Clin. exp. Immunol. (1971) **9**, 809–819.

THE EFFECT OF LUNG REACTIVE ANTIBODIES ON THE PATHOGENESIS OF TUBERCULOSIS

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(*Received* 22 *March* 1971)

SUMMARY

Injections of washed mouse lung-CCT suspended in Freund's complete adjuvant were given to mice and guinea-pigs to stimulate production of homologous and heterologous mouse lung-reactive antibodies which were harvested as peritoneal ascitic fluid and serum, respectively. Reactivity of the homologous antibody was limited to relatively weak, but lung-specific, focal localization in the alveolar septa recipient mice as demonstrated by immunofluorescence studies. Only a very small portion of samples yielded positive results by the antiglobulin consumption test (AGCT). All heterologous sera were strongly active as measured by the AGCT and highly pneumotoxic when injected intravenously in 0·2 ml doses. In addition, the heterologous immune sera were strongly cross-reactive and demonstrated intense, even fluorescent staining in the glomerular basement membranes.

Although the lung density technique did not prove to be useful in these studies, some evidence was obtained with it that seemed to indicate that increased densities or tuberculous involvement were produced in infected animals receiving frequent intravenous homologous lung reactive antibody injections when compared to similar animals receiving saline injections. Due to the difficulty of obtaining sufficient volumes of demonstrably sensitive homologous antibody, no further studies were performed.

The effect of heterologous immune serum on experimental tuberculosis infection was an alteration in the appearance of and an increase in the number of lesions resembling a miliary spread of the organisms not seen in the controls. An index of tuberculous involvement was developed that correlated the enhanced spread of tubercle bacilli with administration of heterologous immune serum and allowed quantitation of the immune response as a function of histopathology.

INTRODUCTION

For many years our laboratories have been concerned with the demonstration and character-

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ization of lung reactive antibodies in humans with chronic lung diseases (Burrell *et al.*, 1964, 1966; Hagadorn & Burrell, 1968) and in experimental animal models of these diseases (Esber & Burrell, 1967). The key question throughout has been concerned with the role, if any, of these antibodies in the pathogenesis of the respective diseases. Are these antibodies harmful, ameliorative, or merely incidental to the disease process?

Early work showed that when rabbits received homologous antilung serum prior to a minimally infective dose of virulent tubercle bacilli, there was an indication that the antibodies contributed to a more invasive, miliary form of the disease when compared to rabbits receiving normal serum prior to infection (Rheins & Burrell, 1960). The extent of the epitheloid reaction, necrosis, and polymorphonuclear neutrophilic infiltration was not as pronounced in the animals receiving anti-lung serum. More importantly, the numbers of tubercle bacilli seen in the lesions were more abundant in the antilung serum treated animals, especially if such serum had been pre-adsorbed with killed tubercle bacilli.

More recently, Iliesco *et al.* (1966) immunized rats with homologous lung suspensions in Freund's complete adjuvant and then infected them with virulent tubercle bacilli. Such animals demonstrated an enhanced susceptibility to the infection that was attributed to isoimmunization. A criticism of this rationale could be that, through the mediation of the tubercle bacilli in the adjuvant, the animals were rendered sensitive to tuberculoprotein, and it thus could be predicted that tuberculous infection would be enhanced in them.

Salomone & Coppola (1967) injected hamsters with rabbit anti-lung serum and then infected them with atypical *Mycobacteria*, but could not find any evidence of enhanced infection of such strains when compared to animals pretreated with normal serum.

The present report deals with an attempt to refine the above experiments for the purpose of assessing the role of lung-reactive antibodies in the pathogenesis of tuberculosis.

MATERIALS AND METHODS

Cultures. A stock culture of the Erdman strain of *Mycobacterium tuberculosis* var *hominis* was grown on Lowenstein medium. A stock bacterial suspension was made by placing 5·0 mg (dry weight) of bacilli in 5·0 ml of phosphate-buffered saline pH 7·2 (PBS) containing 0·05% Tween 80 and homogenizing with a ten Broeck tissue grinder. Appropriate log serial dilutions were prepared up through 10^{-6} mg/0·2 ml.

Antigen. Insoluble mouse lung crude connective tissue (CCT) was prepared according to earlier specifications with minor modifications. After mincing the dissected lung tissue and rinsing in cold PBS, the tissue was washed four times in cold PBS, eight times in cold saline containing 3·3 g/l Na_4-EDTA and finally four times in cold distilled water. The washed CCT was then lyophilized and ground in a Wiley mill with copious amounts of dry ice to prevent denaturation. The antigen was stored at −20°C in sealed containers.

Homologous anti-lung globulin. Mouse anti-CCT was prepared by intraperitoneally injecting varying amounts of a saline suspension of CCT mixed with equal volumes of Freund's complete adjuvant into adult Swiss-Webster albino mice. Inoculations containing 2·5–10 mg of CCT were given weekly for a total of 4 weeks. At 7–10 days after the last injection, ascitic fluid was harvested from the abdomen of each mouse by means of an 18-gauge needle. Pools of three to four animals each were made on samples less than 5·0 ml. All fluids were centrifuged to remove cellular debris and adjuvant oil and then assayed for protein content and antibody activity.

