A Physiologic Differentiation between Delayed and Immediate Hypersensitivity

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ABSTRACT Studies have been made of movement of various macromolecules into and out of the pleural space of guinea pigs during the course of a delayed hypersensitivity reaction to purified protein derivative (PPD), and a passively transferred immediate hypersensitivity reaction to ovalbumin. While the immediate hypersensitivity reaction transiently alters vascular permeability as shown by increased movement of macromolecules into the chest, the delayed hypersensitivity reaction is marked by a decreased capacity to resorb macromolecules from the pleural space. The data suggest that the two hypersensitivity reactions may be distinguished by these physiologic differences. Additional data from studies of a chemically induced pleural effusion in these animals suggest that some type of outflow obstruction is necessary for the development of effusion, but that the outflow defect caused by the irritating chemical is based on a different mechanism than that seen during the delayed hypersensitivity reaction.

INTRODUCTION

Vascular damage and an increase in vascular permeability are essential features of the immediate hypersensitivity reaction (1, 2). The effects of delayed hypersensitivity reactions on vascular integrity have been, however, less well defined (3). It has recently been demonstrated in this laboratory that pleural effusions developing in the tuberculin-sensitized guinea pig after the intrapleural injection of tuberculin purified protein derivative (PPD) are due primarily, if not exclusively, to delayed type of hypersensitivity (4). This suggested the possibility of defining the effects of hypersensitivity reactions occurring in vivo in terms of alterations in macromolecular and water movement within the pleural space, thereby gaining insight into some physiologic consequences of these reactions. Experiments described in this report have suggested that delayed hypersensitivity reactions may be distinguished from immediate hypersensitivity and a nonspecific (chemical) inflammation by differences in macromolecular and water kinetics.

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METHODS

Molecular markers. Guinea pig albumin and γ-globulin were prepared from pooled guinea pig serum by the stepwise processes of ammonium sulfate precipitation, starch block electrophoresis (5), and Sephadex G-200 chromatography (6). These proteins were labeled with ^{125}I by the method of Hunter and Greenwood (7). Residual free iodine was removed by dialysis against cold saline for 48 hr and passage through G-25 Sephadex. Polyvinylpyrrolidone (PVP) was obtained commercially as grade K-30. This material was placed on a 50 × 3.2 cm Sephadex G-200 column with 0.1 μc of γ-globulin-^{125}I. The PVP which eluted with the isotope peak (hereafter referred to as PVP of γG size) was pooled, concentrated, and labeled with ^{125}I by the method of Briner (8). Residual free iodine was removed in the manner previously described. The labeled macromolecules contained less than 1% free iodine when used in these studies. The rate of water outflow from the pleural space was studied with hippuran-^{125}I obtained commercially. Isotope counting was performed in a gamma well counter, the results expressed as counts per minute (cpm) per unit of fluid.

Choice of experimental models. Previous studies (4) have shown that guinea pigs when immunized by the footpad injection of 0.2 mg of heat-killed H37Ra tubercle bacilli in Freund's adjuvant will subsequently produce a pleural effusion after the intrapleural injection of 0.1 μg or more of tuberculin PPD. This reaction is highly reproducible with maximum effusion volumes occurring 12–24 hr after intrapleural stimulation, and may be transferred to nonimmunized guinea pigs by the intravenous injection of peritoneal exudate cells but not serum from immunized donors (4). For studies of the delayed hypersensitivity reaction, guinea pigs were accordingly immunized with H37Ra tubercle bacilli as indicated above and a delayed hypersensitivity reaction elicited within the pleural space 3–6 wk after immunization by the intrapleural injection of 10 μg or more of commercially obtained PPD suspended in 0.5 ml of the supplied diluent. Control groups consisted of nonimmunized animals given the same dose of PPD intrapleurally. The basic

model of delayed hypersensitivity in these studies, therefore, utilized *actively* immunized guinea pigs, but in a limited number of experiments passive transfer of hypersensitivity was accomplished by the intravenous injection into a normal guinea pig of 10^7 cells harvested from the peritoneal cavity of an immunized guinea pig after the injection of sterile mineral oil. Intrapleural challenge in these instances was with 100 μg of PPD. Control animals received peritoneal exudate cells harvested from normal pigs. Techniques of passive transfer were described in more detail in a previous communication (4). PPD was obtained commercially as the preparation for skin testing in man.

