

Inhalation of Horseradish Peroxidase by Rabbits with Specific IgE Antibodies Results in Release into the Circulation of Platelet-Activating Factor and in Lung Lesions

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To further assess the role "in vivo" of platelet activating factor (PAF) in immediate hypersensitivity reactions in the lung, rabbits with either an IgE or an IgG response against horseradish peroxidase (HRP) were challenged with the antigen by aerosol for 1 hour. The animals were sacrificed 30 minutes after termination of the inhalation.

The following observations were made:

- 1) Upon inhalation of HRP, rabbits with circulating IgE antibodies showed signs of respiratory distress and released PAF into the circulation. The highest levels of circulating PAF were measured 20 to 30 minutes after the start of inhalation. PAF may contribute to the lung lesions found in these animals.
- 2) Rabbits with IgG antibodies did not develop any early abnormalities upon challenge with HRP, this despite the fact that in some animals circulating specific IgE-sensitized basophils could be demonstrated.
- 3) Anti-HRP IgG was able to block the "in vitro" degranulation of specific IgE-sensitized basophils when challenged with HRP.

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INTRODUCTION

In the lung, cross-linkage of IgE molecules present on the surface of mast cells, basophils, and alveolar macrophages (1) by corresponding antigens, results in the release of a great variety of mediators of inflammation. The relative contribution of each individual mediator to an ensuing immediate hypersensitivity reaction in the lung has not yet been precisely defined (2).

A mediator that has been the focus of many recent investigations is platelet activating factor (PAF). PAF has a broad spectrum of biological activities. They include platelet and polymorphonuclear leukocyte aggregation, polymorphonuclear leukocyte and monocyte chemotaxis, lysosomal enzyme release, generation of oxygen radicals, increase in vascular permeability, and, in the lung, bronchoconstriction (3). Intravenous administration of PAF to rabbits initiates circulatory and pulmonary alterations identical to those occurring during IgE-induced systemic anaphylaxis (4, 5). Local instillation of PAF in the alveolar space of the rabbit causes acute pulmonary inflammation (6). All these observations are con-

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sistent with the notion that PAF may be an important mediator of immediate hypersensitivity reactions in the lung.

It has been reported that IgE-sensitized rabbit lung, upon incubation *in vitro* with the antigen, liberates a PAF (7). The present investigation was undertaken to assess the possibility that in IgE-sensitized rabbits that inhale the antigen, an *in vivo*, local release of PAF in the lung is reflected in measureable levels of this mediator in the systemic circulation.

MATERIALS AND METHODS

Animals. New Zealand White female rabbits (Beckens Research Animal Farm, Sanborn, N.Y.) weighing 2 to 2.5 kg were used in this study.

Design of the experiments. Two sets of experiments were performed.

(1) To study the effect of antigen inhalation by sensitized animals, rabbits were immunized with horseradish peroxidase (HRP) (Type II; Sigma Chemical Co., St. Louis, Mo.). To induce an IgE response against HRP, rabbits ($n = 10$) received a subcutaneous injection of 20 mg of HRP in physiologic saline; 3 weeks later a subcutaneous injection of 10 mg of HRP was administered (8). To induce an IgG response, rabbits ($n = 11$) were injected subcutaneously on Day 1 with 20 mg of HRP in physiologic saline and intravenously with 10 μ g of LPS coli; on the 3 days that followed they received 20 mg daily of HRP subcutaneously; on the fifth day, 20 mg of HRP was given intravenously. Three weeks later, the same immunization schedule was repeated with the exception of the administration of LPS coli. After characterization of the immune response against HRP (see below), 1 week after termination of the immunization, all immunized rabbits were subjected to an HRP aerosol challenge. In addition, nonimmunized animals ($n = 8$) were also submitted to this procedure. The aerosol was generated by an ultrasonic nebulizer (DeVilbiss Co., Somerset, Pa.). The nebulizer was connected with a small plastic bag that tightly surrounded nostrils and mouth of the animal challenged. Over a period of about 1 hr, 3 ml of physiologic saline containing 10 mg of HRP/ml was aerosolized. Arterial blood samples were obtained from the ear before and 5, 10, 15, 30, 40, and 60 min after the start of the aerosol challenge. The rabbits were sacrificed half an hour after termination of the aerosol challenge.

