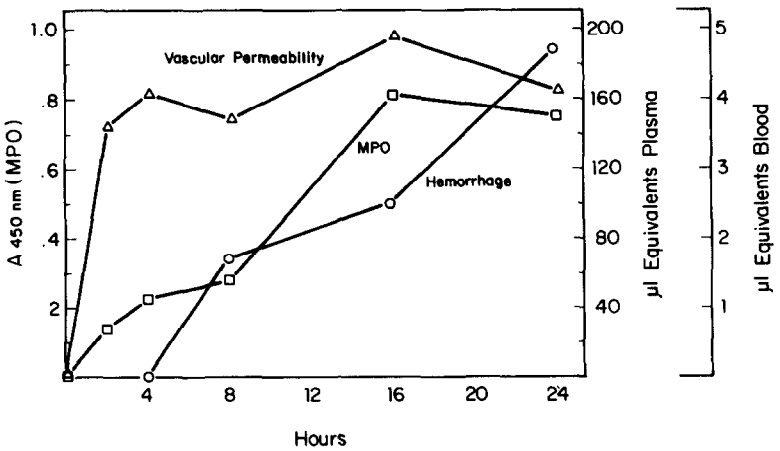

Fig. 2. Extended time course for the cutaneous RPA. Conditions were the same as those described for Fig. 1 except that the antiserum concentration was halved, and the rat weights were 40% lower.

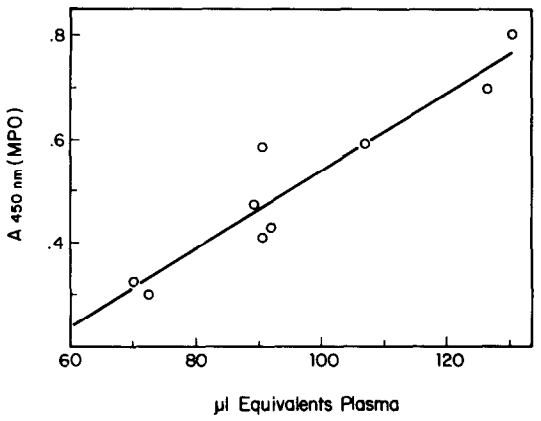

Fig. 4. Linear regression plot of PMN accumulation vs vascular permeability increase in response to development of the RPA in the skin of rats. Conditions were the same as described for Fig. 3. Correlation coefficient = 0.89.

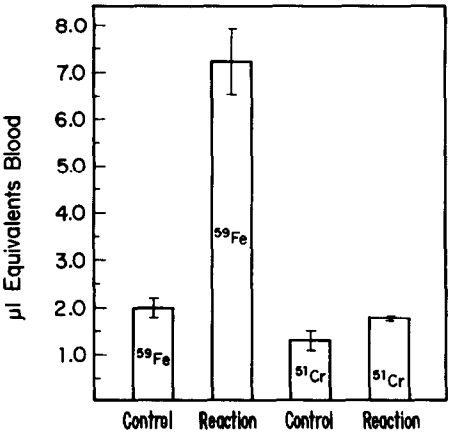

Fig. 5. Verification of hemorrhage readout. ^{51}Cr-labeled erythrocytes were injected into rats and allowed to disperse in the circulation for 5 min before sacrifice and measurement of the RPA variables as described in the text. ^{51}Cr content was then used to calculate the μl equivalents of blood in each skin site in the same manner as used for ^{59}Fe. Controls received antiserum intradermally but no ovalbumin in the intravenous injection. Results are expressed as mean ±S.E.M. (N = 4).

noted in Table 1. Since the survival times of most of these agents in circulation were not known, multiple injections were given to increase the chance of an effect over the 6-hr RPA reaction period. Indomethacin administered by intraperitoneal injection prior to initiating the RPA had little effect on vascular permeability but suppressed PMN accumulation and hemorrhage development. Given 2 hr

Table 1. Inhibition of the cutaneous RPA by various enzyme inhibitors and anti-inflammatory agents