Choice of a model for study of immediate hypersensitivity within the pleural space was more difficult. Passive immunization of the experimental animal by transfer of antibody would be necessary to assure that delayed hypersensitivity, which might develop simultaneously with antibody during active immunization with a protein antigen, would play no role in the reaction. Preliminary experiments with serum from H37Ra-immunized animals indicated that moderate titers of antibody were detectable against PPD by a complement fixation test, but skin fixation of this antibody was not detectable using the passive cutaneous anaphylaxis (PCA) reaction with PPD. This is in agreement with the findings of Ovary (2) who also could not demonstrate positive PCA tests with PPD and guinea pig anti-tubercle bacillus antiserum. The assumption that antibody which fixes to tissue was necessary for the intrapleural reaction has not been subjected to rigorous test, but was felt to be reasonable based on the necessity for such an antibody to demonstrate immediate hypersensitivity reactions in the skin (2). Accordingly, an ovalbumin, rabbit anti-ovalbumin antigen-antibody system was chosen, as high titers of antibody which effectively fix to guinea pig skin are known to develop in rabbits actively immunized with this antigen (2). Such a system also allowed verification that the antiserum in the dosages used was effective in passively transferring hypersensitivity by use of the PCA reaction. Albino rabbits were immunized with commercially obtained, 3X crystallized ovalbumin over a period of several weeks, and a pool of antiserum prepared. In vitro analysis of this antiserum by precipitin curves revealed it to contain about 4.3 mg/ml of precipitating antibody against ovalbumin. Because of generalized increase in vascular permeability in the guinea pig known to occur after the administration of rabbit serum, the pooled rabbit anti-ovalbumin serum used in our studies was handled after the method of Ovary, Spitz, and DeSzalay (9): normal guinea pig serum 0.05 ml/ml of rabbit antiserum were mixed and then spun for ½ hr at 20,000 *g*, 4°C in an ultracentrifuge (model L, Beckman Instruments, Inc., Fullerton, Calif.). Initially, 400–500 g guinea pigs were passively sensitized by intravenous injections of 2- or 4-ml aliquots of this pooled, rabbit anti-ovalbumin serum. After comparative studies (see Results) sensitizing injections of 0.5 ml of the same antiserum into the pleural space were used for the majority of experiments reported here. Stimulation of the hypersensitivity reaction was accomplished by the intrapleural injection of 100 μg of ovalbumin in 0.5 ml of pH 7.4 phosphate-buffered saline 18 hr after antibody administration. Control animals received normal rabbit serum with subsequent challenge by ovalbumin, or anti-ovalbumin antiserum with challenge by saline. Data from these control groups were statistically identical and were combined.

In addition, it was felt that comparative experiments with some form of nonimmunologically induced effusion would be desirable. Arbitrarily, turpentine was chosen as a nonspecific chemical irritant because of precedent for the use of this material (10, 11). Previous experiments in this laboratory (4) had shown that pleural effusions induced by this irritant reached a maximal volume 24–48 hr after injection. A number of experiments were done utilizing different doses of turpentine. Only a third of animals developed effusions after intrapleural injection of 0.05 ml of turpentine; all animals developed effusions at 0.1 ml injection volume, and when 0.2 ml or more was given at least 50% of the animals died. Thus, the usable dosage range with which effusions developed was narrow. Routinely, 0.1 ml of turpentine was suspended as a bubble in 0.4 ml of saline and injected into the pleural space.

Techniques of injection. Intrapleural injections and harvesting of effusions were performed in a standard way as previously recorded (4). In brief, the unanesthetized animal was hand-held and a 20 gauge blunted needle with trochar was introduced by blunt dissection through the intercostal muscles via a 2 mm nick in the skin. The trochar was removed and injections, routinely 0.5 ml in volume were made with a tuberculin syringe. At the time of effusion harvest the animal was killed by a blow to the head and, after cessation of palpable heart beat, the thoracic cavity was opened and effusion harvested into preweighed containers. The thoracic cavity was finally swabbed with preweighed sponges. Effusion weights were obtained by difference. Blood from experimental animals was obtained by aspiration from the exposed abdominal aorta just before death of the animal and before opening of the thoracic cavity.

Experimental design. In each of the models, measurement of vascular permeability was obtained by determining macromolecular flow from the vascular to the pleural space ("inflow") during serial 3-hr intervals after stimulating (intrapleural) injection by a method which was a modification of that of Chien and associates (12). Approximately 0.1 μc of labeled macromolecule representing 10 μg of protein or PVP was injected intravenously 3 hr before animal sacrifice at 3, 6, 12, 18, and 24 hr after intrapleural stimulus. The ratio (cpm 1 g pleural effusion)/(cpm 1 ml serum) was calculated and formed the basis of the measurement of changes in permeability. This ratio in some instances exceeded unity during periods of increased macromolecular inflow due to two factors: a rapid decline in serum marker concentration, and the comparatively slow clearance of macromolecules from the pleural space. Chien et al. made a similar observation (12). A zero-time ratio for each macromolecule was obtained by injecting normal animals intravenously with labeled marker, sacrificing them 3 hr later, and calculating the ratio as previously described.

In each of the models resorption of macromolecules from the reaction site ("outflow") was determined by injecting 0.01 μc of labeled material representing 1 μg of protein or PVP into the pleural space at the time of stimulus and sacrificing groups of animals at 6, 12, 18, and 24 hr. The percentage of labeled macromolecule remaining at each time period was calculated, and the disappearance curve of marker from the pleural space plotted. These outflow experiments measured cumulative changes occurring over the entire experimental period. To measure changes in macromolecular outflow during different phases of the reaction, we used the following technique. Groups of control and experimental animals were injected intrapleurally with 0.01 μc γ-globulin-^{125}I at the time of intrapleural stimulus, or at 6, 12, and 18 hr thereafter, and sacrificed 6 hr later. The percentage of γ-globulin-^{125}I remaining at the end of the 6-hr interval was calculated. The possibility was considered that effusion volume would affect the rate of macromolecular

outflow as studied by these techniques. When various volumes of isotonic saline mixed with trace amounts of γ-globulin-^{125}I were injected into the chest of normal guinea pigs, percentage retention of isotope at 24 hr was identical with 0.5 ml injection controls up to a volume of 5 ml. Above that volume, 24 hr retention of marker differed from the 0.5 ml controls by less than 5%. Further, when the data were plotted as a function of the volume of effusion present, no statistically significant differences could be shown. The data also demonstrated that effusion radioactivity was always 10–60 times greater than serum radioactivity obtained at the same time. On this basis it was felt that recirculation of marker would have little, if any, effect on the data as presented.