(2) To study the development of plasma factors blocking the degranulation of specific IgE-sensitized basophils in the basophil degranulation test (see below), rabbits ($n = 3$) were first immunized for an IgE and then for an IgG response against HRP. At regular intervals the immune response of these rabbits against HRP was evaluated. Furthermore, in order to determine the nature of the blocking factors present in some of the plasma samples of these rabbits, the effect of γ -globulin before and after absorption on an HRP immunoabsorbent (9), of antibodies eluted from the HRP-immunoabsorbent (10), and of γ -globulin after removal of IgG by immunoprecipitation (11) on *in vitro* basophil degranulation was tested.

Characterization of the immune response against HRP. The characteristics of the immune response against HRP were assessed by the following techniques:

For the detection of circulating IgE antibodies the homologous passive cutaneous anaphylaxis (PCA) test in rabbits was used (12). The results were expressed

TABLE 1
RESULTS OF INHALATION OF HORSERADISH PEROXIDASE IN PREVIOUSLY IMMUNIZED OR NONIMMUNIZED RABBITS

Characteristics of the immune response against HRP				In vivo effects of inhalation				
PCA	Precipitating antibody titer	Maximum basophil degranulation ^a (%)	Maximum PAF release BCC (ng/ml)	Maximum reduction basophil count (%)	Maximum reduction WBC count (%)	Maximum reduction platelet count (%)	Maximum PAF release (ng/ml)	Lung lesions ^c
Homologous	Heterologous							
Rabbits immunized for an IgE Response								
1/60	0	61	11.0	51 (30) ^b	22 (40) ^b	22 (30) ^b	3.9 (30) ^b	3
1/60	0	70	14.0	71 (20)	40 (60)	40 (20)	2.5 (20)	3
1/40	0	77	12.8	42 (20)	50 (30)	30 (20)	3.6 (20)	2
1/40	0	54	10.5	68 (20)	26 (20)	32 (30)	2.8 (30)	2
1/40	0	75	10.0	48 (30)	0	25 (30)	0	1
1/20	0	44	6.5	57 (30)	36 (20)	19 (30)	0	1
1/20	0	91	11.0	46 (30)	28 (30)	38 (30)	3.2 (30)	1
1/10	0	75	8.1	52 (30)	ND	ND	0	0.5
1/10	0	84	9.8	52 (20)	20 (30)	19 (30)	0	0.5
0	0	65	9.5	48 (30)	2 (60)	3 (10)	0	0
Mean	—	70 ± 14	10.0 ± 2.1	54 ± 9 (26 ± 5)	25 ± 16 (36 ± 16)	25 ± 11 (26 ± 7)	1.6 ± 1.7 (25 ± 5)	1.4 ± 1.0
± SD	—							
Rabbits immunized for an IgG Response								
0	1/100	10	7.8	7 (40)	8 (10)	3 (10)	0	0
0	1/100	10	2.2	15 (10)	7 (30)	3 (30)	0	0
0	1/60	7	0	11 (30)	5 (30)	5 (30)	0	0
0	1/32	ND	ND	ND	ND	ND	0	0
0	1/32	ND	ND	ND	ND	ND	0	0
0	1/32	ND	ND	ND	ND	ND	0	0
0	1/32	0	0	21 (60)	8 (60)	10 (60)	0	0.5
0	1/60	9	0	20 (30)	6 (20)	3 (20)	0	0
0	1/60	25	9.5	6 (60)	5 (60)	5 (40)	0	0
0	1/16	ND	ND	ND	ND	ND	0	0
0	1/16	0	0	17 (10)	7 (60)	2 (60)	0	1
0	1/40	20	0	10 (60)	3 (30)	3 (30)	0	0
Mean	—	10 ± 9	2.4 ± 3.9	13 ± 6 (38 ± 21)	6 ± 2 (38 ± 20)	4 ± 3 (35 ± 18)	0	0.1 ± 0.3
± SD	—							
Nonimmunized rabbits (n = 8)								
Mean	0	13 ± 8	0	10 ± 6 (33 ± 25)	8 ± 5 (30 ± 22)	3 ± 3 (26 ± 12)	0	0.1 ± 0.2
± SD	0							

Note. BCC, basophil-rich buffy coat cells; HRP, horseradish peroxidase; ND, not done; PAF, platelet-activating factor; PCA, passive cutaneous anaphylaxis; WBC, white blood cells.

^a Determined *in vitro* in whole blood.

^b Time (in min) of the occurrence of maximal reduction in cell or platelet count or of maximal release of platelet-activating factor after the start of the horseradish peroxidase inhalation.