Compound	Dose (mg/kg)	Dosing regimen*	% Inhibition		
			Vascular permeability	Hemorrhage	PMN accumulation
Indomethacin	6	30 min prior (i.p.)	29 ± 7 8 ± 8	62 ± 20 25 ± 6	59 ± 7 45 ± 6
	3	2, 4, 6 hr (i.p.)	−6 ± 2 −13 ± 5 6 ± 3	−6 ± 2 −8 ± 21 2 ± 18	11 ± 7 −18 ± 3 −7 ± 7
Dexamethasone	0.4	30 min prior (i.p.)	56 ± 10 49 ± 11	100 ± 15 79 ± 5	80 ± 10 76 ± 7
		2, 4, 6 hr (i.p.)	31 ± 3	74 ± 14	35 ± 8
Catalase (10, 280 units/mg)	50	2, 4, 6 hr (i.v.)	30 ± 0.3	77 ± 18	52 ± 8
	50	2 hr (i.v.)	−11 ± 11 28 ± 5	53 ± 11 61 ± 6	73 ± 8 37 ± 7
	25	2 hr (i.v.)	14 ± 7	55 ± 7	63 ± 4
	10	2 hr (i.v.)	8 ± 4	7 ± 7	26 ± 7
Soybean trypsin inhibitor	25	2, 4, 6 hr (i.v.)	8 ± 3	32 ± 9	27 ± 3
Trasylol (5,000 units/mg)	8	2, 4, 6 hr (i.v.)	7 ± 3	36 ± 16	8 ± 5
Alpha-1-antitrypsin	40	2, 4, 6 hr (i.v.)	33 ± 9	83 ± 16	46 ± 13
	50	2 hr (i.v.)	−6 ± 7 8 ± 7	58 ± 6 75 ± 13	76 ± 8 46 ± 10
	25	2 hr (i.v.)	6 ± 4	49 ± 14	55 ± 2
	10	2 hr (i.v.)	−1 ± 7	−27 ± 4	21 ± 6

Compounds were injected at the times and by the routes indicated. RPA variables were measured 8 hr after initiation of the reaction. Control and reaction standards were run simultaneously with each test group as described in the text. Values are expressed as a percentage reduction from the reaction group after subtracting the controls. Values are given as mean ±S.E.M. for each experiment (N = 4).

* Compounds were given by single or multiple injections at the times indicated after, or prior to, initiation of the RPA. Intravenous, i.v.; intraperitoneal, i.p.

after beginning the RPA, indomethacin produced no effects on any of the variables. Dexamethsone, a steroidal anti-inflammatory drug, suppressed the entire RPA response when given 30 min before or 2 hr after initiating the RPA.

Effects of enzyme inhibitors. In general, catalase, as well as the proteinase inhibitors alpha-1-antitrypsin, soybean trypsin inhibitor and trasylol, inhibited both PMN accumulation and hemorrhage development. No effect was seen on vascular permeability change using any of the enzyme inhibitors. Animals were injected with PMN proteinase inhibitors 2 hr after beginning the RPA in order to minimize their effect on the enzyme systems necessary for initiating the inflammatory response, such as complement activation and liberation of chemotactic factors, but early enough to be present in the circulation before tissue damage was severe enough to cause hemorrhage.

DISCUSSION

The purpose of this work was to provide a model in which to obtain direct evidence for the involvement of PMN enzymes in the tissue damage that occurs during inflammatory reactions. The cutaneous RPA reaction has been investigated extensively as a model of the inflammatory response and has been shown to involve formation of immune complexes in the blood vessel walls of the skin [18], release of chemotactic factors as a consequence of complement activation [19], and infiltration of polymorphonuclear cells into the RPA site [20]. Previous studies have mainly focused on the use of edema and vascular permeability determinations as a means of measuring the physiological consequences of this series of events, although hemorrhage and necrosis have also been shown to occur. Both are normal, reversible responses of tissue to inflammatory stimuli. Therefore, we decided to study the possibility of using hemorrhage as an indicator of irreversible tissue damage, which may be more representative of the type of connective tissue damage that occurs in chronic inflammatory disease states. Such a model has been developed in the rabbit by Crawford *et al.* [2, 3] to study the inflammatory responses occurring during a cutaneous RPA reaction. We have adapted their technique of measuring skin hemorrhage to our rat model.