Outflow of water was approximated by measuring the disappearance of ^{125}I-labeled hippuran from the pleural space. Approximately 0.025 μc of hippuran-^{125}I was injected intrapleurally into each member of five groups of 16 animals, one group each at 0, 5, 11, 17, and 23 hr after intrapleural stimulus. Four animals from each group were sacrificed 15, 30, 45, and 60 min after each hippuran injection, and the percentage of labeled hippuran remaining was determined. From the mean value determined at each 15-min interval, a hippuran clearance curve was constructed by the least squares method of regression analysis, and from this the half-life of hippuran ($t_{\frac{1}{2}}$) calculated. Thus a $t_{\frac{1}{2}}$ ($t_{\frac{1}{2}}x$) of hippuran was determined at 0, 5, 11, 17, and 23 hr after intrapleural stimulus. Analysis of these data indicated that the volume of effusion definitely influenced the $t_{\frac{1}{2}}$ of hippuran. Accordingly, a "control curve" was constructed by injecting volumes of saline at 1 ml increments from 1 to 7 ml (corresponding with the extremes of effusion volume seen in these experiments) into the pleural space of normal animals. The rate of hippuran-^{125}I disappearance was calculated over a 1 hr time period for each volume in the same manner as above, and a $t_{\frac{1}{2}}$ for each effusion volume determined ($t_{\frac{1}{2}}c$). Results of hippuran outflow in the experimental animals were then expressed as the ratio of the control $t_{\frac{1}{2}}$ ($t_{\frac{1}{2}}c$) to experimental $t_{\frac{1}{2}}$ ($t_{\frac{1}{2}}x$), with the control valume representing the same effusion volume as that observed in the experimental animal. A ratio $(t_{\frac{1}{2}}c)/(t_{\frac{1}{2}}x)$ greater than one, therefore, indicates a water outflow greater than control, a ratio equal to one a water outflow equal to control, and a ratio less than one a water outflow less than control.

Statistical analysis was performed after the methods of Stearman (13). Each point given in the results represents the mean of results from at least four animals.

RESULTS

Figs. 1 and 2 show results of inflow of γ-globulin-^{125}I into the pleural space during the delayed hypersensitivity reaction, immediate hypersensitivity reaction and the acute inflammatory response to turpentine. In the delayed hypersensitivity reaction the ratio (cpm 1 gm pleural effusion)/(cpm 1 ml serum) is never significantly greater than that of control ($P > 0.1$). This is in contrast to the high ratio seen in the 1st 3 hr in both the acute inflammatory reaction to turpentine ($P < 0.001$) and the immediate hypersensitivity reaction ($P < 0.01$). In the acute inflammatory reaction the ratio remains significantly elevated ($P < 0.01$) over that of control during the entire experiment, while in

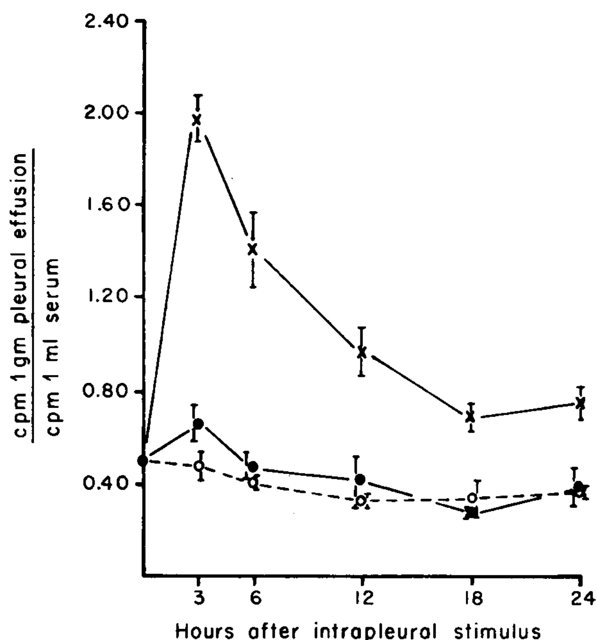

FIGURE 1 Serial measurements of the γ-globulin-^{125}I inflow ratio (cpm 1 g pleural effusion)/(cpm 1 ml serum) in control (O---O), delayed hypersensitivity reaction (●——●), and turpentine pleurisy (X——X). Each point represents the mean value from at least four animals and the vertical lines represent 1 SEM.

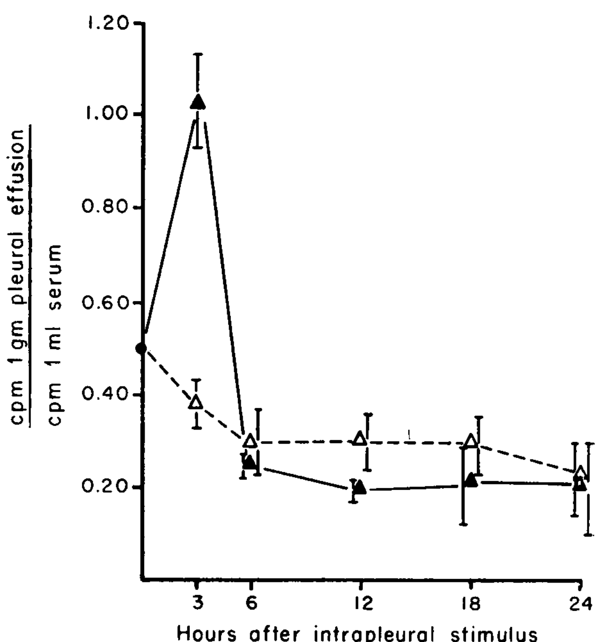

FIGURE 2 Serial measurements of the γ-globulin-^{125}I inflow ratio (cpm 1 g pleural effusion)/(cpm 1 ml serum) in control (△---△), and immediate hypersensitivity reaction (▲——▲). Each point represents the mean value from at least four animals and the vertical lines represent 1 SEM.

the immediate hypersensitivity reaction the rise does not persist past 3 hr ($P > 0.1$).