^c Scored on a scale of 0–3.

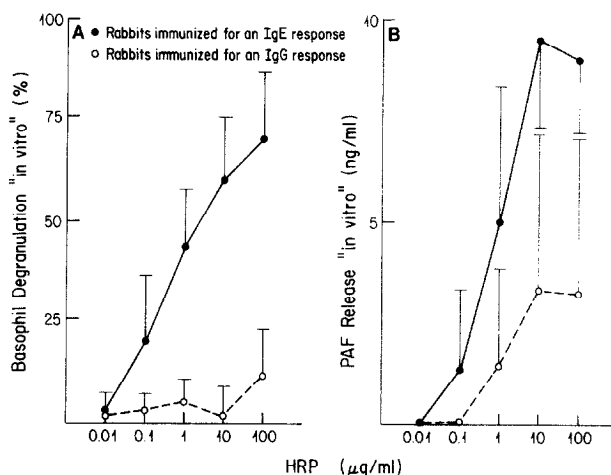


FIG. 1. (A) The results of basophil degranulation tests performed *in vitro* on whole blood of rabbits immunized for either an IgE ($n = 10$) or an IgG response ($n = 11$) against HRP. (B) The *in vitro* PAF release upon challenge with HRP from BCC of rabbits of the same two groups. Mean values are given ± 1 SD.

as the highest serum dilution giving a blueing area of more than 5 mm. Positive sera were also tested after previous heating for 2 hr at 56°C to inactivate IgE.

To detect specific basophil sensitization, two tests were done. In one, the percentage of degranulated blood basophils after incubation with various amounts of HRP was determined (8). Basophils were counted on the basis of their metachromatic staining by toluidine blue. This test was performed on whole blood, and on blood submitted to centrifugation and washing in order to remove blocking factors possibly present in plasma (8). In the second, the release *in vitro* of PAF from basophil-rich buffy coat cells (BCC) (13) after challenge with HRP was measured. BCC (1×10^7) were incubated for 10 min at 37°C with various concentrations of HRP in 1 ml of Tris-buffered Tyrode bovine serum albumin solution with or without Ca^{2+} and Mg^{2+} (13). PAF was extracted from the supernatant, purified, and assayed (see below).

For determining the presence of circulating IgG antibodies, the heterologous PCA test (reserve passive Arthus reaction) in rats (14), and two-dimensional gel diffusion were employed.

Assessment of the in vivo effect of inhalation of HRP. Blood samples taken before and during the inhalation of HRP were used to determine the number of circulating basophils, white blood cells, and platelets. PAF was extracted from plasma samples, purified, and assayed.

Lung tissue obtained at sacrifice was studied by light, electron, and immunofluorescence microscopy (see below).

Extraction, purification, assay, and characterization of PAF. PAF was extracted and purified from samples using methods described in detail elsewhere (15, 16).

PAF was detected by aggregation of washed rabbit platelets using previously

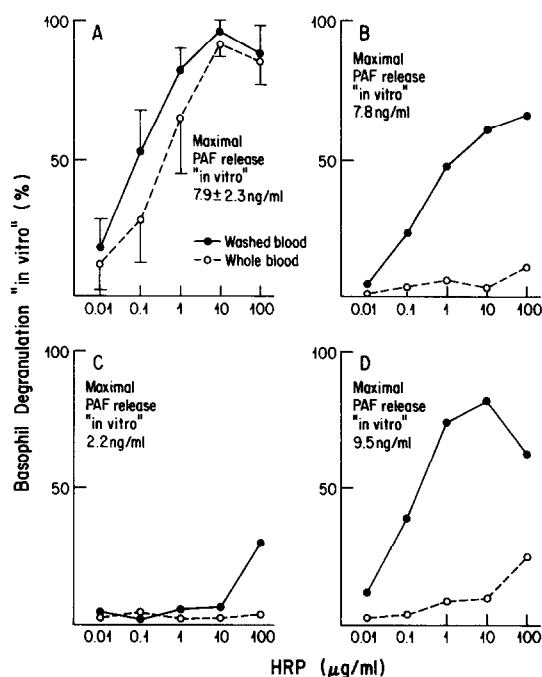


FIG. 2. (A) No difference in results were obtained when the basophil degranulation test was performed with whole blood or with washed blood of three rabbits immunized for an IgE response. Mean values $\pm$ 1 SD. In contrast, marked enhancement of basophil degranulation was observed (B–D) when, instead of whole blood, washed blood was used of the three rabbits immunized for an IgG response and showing PAF release *in vitro* from BCC (Table 1).

published techniques (16, 17). PAF concentrations were expressed as nanograms/milliliter (17).

