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Acute-Phase Reactants in Experimental Inhalation Lung Disease[1] (41233)

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Abstract. An investigation on the occurrence and role of acute-phase reactants in experimental inhalation lung disease (ILD) was undertaken. Using an experimental model of ILD in which rabbits are exposed to aerosols of appropriate fungal spores, haptoglobin analysis was compared with depressions in arterial oxygen tension (P_aO_2) with time following challenge. Haptoglobin values of rabbits exposed to single 30-min aerosol challenges of *Aspergillus terreus*, demonstrated a significant (two- to three fold) increase 24–48 hr after challenge. Haptoglobin elevation was found to be a more reliable and consistent indicator of pulmonary inflammation than depression of P_aO_2. In an effort to determine the role of haptoglobin in this response, acute phase reactant levels were elevated either actively or passively. When rabbits with augmented haptoglobin levels were exposed to aerosol challenges with *A. terreus*, the typical depressions in P_aO_2 at 1–4 hr postchallenge were not observed. This indicated that plasma containing elevated acute-phase reactants may be involved in limiting the pulmonary response that normally occurs following challenge. Haptoglobin was not found to bind to *A. terreus* spores *in vitro*.

Hypersensitivity pneumonitis is a term used to describe a group of environmental, pulmonary diseases resulting from the inhalation of organic dusts, the prototype of which is farmer's lung. The underlying problem in determining the pathogenic mechanism(s) involved in hypersensitivity pneumonitis is that it behaves as a disease spectrum and that its manifestations depend on variations in the host's immune responses and site of reaction with antigens (1). All four major types of immune injury, as well as nonspecific complement activation, have been implicated in the pathogenesis of hypersensitivity pneumonitis (2), and there exists the possibility that the involvement or importance of one of these mechanisms may change with time, even in the same individual (3).

In the course of studies on the mitogenic effect of *Micropolyspora faeni* antigen on human lymphocytes, we found a patient with acute farmer's lung disease whose serum was inhibitory for the control mitogens, phytohemagglutinin and concanavalin A. Subsequent investigations revealed that this serum also possessed exceptionally high levels of acute-phase reactants. Analysis of several other farmer's lung patients' sera showed similar elevations and some abnormally low values as well. The role of acute-phase reactants in lung diseases due to inhalants has not been evaluated, but the occurrence of the abnormal levels in these patients suggested a connection.

The purpose of this paper is to report on studies of the behavior of acute-phase reactants, as monitored by haptoglobin content, in an experimental model of inhalation lung disease (ILD) and to present experiments designed to assess the role, if any, of such an acute-phase reactant on the pathogenesis of this disease.

Materials and Methods. *Experimental animals.* New Zealand white rabbits, male and female, weighing approximately 2.5 kg were obtained from Hilltop Laboratories, Scottdale, Pennsylvania. The rabbits were maintained on water and routine chow *ad libitum* in American Laboratory Animal Care-approved facilities.

Determination of plasma haptoglobin. Haptoglobin levels, expressed as milligram percentage hemoglobin binding capacity

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(HBC), were determined by a modification of the method of Tarukoski (4). The modifications consisted of using carbonmonoxyhemoglobin, in place of hemoglobin, as the haptoglobin binding target due to its greater stability over long storage times (5).

Determination of arterial blood gas levels. The method and rationale behind using arterial blood gas levels as an indicator of pulmonary damage have been given in detail previously (6). All P_aO_2 and P_aCO_2 determinations were made within 20 min of sampling on a Corning Model 160 pH and blood gas analyzing system (Corning Scientific Instruments, Corning N.Y.). Determinations were made on each rabbit immediately before aerosol challenge and 1, 4, 6, and 24 hr after. Previous work with this model showed that the P_aCO_2 levels remain stable during such experiments and serve only to confirm hyperventilation (6).

Plasma samples. A 3.0-ml blood sample was obtained from the medial ear artery for both the haptoglobin determination and the arterial blood gas determination (6). After blood gas analysis, the remaining blood was centrifuged and the plasma removed for storage in separate 1-ml aliquots at −70° for later use in haptoglobin determinations.