Differences in the outflow of γ-globulin–^{125}I from the pleural space are seen in Fig. 3. There is a delay in resorption of γ-globulin in acute inflammatory reaction to turpentine and the delayed hypersensitivity reaction. This delay becomes significantly greater than control at 12 hr ($P < 0.02$) in the delayed hypersensitivity reaction and at 18 hr ($P < 0.02$) in the acute inflammatory reaction. The outflow of γ-globulin–^{125}I in the immediate hypersensitivity reaction remains the same as control ($P > 0.1$). As mentioned in Methods, analysis of the data to determine the effect of effusion size on resorption of macromolecules revealed no such effect within the effusion volume limits seen in these animals. To assess macromolecular outflow during different phases of the reaction serial, 6 hr measurements in the resorption of γ-globulin–^{125}I in the delayed hypersensitivity and acute inflammatory reactions were determined and are shown in Fig. 4. Gamma globulin outflow during the delayed hypersensitivity reaction is progressively impaired, attaining maximal values after 6 hr ($P < 0.02$). During the acute inflammatory reaction, on the other hand, γ-globulin outflow impairment is maximal during the 1st 6 hr after stimulus ($P < 0.02$). Comparison of the mean interval percentage retention of γ-globulin 6 hr or more after stimulus in the delayed reaction with

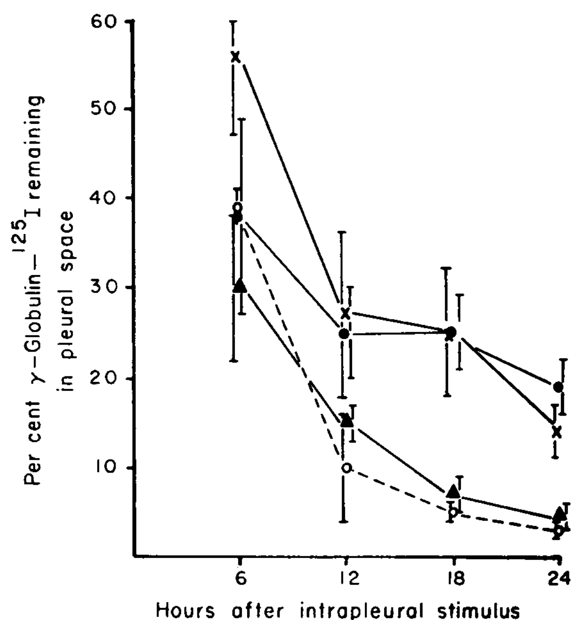

FIGURE 3 Macromolecular outflow: serial measurements of the per cent of γ-globulin–^{125}I remaining the the pleural space in control (O- - -O), immediate hypersensitivity (▲——▲), delayed hypersensitivity (●——●), and turpentine pleurisy (X——X). Each point represents the mean value from at least four animals and the vertical lines represent 1 SEM.

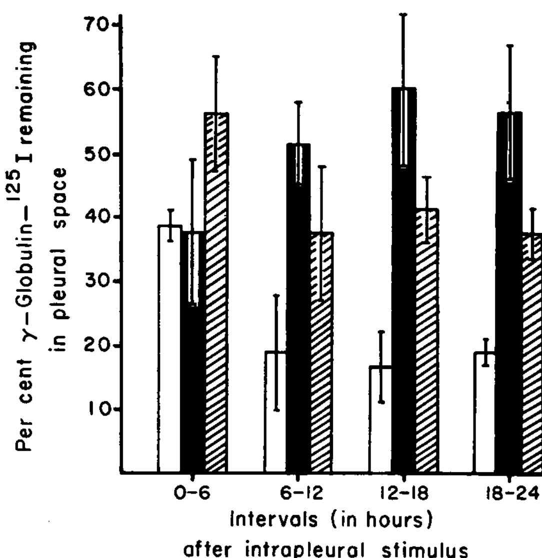

FIGURE 4 Macromolecular outflow: serial measurements of the percentage of γ-globulin–^{125}I remaining in the pleural space 6 hr after intrapleural isotope injection in control (open bar), delayed hypersensitivity reaction (closed bar), and turpentine pleurisy (cross-hatched bar). Each bar represents the mean value from at least four animals and the vertical lines represent 1 SEM.

that 6 hr or more after stimulus in the acute inflammatory reaction also reveals a high degree of statistical difference ($P < 0.001$).