The biologically active material released from BCC challenged *in vitro* with HRP or extracted from plasma was identified as PAF based on sharing the following properties with synthetic PAF (AGEPC) (Bachem, Feinchemikalien AG, Switzerland) (3, 15–18): (1) It had the same biological activity on washed rabbit platelets which was independent of ADP and arachidonic acid-thromboxane A_2 -mediated pathways. (2) It had the same physicochemical characteristics such as stability to acid and weak basic conditions, and to boiling, but was inactivated by strong bases. (3) As AGEPC, the material was inactivated by phospholipase A_2 . (4) Its R_f determined on silica gel thin layer chromatography was the same as that of AGEPC.

Histologic and immunohistologic studies. At sacrifice of the rabbits, the lungs were removed from the thoracic cavity and inflated at 25 cm H_2O pressure with 1% bovine serum albumin in phosphate-buffered saline. Tissue specimens of lower and upper lobes were processed for light and electron microscopy as previously described (6). For immunohistologic study, 4-μm-thick sections of unfixed lung tissue, snap-frozen in liquid nitrogen, were stained with fluorescein-conjugated goat anti-rabbit IgG, C3 (Cappel Laboratories, Cochranville, Pa.), or anti-HRP as described elsewhere (19).

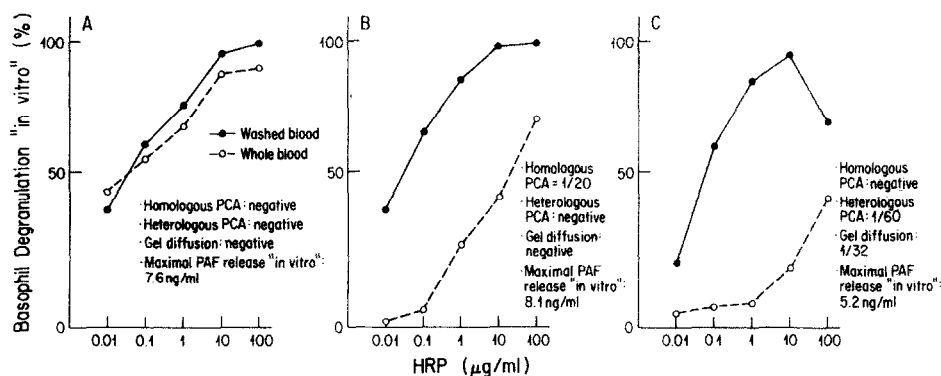


FIG. 3. The sequential immune response of a representative rabbit out of a group of three, immunized first for an IgE response (A), and then for an IgG response (B and C, 2 and 4 weeks after A respectively). The basophil degranulation test is increasingly more positive when performed with washed blood instead of with whole blood. The maximal difference coincides with the time of appearance of circulating anti-HRP antibodies of the IgG class. Note in A that the basophil degranulation test is already positive when circulating specific IgE antibodies are not yet demonstrated by the homologous PCA technique.

RESULTS

Characteristics of the Immune Response against HRP

As shown in Table 1 and Fig. 1, in rabbits immunized for an IgE response, specific IgE antibodies and no IgG antibodies could be demonstrated. In animals immunized for an IgG response, anti-HRP antibodies only of the IgG class were detected in the circulation. However, in three rabbits of this latter group specific IgE-sensitization of basophils was suggested by a release *in vitro* of PAF from BCC upon challenge with HRP (Table 1). This observation, together with the negative results of the basophil degranulation test performed with whole blood, raised the possibility of the presence of blocking factors in the plasma of these animals. Blocking factors were documented and their nature clarified in the following experiments:

(1) In rabbits immunized for an IgE response (Fig. 2A) and in rabbits immunized for an IgG response not showing *in vitro* PAF release from BCC, no difference was found in the results of the basophil degranulation test performed either with whole blood or with washed blood. In contrast, in the three rabbits with an IgG response in which a release of PAF from BCC was found, the basophil degranulation test while negative with whole blood, became positive when done with washed blood (Figs. 2B–D). From these results it was concluded that these three animals, in fact, had blocking factors in their circulation.

(2) In rabbits immunized first for an IgE response and then for an IgG response against HRP, the level of plasma factors blocking the degranulation *in vitro* of specific IgE-sensitized basophils progressively increased (Fig. 3).