Culture preparations. Aspergillus terreus obtained from a West Virginia University stock culture was grown on 1% glucose−1% polypeptone agar. The plates were incubated at room temperature for 2−3 weeks at which time the surfaces of the plates were covered with confluent growth consisting of well-developed conidiophores with mature conidia. At this time the plates were stored at 4° until further use.

Inhalation challenge protocols. Aerosols were administered to four rabbits at a time using previously described aerosol chambers (6). In the *A. terreus* aerosol experiments, three mature agar plate cultures of the fungus were placed uncovered in the chamber. *A. terreus* spores were aerosolized by playing a jet of air at 2−3 psi delivered through a Pasteur capillary pipet over the agar surface for 30 min. The amount of spores deposited in the lungs by the rabbits was determined by standard surface colony count of 10-fold dilutions of lung homogenate on glucose-peptone agar and was consistently found to be $3-6 \times 10^5$ viable spores per gram lung homogenate.

Controls for the above experiments consisted of sham-treated rabbits subjected to exactly the same conditions except that no cultures were placed into the aerosol chamber. Aerosols of saline or human serum albumin given to unimmunized animals result in no blood gas changes (6).

Production of haptoglobin-rich plasma. Elevation of control plasma haptoglobin levels was accomplished by 1.0-ml intramuscular injection of turpentine (Aldrich Chemical Co., Milwaukee WI) into each hind leg of the rabbits. This is a standard, convenient, and reliable method most often used for haptoglobin induction (7). The turpentine was defined as a mixture of 70% α-pinene and 30% β-pinene. Forty-eight hours later, plasma samples that contained elevated haptoglobin levels that were used in later experiments were drawn from the rabbits by intracardiac exsanguination into plastic syringes containing heparin. The plasma was removed by centrifugation and frozen at −20° until needed.

Passive transfer of haptoglobin-rich plasma. Haptoglobin-rich plasma, derived by the above method, was passively transferred to recipient rabbits. Prior to the transfer, blood samples taken from the donor and recipient rabbits were checked for serologic compatibility by direct and indirect cross-matching. In order to prevent physiologic response to hypervolemia, attempts to equalize blood volumes were made by first taking 50 ml of blood from each of the recipient rabbits medial ear arteries into 60-ml heparinized, plastic syringes. The whole blood was centrifuged at 1000 *g* for 20 min at which time the recipient's plasma and the donor's cells were removed and discarded. The recipient cells were then resuspended with 60 ml of haptoglobin-rich, donor plasma. The reconstituted blood was reintroduced back into the recipient rabbits by lateral ear vein injection. After an 18- to 20-hr resting period, the recipient rabbit underwent one of the aerosol challenges. Control rabbits were transfused in the same manner using normal donor plasma.

Haptoglobin binding of A. terreus spores. A. terreus spores were collected from mature cultures by washing the surface of the cultures with phosphate-buffered saline plus 0.1% Tween 80. Equal volumes of a standard spore suspension were mixed with various dilutions of haptoglobin-rich plasma. After incubation at either 37°C for 1 hr or 4°C for 24 hr, the supernatants were removed and haptoglobin determinations performed.

Results. *Effects of single aerosol challenges on the plasma haptoglobin levels of rabbits.* Three groups of four rabbits each were exposed to single aerosol challenges with *A. terreus* spores.

Figure 1 represents the relative changes in P_aO_2 in the three groups performed on different days, following the standard aerosol challenge. The changes from baseline values were analyzed for each group of rabbits, by the Dunnett's test (8). The critical differences from the prechallenge value, represented by the shaded areas, indicate statistical significance at the 0.05 level and was different for each group of rabbits tested. Any decrease in P_aO_2 below the shaded area is significant. Eight out of twelve rabbits demonstrated a significant decrease in P_aO_2 following aerosol challenge. Four rabbits either did not respond or demonstrated increases in P_aO_2 due to hyperventilation.