To assure that the delayed outflow of the macromolecular markers seen in the H37Ra-immunized animals was a function of the delayed hypersensitivity reaction, we performed a limited number of experiments on guinea pigs passively immunized to PPD by the transfer of cells from actively immunized donors. These gave qualitatively identical results. Two animals which received cells from immunized donors and developed effusion (1.6 and 3.3 g) after the intrapleural injection of 100 μg of PPD had 9.3 and 8.6% of the γ-globulin–^{125}I left at 24 hr. Two animals which received cells from normal donors and did not develop effusions (0.9 and 0.7 g) had 1.9 and 1.6% of the same marker left at 24 hr after PPD injection.

To assess any role of the chemical nature of the marker in the results of these experiments, different marker molecules were also used. Tables I and II show results of outflow and inflow studies with albumin-^{125}I and PVP-^{125}I of γG size. The same changes as seen with γ-globulin are again noted. In the delayed hypersensitivity reaction, the rate of inflow of these macromolecules is never significantly greater than control ($P > 0.1$). Both turpentine and immediate hypersensitivity reactions demonstrate a significantly increased inflow ($P < 0.001$) beginning at 3 hr which is transient in the immediate reac-

Table I

Albumin-^{125}I

Time after intra-pleural stimulus	Control	Delayed hyper-sensitivity reaction	Turpentine pleurisy	Immediate hypersensitivity reaction: Control	Immediate hypersensitivity reaction: Experimental
			INFLOW Mean ratio (cpm 1 g pleural effusion)/(cpm 1 ml serum)		
hr					
3	0.23 ±0.03*	0.36 ±0.06	0.74 ±0.05	0.26 ±0.07	0.69 ±0.04
6	0.21 ±0.06	0.32 ±0.03	0.42 ±0.04	0.29 ±0.07	0.24 ±0.03
12	0.09 ±0.01	0.19 ±0.04	0.24 ±0.04	0.28 ±0.06	0.18 ±0.03
18	0.19 ±0.09	0.21 ±0.05	0.13 ±0.01	0.28 ±0.04	0.21 ±0.09
24	0.16 ±0.07	0.21 ±0.05	0.10 ±0.01	0.21 ±0.10	0.23 ±0.08
			OUTFLOW Mean percent albumin remaining		
6	47 ±4*	49 ±2	46 ±5	31 ±8	
12	20 ±2	29 ±3	28 ±4	17 ±3	
18	6 ±2	22 ±2	19 ±1	7 ±2	
24	3 ±1	15 ±3	13 ±1	2 ±1	

* SEM.

tion and persistent in the turpentine reaction. A decreased rate of outflow of macromolecules ($P < 0.02$) is again seen in the delayed hypersensitivity reaction and the acute inflammatory response to turpentine. There is no delay in the resorption of albumin during the immediate hypersensitivity reaction. An apparently deviant value for control albumin inflow ratio at 12 hr shown on Table I is due to the occurrence of high ratios in one animal of each group at 18 and 24 hr. Thus, of the 15 animals at these three points all but two fell within a range of 0.05–0.19; comparison of the mean values at 12, 18, and 24 hr in the control column by the Student's t test revealed no significance to this difference ($P > 0.2$).

It was considered possible that some alteration might have occurred in the state of the markers while they were within the pleural space. Careful analysis of effusions harvested from these reactions by Sephadex G-200 chromatography, zone electrophoresis in starch, and sucrose density gradient showed the ^{125}I labeled molecules to be identical in behavior with the parent unlabeled material, and with the labeled material when studied before its intrapleural injection. Separation of the effusions into cellular and noncellular fractions by centrifugation and membrane filtration revealed that 1% or less of the ratioactivity was associated with the cellular elements.

To explore the possibility that differences in intensity rather than qualitative differences existed between the delayed and immediate hypersensitivity models which would explain the differences in macromolecular movement observed, we performed experiments with different degrees of antigenic challenge or passive immunization. In these studies, one apparently distinguishing aspect of the two reactions was explored: the marker inflow leak observable in the immediate hypersensitivity reaction at 3 hr. Data from these studies are presented in Tables III and IV. Increasing the intrapleural stimulating dose of PPD from 10 to 100 μg in the animal

Table II

PVP-^{125}I of γG Size

Time after intra-pleural stimulus	Control	Delayed hyper-sensitivity reaction	Turpentine pleurisy
		INFLOW Mean ratio (cpm 1 g pleural effusion)/(cpm 1 ml serum)	
hr			
3	0.36(±0.01)*	0.48(±0.05)	1.17(±0.04)
6	0.20(±0.05)	0.32(±0.06)	0.73(±0.03)
12	—	—	0.53(±0.04)
18	0.06(±0.01)	0.10(±0.06)	0.43(±0.05)
24	0.08(±0.03)	0.08(±0.04)	0.34(±0.02)
		OUTFLOW Mean percent remaining	
6	61(±4)*	82(±7)	66(±4)
12	45(±7)	63(±6)	53(±7)
18	25(±3)	50(±4)	41(±5)
24	13(±4)	41(±6)	42(±6)

* SEM.

TABLE III

Effect of PPD Dosage on Pleural Inflow of γ-Globulin-^{125}I during Delayed Hypersensitivity Reaction

Dose of PPD	Mean ratio	
	Experimental	Control
	cpm/g effusion: cpm/ml serum at 3 hr	
μg		
10	0.67(±0.06)	0.47(±0.10)
100	0.71(±0.12)	0.69(±0.12)

actively immunized with H37Ra tubercle bacilli did not elicit an inflow leak of γ-globulin-^{125}I detectable by these techniques (Table III).