(3) Addition of plasma- or γ -globulin-containing anti-HRP IgG depressed the degranulation of specific IgE-sensitized basophils (Figs. 4A and B). This effect could be abrogated by treatment of the γ -globulin with anti-rabbit IgG or by

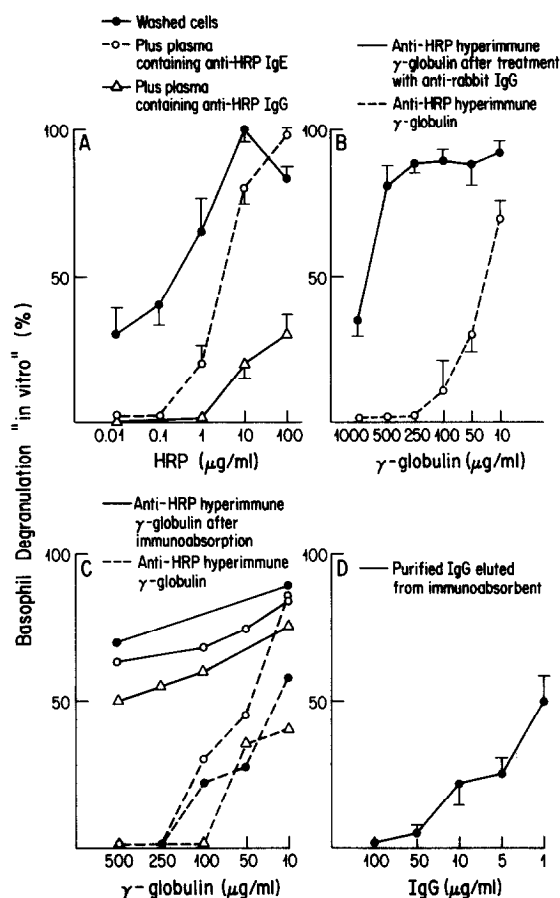


FIG. 4. Depression of basophil degranulation when plasma containing anti-HRP IgG is added to specific IgE-sensitized basophils is depicted (A). This effect is abrogated when hyperimmune γ -globulin is treated with anti-rabbit IgG (B) or is passed on an HRP-immunoabsorbent (C). Depression of basophil degranulation is observed when IgG eluted from the HRP-immunoabsorbent is added to sensitized basophils (D). In A, representing three experiments, and in B and D, each representing four experiments, mean values $\pm$ 1 SD are indicated; in C, three experiments are graphed individually.

passing the γ -globulin over an HRP-immunoabsorbent (Figs. 4B and C). On the other hand, IgG eluted from the HRP-immunoabsorbent did have a blocking effect on the degranulation of basophils (Fig. 4C). Last, rabbit plasma with anti-HRP antibodies of the IgE class and anti-bovine serum albumin antibodies of the IgG class, did not exert an inhibitory effect on the degranulation of specific IgE-sensitized basophils when challenged with HRP.

In Vivo Effects of Inhalation of HRP

As of the start of HRP inhalation, rabbits immunized for an IgE response developed respiratory distress with rapid and shallow breathing, which lasted till they were sacrificed. The other effects of inhalation of HRP in these animals are summarized in Table 1. They showed a decrease in the number of circulating