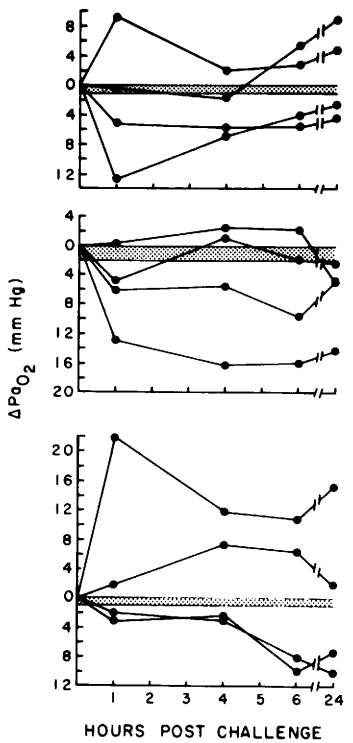

FIG. 1. Comparison of relative changes in arterial oxygen tensions in three sets of normal rabbits after aerosol challenge with *A. terreus* spores. The shaded area represents depressions of chance variation beyond which any changes are significant at the 0.05 level (critical difference).

The mean haptoglobin levels of 8 control rabbits exposed to a sham aerosol are presented in Table I and indicate no significant differences between the pre- and post-aerosol levels of haptoglobin. Table I also presents the mean plasma haptoglobin levels of 16 rabbits following an aerosol challenge with *A. terreus* spores. The mean haptoglobin levels of the experimental rabbits were evaluated by Student's *t* test (9) in two different ways. First, the mean haptoglobin levels of the experimental rabbits were compared with the mean haptoglobin levels of the controls. In addition, the mean haptoglobin levels of the experimental rabbits, after challenge, were compared against their own opening mean haptoglobin level. Both comparisons demonstrated a significant increase ($P < 0.05$) in mean haptoglobin levels of the experimental rabbits at 24 and 48 hr after aerosol. The changes at earlier sampling times were insignificant.

When the individual blood gas and haptoglobin data from the *A. terreus*-exposed animals were compared, a striking observation was noted. In three out of four animals that hyperventilated, a marked elevation in haptoglobin was produced later, averaging 11.3 mg% HBC, indicating that the hyperventilation may have masked depressions in P_aO_2.

Effects of elevating the plasma haptoglobin levels of rabbits prior to challenge with A. terreus spores. The mean normal plasma haptoglobin level, determined from the plasmas of 31 untreated, healthy rabbits, was 5.4 ± 2.5 mg% HBC.

Table II summarizes the methods of haptoglobin augmentation and treatment of each experiment and gives the haptoglobin

TABLE I. COMPARISON OF MEAN PLASMA HAPTOGLOBIN BETWEEN RABBITS GIVEN A SHAM AEROSOL OR A 30-min INHALATION CHALLENGE WITH *Aspergillus terreus* SPORES

Aerosol	Hours after challenge					
	0	1	4	6	24	48
Sham	7.37[a]	4.50	4.50	5.27	6.87	8.00
(n = 8)	± 6.30	2.61	2.32	2.82	4.35	5.80
A. terreus spores	5.93	4.87	6.00	5.75	13.06[b]	14.00[b]
(n = 16)	± 2.54	2.24	4.95	4.62	12.48	7.74

[a] Results are expressed as the mean mg% hemoglobin binding capacity (HBC) ± SD.
[b] $P < 0.05$ as calculated by Student's t test.

level of each animal following such augmentation, before and 24 hr after *Aspergillus* aerosolization.

Figure 2 shows that three rabbits with elevated haptoglobin levels in Experiment 1 did not have significant decreases in their P_aO_2 levels, from the baseline over the time periods sampled, while one rabbit (actively elevated) showed only a moderate depression at 6–24 hr postchallenge.

Figure 3 shows that both rabbits, which had elevated plasma haptoglobin levels prior to challenge in Experiment 2, showed no significant decreases in their P_aO_2 levels after challenge. However, the unaugmented control rabbits did show significant decreases in their P_aO_2 levels and increases in their haptoglobin levels after challenge, as in earlier experiments.