Because of limitation in the amount of serum which can be administered intrapleurally without induction of residual effusion at 24 hr, comparative studies were made between groups of animals receiving 2.0 and 4.0 ml of the anti-ovalbumin serum *intravenously*, and stimulated with 100 μg of ovalbumin intrapleurally. These animals had positive immediate skin tests to 10 μg (average diameter 14 mm) as well as to 100 μg ovalbumin (17 mm with central ischemia). As shown in Table IV, no increase in the inflow defect accompanied this increased antibody dosage. The data also indicate that comparable effects were produced by sensitization with 0.5 ml of antiserum injected intrapleurally, the procedure used in the majority of experiments reported here. Challenge of guinea pigs actively immunized with ovalbumin, having demonstrable complement-fixing antibodies against ovalbumin and positive skin tests of the Arthus type, resulted in an inflow leak of the γ-globulin-^{125}I comparable to that seen with the passively immunized animals (Table IV). The value of 1.24 seen with the actively immunized animal is not significantly different from those attained by passive immunization (all P values > 0.2). No effusions were observed during the course of any of these immediate hypersensitivity reactions.

TABLE IV

Effect of Degree of Sensitization on Pleural Inflow of γ-Globulin-^{125}I during Immediate Hypersensitivity Reaction to Ovalbumin

Type of sensitization	Antibody dosage and route	Mean ratio	
		Experimental	Control
	ml	*cpm/g effusion: cpm/ml serum at 3 hr*	
Passive	0.5 intrapleural	1.03(±0.10)	0.38(±0.05)
	2.0 i.v.	1.08(±0.20)	0.42(±0.01)
	4.0 i.v.	0.94(±0.05)	0.38(±0.02)
Active	—	1.24(±0.36)	0.47(±0.10)

TABLE V

*Rate of Water Resorption from the Pleural Space during Delayed and Immediate Hypersensitivity Reactions Expressed as $t_{\frac{1}{2}}c/t_{\frac{1}{2}}x$**

Interval after intrapleural stimulus	Immediate hypersensitivity reaction	Delayed hypersensitivity reaction
hr		
0–1	1.0	3.0
5–6	1.06	1.36
11–12		0.76
17–18	0.94	0.64
23–24	0.88	0.75

* See text for explanation.

The disappearance of hippuran-^{125}I as a marker of the behavior of water during the course of the delayed and immediate hypersensitivity reactions was studied, and the results presented in Table V. During the delayed hypersensitivity reaction there is an increased rate of hippuran outflow compared with control during the 1st 6 hr after intrapleural stimulus, ($t_{\frac{1}{2}}c/t_{\frac{1}{2}}x > 1$), whereas hippuran outflow is equal to control during this period in the immediate hypersensitivity reaction ($t_{\frac{1}{2}}c/t_{\frac{1}{2}}x = 1$). From 18 to 24 hr, however, the ratio falls significantly in the delayed hypersensitivity when compared with the immediate hypersensitivity reaction.

DISCUSSION

Immediate and delayed hypersensitivity reactions have been compared in a number of ways including the agents carrying their activity, temporal characteristics of their reactions, histologic effects, characteristics of the antigenic determinants necessary for each, and the pharmacologic agents responsible for their mediation. A comparison of the physiologic effects of these reactions in the living organism has largely involved qualitative or semiquantitative evaluations of skin reactivity. This mode of comparison is somewhat limited by difficulty in clearly differentiating delayed from immediate reactions. Our results suggest that by studying these reactions as they occur within the pleural space a differentiation of the physiologic effects of these two types of reaction can be made, and a quantitative estimation of a physiologic defect of each is possible.

Previous studies of circulation through the pleural space have indicated that proteins and water enter primarily by way of the capillaries; egress of proteins is via lymphatics while water exits primarily by passing back into the capillary circulation (14, 15). Based upon such a differentiation of macromolecular inflow from outflow, the data presented in our study may be interpreted to suggest two things. The immediate hypersensitivity reaction causes a measurable increase in capil-

lary permeability to macromolecules during the 1st 6 hr after immunologic challenge. This interpretation is compatible with qualitative observations on immediate hypersensitivity reactions in the skin (1, 2). The delayed hypersensitivity reaction, on the other hand, lacks demonstrable capillary permeability to macromolecules during any phase of the reaction; it is characterized instead by a striking delay in the resorption of macromolecules from the pleural space most marked 12 hr or more after onset of the reaction. Previous qualitative observations on delayed hypersensitivity reactions based on the appearance of dye in the skin have been interpreted to indicate increased permeability of the capillaries of a limited degree, but have made no recognition of the possibility that outflow delay might be responsible for the findings (16).