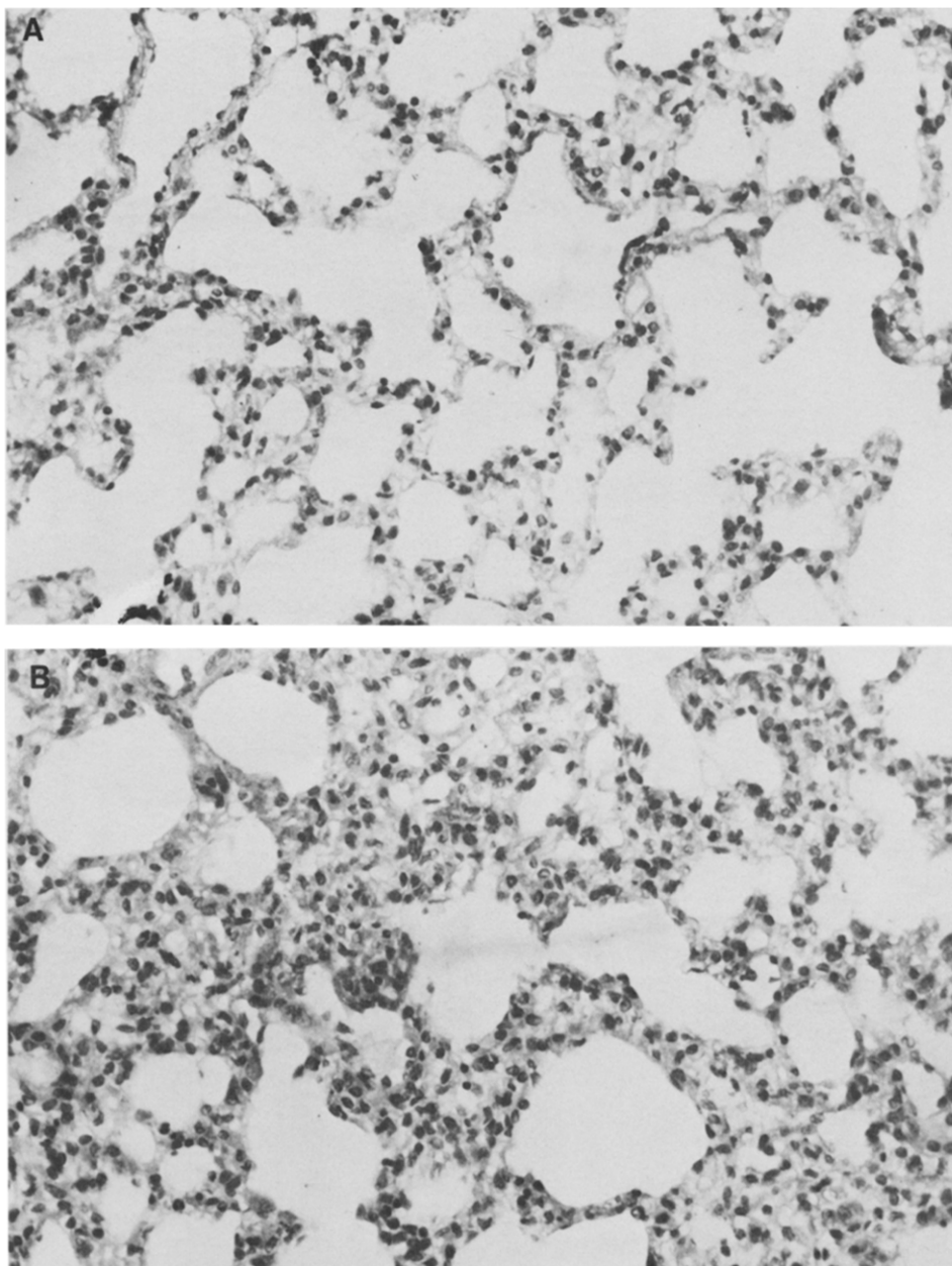


FIG. 5. (A) A light micrograph of lung of an IgE-sensitized rabbit that inhaled HRP, showing 1 + lesions (on a scale of 0 to 3). (B) The lung of a similarly treated rabbit with 3+ lesions. (C) The lung of a rabbit immunized for an IgG response, showing no lesions after inhalation of HRP ($\times 80$).