In this report, we have used the cutaneous RPA model to present evidence that the tissue damage that results in hemorrhage during the RPA reaction is dependent on the infiltration of PMN. PMN accumulation always preceded hemorrhage development, and the total amount of hemorrhage always reflected the quantity of PMN infiltrating the skin site. Steroidal as well as nonsteroidal anti-inflammatory drugs can suppress PMN infiltration of the RPA skin site [21, 22]. We have found similar results. In addition, these compounds, indomethacin and dexamethasone, which suppressed PMN infiltration, suppressed hemorrhage development as well. Therefore, a strong correlation exists between PMN accumulation and the development of tissue damage as measured by hemorrhage.

A correlation also existed between the maximum increase in vascular permeability and the final amount of PMN infiltration of the skin sites during the RPA reaction. Indeed, the final increase in permeability at 2 hr could be used to predict the ultimate severity of the reaction, in terms of how much PMN accumulation and hemorrhage would occur. PMN play a role in the increased leakage of plasma fluids and proteins into tissue during inflammatory reactions [23, 24]. However, since vascular permeability in the RPA always peaked much earlier than PMN accumulation, these permeability changes must require only a small fraction of the PMN that eventually accumulate at the RPA site. Our results are similar to those of Crawford *et al.* [2, 3]. They showed that the rate of albumin leakage from the blood vessels, during the RPA, had peaked at 2 hr and was minimal at 4 hr. PMN continued to accumulate up to 8 hr, the point at which total hemorrhage was maximum. Kopaniak *et al.* [4, 5] have shown that, in an inflammatory response in the rabbit, the maximum rate of PMN infiltration coincides with the peak of the vascular permeability increase, occurring about 2 hr after injection of *E. coli* into the skin. However, PMN continues to accumulate for several more hours in this model. Also, Wedmore and Williams [24] have shown vascular permeability changes occurring within a few minutes in rabbit skin in response to zymosan injection, while PMN accumulation in high numbers required several hours.

Indomethacin had no effect on vascular permeability when animals were injected intraperitoneally prior to initiating the RPA. This result is similar to that reported by Bailey and Sturm [21]. Issekutz and Bhimji [22] have reported that direct injection of indomethacin into the RPA skin site did result in inhibition of vascular permeability as well as of PMN infiltration and blood flow. The effect on leukocyte infiltration appeared to be secondary to inhibition of the vascular permeability increase. That is, indomethacin appeared to inhibit the vascular permeability increase during the inflammatory response by its effect on prostaglandin-mediated blood flow increase [25, 26]. Since these vascular changes facilitate PMN accumulation, their suppression by indomethacin resulted in suppression of PMN accumulation in the inflammatory sites [22]. The discrepancy between these results and ours may reflect the systemic versus local response to indomethacin in these models of inflammation.

PMN contain a variety of enzymes capable of degrading tissue components. Many of these have been shown to be released extracellularly upon stimulation of the cells with appropriate agents or during "frustrated phagocytosis" [10]. The presence of immobilized immune complexes in the vessel walls during an RPA reaction could conceivably result in such "frustrated phagocytosis" and release of PMN enzymes [13]. These enzymes, mainly the neutral serine proteinases, have activity against a wide variety of substrates, being capable of cleaving elastin, collagen and other constituents of the blood vessels and connective tissue of the skin [27]. Such degradation occurring *in vivo* would result in the loss of integrity of blood vessel walls and subsequent hemorrhage. However, no direct evidence has been reported implicating any PMN enzymatic activity in

the degradation of tissue during *in vivo* inflammatory reactions. If PMN granular enzymes are important in causing blood vessel damage during the RPA, their inhibition should result in decreased hemorrhage. Therefore, enzyme inhibitors were tested in our model in the hope of demonstrating a correlation between the use of proteinase inhibitors and decreased hemorrhage. Our results were inconclusive on this point. Compounds which affected hemorrhage development invariably affected PMN accumulation. Therefore, a direct relationship between PMN proteinase activity and hemorrhage production could not be demonstrated. This includes the effect of catalase, which may decrease the RPA response by eliminating peroxides necessary for its continuation or amplification [7]. Serine esterases, shown to be present on the PMN cell surface and necessary for response to chemotactic factors [28], may be affected by these inhibitors. The use of more specific inhibitors of PMN lysosomal enzymes, as they become available, may prove useful in differentiating the effects of inhibition of PMN proteinases from the generalized effects on other enzyme systems associated with continuation of the RPA response.

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