Before accepting the validity of such a comparison between the immediate and delayed hypersensitivity reactions, however, certain questions must be considered about the experiments by which it was derived. It is conceivable, for instance, that intensity of the reactions alone might account for the differences observed. We feel that such a possibility is unlikely, however, for the following reasons. In the actively immunized animal subjected to studies in macromolecular inflow during the delayed hypersensitivity reaction, increasing the antigenic strength by 10-fold gave no detectable evidence for an inflow defect. This is an antigen dose 1000 times the minimal amount necessary to cause an effusion due to delayed hypersensitivity in these guinea pigs. By quantitative as well as qualitative studies of the effusions produced, this dose of PPD is eliciting the maximal intensity of reaction possible in this model (4). Similarly, increasing the amount of antibody used in passively immunized animals for these studies, or the use of actively immunized animals, had no demonstrable effect on the intensity of inflow leak demonstrable in the immediate hypersensitivity model. At this level of reaction no delay in the outflow of macromolecules could be demonstrated. Different amounts of antigen were not used in quantitative comparison of the immediate hypersensitivity model. It has been demonstrated by Ovary, however, that the passive cutaneous anaphylaxis reaction in guinea pigs utilizing intravenous administration of rabbit anti-ovalbumin antibody may detect as little as 0.02 μg of ovalbumin (2), an amount 5×10^{-3} smaller than the dose used in these studies. Such sensitivity was achieved by administration of 0.13 mg of antibody/100 g body weight; doses of antibody used in our studies have been greater than 1 mg antibody/100 g of body weight. Since our studies used 10 times as much antibody and 1000 times as much antigen as that shown adequate in a comparable system, we feel it unlikely that dose of antigen or antibody is a limiting factor in our studies. The data would seem to indicate, in fact, that within the limits of the models used, near maximal defects were being produced in both the immediate and delayed hypersensitivity reactions. This raises the possibility that the physiologic manifestations demonstrated are threshold effects, occurring beyond a certain degree of hypersensitivity stimulus and not augmented by increasing the stimulus beyond that point. Data presented in this paper are not adequate to speak to this suggestion, but it is in keeping with observations on the development of effusions during intrapleural delayed hypersensitivity reactions. At an antigen (PPD) dose of 0.1 μg, effusions appear which are quantitatively, qualitatively and the same incidence as effusions resulting from the injection of 100 μg of PPD (4).

Interpretation of data evolved from intrapleural injections of turpentine is more difficult due to inability to give meaningful quantitative evaluation of the inflammatory response. The data derived from study of the turpentine-induced intrapleural lesion are, however, in no way critical to comparison of the effects of delayed and immediate reactions occurring within the chest, nor is it clear that inflammation induced by different chemical agents would be similar in behavior. For these reasons detailed studies of different doses of turpentine have not been performed, and the data may be used to speak to only a limited number of points. Such an injury clearly begins quickly after initiation of stimulus, as evidenced by the most marked macromolecular inflow and outflow defects occurring within the 1st 6 hr. The contrast between early onset of the most severe phase of outflow defect in turpentine-induced pleurisy and the later onset of the most severe phase of this defect induced by the delayed hypersensitivity reaction is of interest. In the early onset of its effects, the chemical injury more directly compares with the immediate hypersensitivity reaction. The late effects of turpentine injections could possibly be obliterated by smaller stimuli, being (both inflow and outflow) of lesser magnitude than those noted within the 1st 6 hr. Of the three reactions studied, only two were accompanied by the collection of significant amounts of fluid within the pleural cavity: delayed hypersensitivity and the turpentine pleurisy. Both of these reactions *at the levels studied* shared a characteristic which features in part, at least, an impairment of the removal of macromolecules from the pleural space. That such a phenomenon may be pathogenetically important in the collection of interstitial fluid is supported by observations that the accumulation of fluid in cardiac edema in the dog (17) and ascites in the rat with portal hypertension (18) is dependent upon some degree of lymphatic (*sic,* outflow) obstruction.

Nonimmunologic phenomena might play a role in the findings presented. The absence of impaired macromolecular clearance after the intrapleural injection of PPD into nonimmunized controls, or the failure of anti-oval-

bumin antiserum alone to elicit an early increase in capillary permeability are against this possibility. It has been known for some time, however, that immunization of animals with tubercle bacilli makes them hypersusceptible to the effects of endotoxin (19), and also that derivatives of tubercle bacilli in the form of old tuberculin contain detectable amounts of endotoxin (20). Evidence from a number of sources, however, is against the possibility that endotoxin is playing a role in the reaction to PPD described here. First, there is data indicating that PPD does not contain detectable quantities of endotoxin. Fractionation of tuberculin shows a differentiation of endotoxin activity from protein (3), as would be expected from the lipopolysaccharide nature of endotoxin. The antigen used in our study is, of course, a protein derivative of the tuberculous organism. Similarly, though endotoxin has a detectable lethal effect on the normal, nonimmunized guinea pig in appropriate dosage, intravenous or intraperitoneal administration to normal guinea pigs of up to 5000 μg of PPD has shown no detectable endotoxin effect (21). Our studies have routinely utilized 10 μg of PPD per animal, effusions may be evoked in the immunized animal with 0.1 μg of PPD, and no effect on normal animals by the intrapleural administration of 100 μg of PPD could be demonstrated. Finally and most importantly, we have been able to achieve qualitatively similar results in normal animals passively immunized by cell transfer; repeated attempts to transfer sensitivity to endotoxin by cells from endotoxin-sensitive donors have been unsuccessful (19).

Certain characteristics of these immunologic systems have prevented design of a desirable situation in which actively immunized animals were challenged with the same antigen and effects of the two different immunologic systems distinguished. Considerations of the reasons dictating choice of the models have been given in detail in Methods. Complete studies have not been done utilizing different antigen and their respective immediate and delayed hypersensitivity states. Certain observations, however, have been made to suggest that the phenomena observed are characteristic of the immunologic systems and not of the specific antigens used in this study. Active immunization of guinea pigs with formalinized *Histoplasma capsulatum,* heat-killed *Candida albicans,* or picrylated ovalbumin has produced animals with strongly positive skin tests of the delayed type which develop pleural effusions after the intrapleural injection of the appropriate antigen. Active immunization of guinea pigs with human gamma globulin or bovine albumin has produced animals with complement-fixing and precipitating antibody against these antigens as well as strongly positive skin tests of the Arthus type, yet animals which produced no effusions after intrapleural challenge. This aspect of the behavior of the immunologic systems used in the present study would seem, therefore, generic.