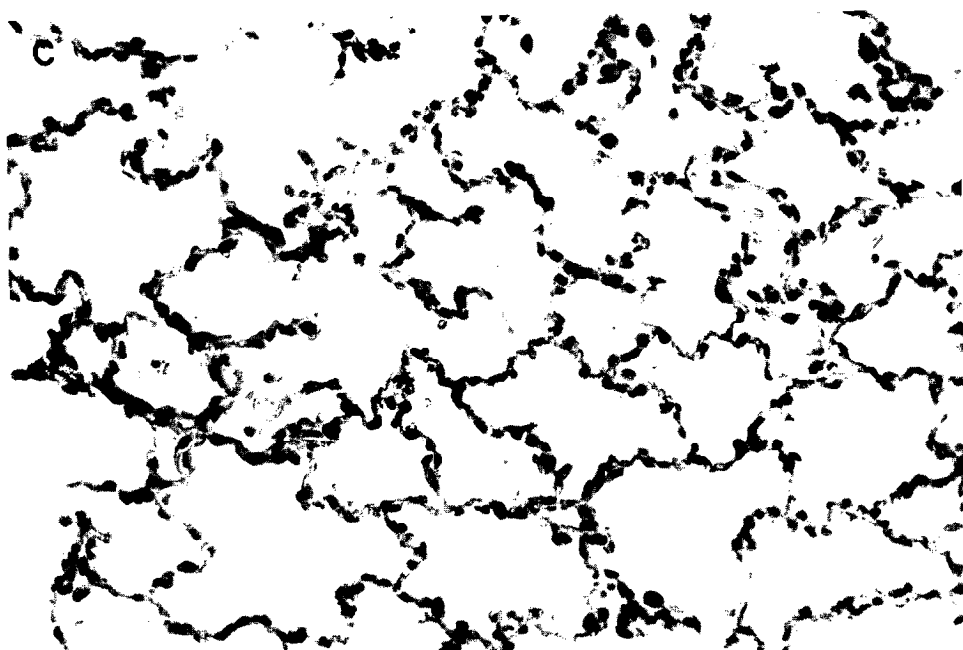


FIG. 5—Continued.

basophils and, to lesser extent, of white blood cells and platelets, which was maximal 20 to 30 min after the start of HRP inhalation. In five of the ten rabbits, a release of PAF into the circulation was found. This measurable systemic release of PAF was of short duration and peaked 20 to 30 min after the beginning of the HRP inhalation. Upon sacrifice, in seven of the rabbits diffuse lung lesions were found. They consisted of interstitial edema and an influx of neutrophils and monocytes resulting in thickening of the alveolar septa (Figs. 5A and B). In addition, swelling and vacuolization of alveolar capillary endothelium, an increased number of endothelial caveolae, and degranulated platelets in alveolar capillary lumina (Fig. 6) were also found. Degranulated mast cells and alveolar macrophages were sought for but not found. Brochoconstriction was also not seen. A positive correlation existed between the level of circulating specific IgE antibodies, as determined by the homologous PCA test, and the extent of the lung lesions (Table 1). Immunofluorescence tests for tissue localization of HRP and rabbit IgG and C3 were negative.

With the exception of focal inflammatory lesions in the lung of one rabbit, no change in any of the parameters studied was observed in rabbits immunized for an IgG response and in nonimmunized rabbits (Table 1; Fig. 5C).

DISCUSSION

Kravis and Henson have described the release of a PAF from IgE-sensitized rabbit lung when challenged *in vitro* with specific antigen (7). In the present study, inhalation of HRP resulted, in five out of ten rabbits with an IgE response against HRP, in the appearance in the systemic circulation of biologically active material