Figure 4 shows that the two rabbits which received haptoglobin-rich plasma transfusions prior to challenge in Experiment 3,

TABLE II. HAPTOGLOBIN DETERMINATION OF RABBITS' PLASMA PRIOR TO AND 24 hr AFTER AEROSOL CHALLENGE WITH *Aspergillus terreus* SPORES

Rabbit No.	Treatment	Haptoglobin level[a]	
		0 hr	24 hr
	Experiment 1		
1	Passive transfer/Hp-rich plasma	65	41
2	Turpentine injection	176	214
3	Passive transfer/Hp-rich plasma	29	22
4	Turpentine injection	212	207
	Experiment 2		
5	Passive transfer/Hp-rich plasma	37	24
6	Passive transfer/Hp-rich plasma	24	29
7	No treatment	6	15
8	No treatment	5	11
	Experiment 3		
17	Passive transfer/Hp-rich plasma	38	17
18	Passive transfer/Hp-rich plasma	62	44
19	Passive transfer/normal plasma	7	18
20	Passive transfer/normal plasma	8	19
	Experiment 4		
21–52	No treatment (baseline controls)	5.4 ± 2.5[b]	N.A.[c]

[a] Haptoglobin expressed in mg% HBC after augmentation immediately before aerosolization (0 hr) and 24 hr later after aerosol challenge.
[b] Mean ± SD.
[c] Not applicable.

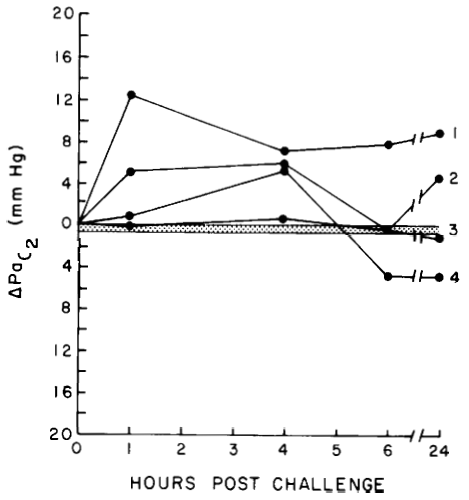

FIG. 2. Comparisons of relative changes in arterial oxygen tensions of four rabbits with elevated plasma haptoglobin levels following aerosol challenge with *A. terreus* spores. Prior to the aerosol challenge, the plasma haptoglobin levels of rabbits 2 and 4 were elevated by turpentine injections, while rabbits 1 and 3 received passive transfer of haptoglobin-rich plasma. For explanation of the shaded area see Fig. 1.

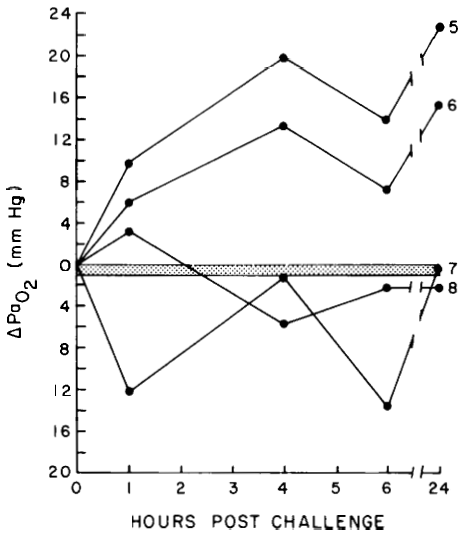

FIG. 3. Comparisons of relative changes in arterial oxygen tensions of four rabbits after an aerosol challenge with *A. terreus* spores. Prior to the aerosol challenge, the plasma haptoglobin levels of rabbits 5 and 6 were elevated by passive transfer of haptoglobin-rich plasma, while rabbits 7 and 8 underwent no treatment and had normal levels of plasma haptoglobin. For explanation of the shaded area see Fig. 1.

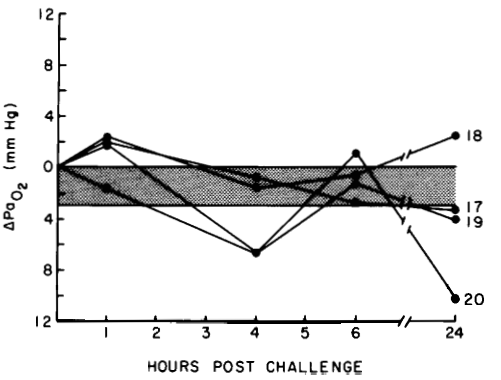

FIG. 4. Comparisons of relative changes in arterial oxygen tensions of four rabbits following an aerosol challenge with *A. terreus* spores. Prior to the aerosol challenge, the plasma haptoglobin levels of rabbits 17 and 18 were elevated by passive transfer of haptoglobin-rich plasma. Rabbits 19 and 20 underwent passive transfer with plasma that contained normal levels of haptoglobin. For explanation of the shaded area see Fig. 1.

again, demonstrated no significant decreases in their P_aO_2 after challenge. However, the control rabbits that underwent passive transfer with normal plasma demonstrated the expected, significant, decreases in their P_aO_2 following challenge. Thus, in all three experiments, no rabbit with an augmented haptoglobin level demonstrated the P_aO_2 decline after aerosol challenge, whereas all rabbits with normal haptoglobin levels exhibited the characteristic depression.