It would seem from comparative data presented on two proteins of different size (albumin and γ-globulin) as well as on a synthetic molecule of comparable size (PVP) that the observations on macromolecular flow are not significantly influenced by the nature of the macromolecule involved. It is possible that size of the molecules is playing some role in the quantitative but not qualitative nature of the defects shown, as suggested by the consistently smaller percentage retention of albumin than of γ-globulin during the course of macromolecular outflow after delayed hypersensitivity reaction. These differences, however, are not statistically significant with the numbers of animals used here and remain suggestive at best.

Both protein and water accumulate in the pleural space during the course of delayed hypersensitivity and turpentine-induced reactions. It is possible that because of limited numbers or accessibility of sites responsible for the inflow and outflow of protein, the presence of unlabeled, competing macromolecules would affect our data. Information concerning the proteins in effusions induced by turpentine when compared with the macromolecular flow defects demonstrated here would seem to offer evidence against such a possibility. From 2 to 24 hr after the intrapleural injection of turpentine the quantity and relative proportions of various proteins in the resulting effusions are constant (4), yet it is during this time period when both inflow and outflow defects have been demonstrated to appear and quantitatively improve. Similarly, 3 hr after stimulation of the delayed hypersensitivity reaction the effusion protein concentration is low (4) and our studies have shown no macromolecular inflow defect. In turpentine-induced effusions at this time, however, the protein concentration is high and such a defect is demonstrable. These results are the opposite of what would be expected if high intrapleural protein concentration impedes inflow of macromolecules.

Observations on the accumulation of protein during effusions developing after the delayed hypersensitivity reaction fit well, in fact, with the defects described here. In the early phase (up to 12 hr) there is an increasing protein concentration as well as an increasing fluid volume within the chest (4), indicating retention of protein in excess of water. Data presented here show a delay in protein clearance from the chest during this period coupled with an increased rate of water outflow, as suggested by a high $t_{\frac{1}{2}}c/t_{\frac{1}{2}}x$. Unfortunately, rapid clearance of hippuran from the guinea pig's blood stream has not allowed the accumulation of valid data for evaluating water inflow, but since its clearance is increased one would assume its entrance is enhanced, necessarily to a greater degree. This combination of lesions could account for the accumulation of protein greater than the

accumulation of water during this period. It would also suggest the possibility that movement of water and movement of macromolecules are under different control (mechanism or site), though quantitative differences (e.g. molecular size) could be responsible. The possibility of differences in control is not at variance with previous studies which have shown differences in routes of egress of water and macromolecules from the pleural space (14, 15). The low ratio $t_{\frac{1}{2}}c/t_{\frac{1}{2}}x$ for hippuran seen late in the course of the delayed hypersensitivity reaction, however, suggests the possibility that water molecules are also subjected to some sort of outflow obstruction. Validity of this interpretation is subject to evaluation of the role of osmotic pressure from the retained macromolecules in holding water within the pleural space. That osmotic effects may not be the sole determinant in the production of pleural effusions in these animals is suggested by the observation that fluid volume and protein concentration are essentially constant between 12 and 24 hr after the induction of the delayed hypersensitivity reaction. If no organic defects were influencing the inflow or outflow of water, one would expect the $t_{\frac{1}{2}}c/t_{\frac{1}{2}}x$ of water to be unity. Since data presented here suggest the ratio to be low during this period, it is possible that some degree of outflow obstruction is holding back water as well. This would require a coincident decrease in the rate of water inflow, and data to speak to this point have not been attained.

If the studies of Courtice and Simmonds (14) indicating that proteins leave the pleural space solely through the lymphatics are valid in these circumstances as well as in the normal animal, the observations of impaired macromolecular outflow in the delayed hypersensitivity reaction as well as in the turpentine pleurisy would suggest a decrease in the resorptive capacity of the pleural lymphatics. The early onset of delay demonstrated in the turpentine reaction suggests a reduction in the effective lymphatic resorptive area, perhaps due to sclerosis of lymphatic channels. Capillary damage demonstrated during turpentine pleurisy by the electron micrographs of Ham and Hurley support this thesis (10). Brann and Nilsson (22) have studied the pleura of sensitized guinea pigs 48 hr after intrapleural tuberculin stimulus and have demonstrated an electron-dense material deposited within the submesothelial space. This material was not found in animals subjected to turpentine pleurisy (11). The role such a material plays in the outflow we have demonstrated during the delayed hypersensitivity reactions is not clear. The differences between it and the turpentine pleurisy models as seen on electron microscopy fit with our demonstration that maximal outflow obstruction occurred early after turpentine stimulus but late after PPD stimulus in the hypersensitive animal, and suggest that a different mechanism of obstruction is involved.

These data suggest that the changes in macromolecular and water kinetics which occur in the immediate and delayed hypersensitivity reaction result from entirely different physiologic effects. The immediate hypersensitivity reaction transiently alters the vascular permeability of the vessels at the site of the reaction. No changes in the outflow of water can be detected. In the delayed hypersensitivity reaction the major alteration occurs not in vascular permeability but rather in a decreased capacity to resorb macromolecules from the site of reaction accompanied by a decreased rate of resorption of water.

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