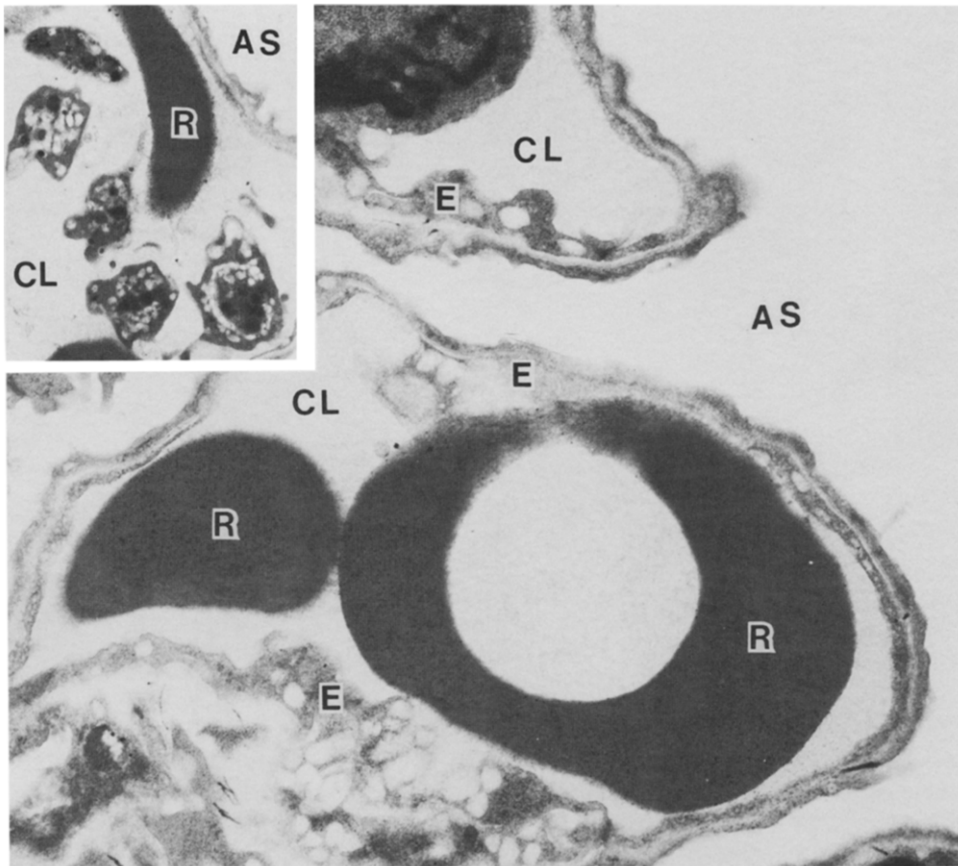


FIG. 6. An electron micrograph of a rabbit immunized for an IgE response and challenged with HRP by aerosol. The endothelium (E) of the alveolar capillary walls is swollen and contains vacuoles of various sizes. AS, alveolar space; CL, alveolar capillary lumen; R, red blood cell ($\times 10,000$). Inset: In the same rabbit, degranulated platelets are present in the lumen of an alveolar capillary. AS, alveolar space; CL, alveolar capillary lumen; R, red blood cell ($\times 6000$).

that could be identified as PAF. The release was short lived and maximal 20 to 30 min after the start of antigen inhalation. In the five remaining rabbits in which, upon HRP challenge, no circulating PAF was detected, clinical and laboratory parameters (Table 1) nevertheless indicated an IgE-mediated hypersensitivity reaction. One may speculate that more frequent measurements would have shown the release of PAF into the circulation also in these rabbits. Other explanations for the negative results in these latter animals include a limited sensitivity of the techniques used for measurement of PAF, and the rapid inactivation of PAF once released *in vivo* (3).

The cells initially involved in the local release of PAF in the present model are assumed to be specific IgE-sensitized alveolar macrophages (1), alveolar basophils (20), and lung mast cells. Activated blood-borne neutrophils and monocytes (21), and damaged lung endothelial cells (16, 17) may secondarily have contributed to the liberation of PAF into the systemic circulations.

To what extent can PAF be held responsible for the *in vivo* manifestations following the HRP aerosol challenge of IgE-sensitized rabbits? Respiratory distress and a drop in circulating basophils, white blood cells, and platelets have been observed in rabbits receiving intravenously PAF (4, 5). Also the lung pathology, characterized by interstitial edema, infiltration of neutrophils and monocytes, endothelial damage, and presence of degranulated platelets, is consistent with PAF-mediated injury ((6); G. Camussi, unpublished observations). More definite proof for a pathogenic role of PAF in this animal model of immediate hypersensitivity of the lung should come from experiments in which the biological action of PAF is specifically blocked. However, assuming the feasibility of such experiments, their results may prove to be difficult to interpret, because other mediators of inflammation may bypass PAF-dependent pathways, causing a partial or even complete expression of the immediate hypersensitivity reaction.

The lung pathology in the rabbits was evaluated half an hour after termination of the HRP inhalation. Studies during and late after inhalation were not performed. Thus, information on the evolution of the lung lesions is not available. Whether lung damage similar to that observed in IgE-sensitized rabbits occurs in patients with asthma is not known, because systematic morphologic studies of lung of patients with nonfatal asthma attacks, have understandably, not been done. Lastly, the relevance of this model of IgE-mediated lung injury for our understanding of extrinsic allergic alveolitis or hypersensitivity pneumonitis is not obvious as symptoms in the latter condition arise not immediately but 6 or more hr after exposure to the eliciting antigen(s) (22).

In rabbits with anti-HRP IgG, no effects of aerosol challenge with HRP were noted, even though in three of the eleven animals circulating specific IgE-sensitized basophils could be demonstrated. The absence of symptoms in these latter animals may be explained by a blocking effect of anti-HRP IgG on the expression of immediate hypersensitivity (7). This interpretation is strengthened by results of experiments showing that at least *in vitro*, anti-HRP IgG did suppress the degranulation by HRP of specific IgE-sensitized basophils. The development of blocking antibodies has been reported in allergic patients undergoing hyposensitization therapy (23, 24). The precise mechanisms leading to hyposensitization are, however, still a matter of debate.

In conclusion, an easily reproducible rabbit model of immediate hypersensitivity of the lung has been developed offering not only the opportunity to do studies on mediators involved in the pathogenesis of this condition but also on the nature of blocking antibodies preventing it.

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