Haptoglobin binding of A. terreus spores. When viable *A. terreus* spores were incubated with haptoglobin-rich plasma either at 1 hr/37° or 24 hr/4°, no significant loss of hemoglobin binding capacity of the plasma was noted.

Discussion. Changes in arterial P_aO_2 in rabbits, following inhalation challenge with *A. terreus* spores or bacterial lipopolysaccharide, have been established as a reliable parameter of physiologic impairment (6, 10). It was later shown (11) that these same responses also occurred in rabbits after aerosol challenge with *M. faeni* or *Thermoactinomyces vulgaris*, both etiologic agents of human hypersensitivity pneumonitis.

Certain disadvantages were encountered

with this system of monitoring lung injury which included the need for more than one person to perform these tests and the infrequent obscuring of P_aO_2 results due to hyperventilation of the test animals. If an animal hyperventilated, following challenge, then a depression in P_aO_2 might not be seen even though lung injury had occurred.

The P_aO_2 levels obtained in our experiments following aerosol challenge, with *A. terreus* spores, indicated that exposure to these spores caused pulmonary responses in a majority of the rabbits tested. Once it was established that physiologic alterations characteristic of pulmonary damage occurred, the effect of this response on acute phase reactants was explored. Haptoglobin measurement was chosen as a collective monitor for these acute-phase reactants.

Our results showed that the normal rabbit plasma haptoglobin level is low, with a very narrow range of variation, such that any significant deviation from which would indicate that an inflammatory event had occurred. Our experiments demonstrated that there were significant increases in the plasma haptoglobin levels 24–48 hr following aerosol exposure to *A. terreus*. The data also showed that most aerosol-treated animals that hyperventilated had high haptoglobin levels. This suggested that an inflammatory reaction had occurred in spite of an increased P_aO_2. Although the haptoglobin production peaked 24–48 hr after exposure, it proved to be a more reliable measurement of insult in those animals in which a hyperventilation might have obscured a P_aO_2 depression.

Measurements of both P_aO_2 and haptoglobin are more sensitive indicators of inflammation than histopathology studies. *Aspergillus* aerosols used in these concentrations induce no detectable histopathological changes in rabbit lungs at any of the short-term intervals used in this study (12). Even when *Aspergillus* spores are intratracheally injected into rabbits, very little cellular infiltrate is seen in the airways, possibly because the spores are phagocytized so quickly (13).

Since haptoglobin was elevated, presumably due to this pulmonary inflammation, the question of the role, if any, of acute-phase reactants on this *in vivo* process arose. In order to begin to answer this question, we designed a series of experiments in which the levels of acute-phase reactants of the rabbits were artificially elevated by active and passive means, prior to an aerosol challenge by *A. terreus* spores. *A. terreus* was used exclusively because it was the organism that had given the most consistent results in the present as well past studies (6, 14).

When the acute-phase reactant levels of rabbits were augmented actively or passively, no 1- or 4-hr depressions in P_aO_2 occurred after a standard *A. terreus* aerosol challenge as are usually seen in normal, nonhyperventilating rabbits exposed to the same aerosol. These results indicate that the response was in some way moderated in those rabbits that had elevated haptoglobin before challenge.

It is important to note that haptoglobin was probably not the only acute-phase reactant elevated in these rabbits. Nor can it be stated from these data that haptoglobin itself brought about the blood gas reductions, since no work was done with isolated material. Haptoglobin was chosen for convenience in measurement and for ease of artificial induction. It was an index in that if it were elevated, then it is likely that other mediators are elevated as well. Further research involving purified haptoglobin is being conducted in order to determine the exact role of this acute-phase reactant in inhalational lung disease.

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