Heterologous anti-lung globulin. Sufficient insoluble mouse lung CCT antigen was suspended in physiological saline and incorporated into an equal amount of Freund's complete adjuvant for injection into young (3–4-month-old) albino guinea-pigs. The injections were carried out as follows for each animal: (a) 0·05 ml distributed into the footpads of each foot; (b) 0·5 ml (IM) into each thigh; (c) 0·5 ml (SC) dorsolaterally on each side at the scapular region. Commencing with day 21, weekly cardiac bleedings were made through the eighth week at which time the animals were exsanguinated. At each bleeding the serum was harvested, heated for 20 min at 56°C and stored at −20°C.

Pneumotoxicity. Pneumotoxicity was determined by injecting single doses of 0·05 ml to 0·3 ml immune mouse ascitic fluid or guinea-pig serum intravenously into previously unchallenged mice and observing them for toxic symptoms. If no reaction was observed within 1 hr of injection the sample was considered non-toxic. Whenever injection was followed by death the lungs and airways were examined grossly. The criteria for pneumotoxic death were essentially those of Hagadorn, Vazquez & Kinney (1969), '. . . marked cyanosis and dyspnea, foaming at the nose and mouth. . . the lungs were distended, morrhagic and edematous and froth exuded from the trachea and cut surfaces'.

Antiglobulin consumption test (AGCT). To detect antibody to the insoluble lung antigen, a modification of the previously described AGCT (Burrell, Wallace & Andrews, 1967) was employed. To avoid the necessity of using fresh erythrocytes, a stock quantity of sheep red blood cells was first formalinized according to the technique of Czsimas (1960). Such treated cells were washed in cold saline and adjusted to 2% prior to mixing with an equal volume of freshly prepared 1:20,000 purified tannic acid for 30 min at room temperature. The tanned cells were washed three times in pH 6·4 PBS and sensitized with an appropriate concentration of either mouse or guinea-pig globulin (usually 0·200 mg/ml) at room temperature for 1 hr. The cells were then washed in pH 7·2 PBS and resuspended in pH 7·4 PBS containing 0·005 M dipotassium versenate.

For use in the test 0·2 ml of the antibody was incubated with 5·0 mg of the globulin free CCT for 30 min at 37°C. Following at least eight PBS washes, the sediment was quickly mixed with the appropriate, prestandardized antiglobulin serum for 5 min and centrifuged. The supernate was then titred against the sensitized cells. An inhibition of the antiglobulin titre by one dilution was considered as one unit of antiglobulin consumption.

Lung density measurements. Lung density measurements to assess the extent of tuberculous involvement were made according to the method of Crowle (1958).

Histologic studies. Tissue for histopathologic study was fixed in neutral phosphate buffered formalin and included all lungs from the lung density studies and selected lungs from each experimental group. In addition selected kidneys and spleens were also removed for sectioning. Companion sections were cut from paraffin blocks at 6 μ and stained with routine haematoxylin and eosin and where appropriate, by the Kinyoun method for acid-fast bacteria.

Immunofluorescence. Sections of lung for immunofluorescent studies were insufflated *in situ* before extirpation by intratracheal injections of a commercial frozen tissue supporting medium, diluted to half-strength with distilled water. Following removal, the lungs and kidneys were stored at −70°C until cutting. Sections were cut at 5–7 μ, mounted on clear glass slides and fixed and stained according to standard methods. The sections were then pre-washed in buffer. Appropriate fluorescein labelled antiglobulin conjugates (Hyland Laboratories, Los Angeles, California) previously adsorbed with washed CCT were placed

on the sections in a moist chamber at room temperature for 30 min. After two washings in FA-PBS and one in triple-distilled water, the sections were mounted in buffered glycerol for viewing in a Leitz Ortholux microscope, model 250, with a Leitz Universal Light Source, HB0200. Sections from lungs of normal mice were always used as controls.

Tuberculous index calculations. H & E stained sections of whole lungs were scanned at 3·5× for the total number of lesions present. All foci of inflammation, consolidation and granulomatous infiltration were considered to be lesions. Once located, all pathologic foci were then examined at high power in companion sections (stained with Kinyoun's stain) for the presence of tubercle bacilli. An index of tuberculous involvement (T.I.) was calculated for each group of infected mice in the following manner: (1) The average number of lesions per whole lung section was ascertained. (2) The percent of tuberculous lesions was calculated by dividing the total number of lesions in which acid-fast organisms were found by the total number of lesions seen. (3) The percent of tuberculous lesions (2) was multiplied by the average number of lesions per whole lung section (1).

RESULTS

Homologous lung reactive antibody. Of seventy-five mice immunized variously with homologous lung antigen, the ascitic fluid from only five demonstrated sufficient AGCT reactivity (more than 4 units of consumption) for further study. Pneumotoxicity from single or multiple I.V. injections was never demonstrated with any of these fluids. However, when normal recipient mice were injected with 3–6 daily 0·2 ml doses of the AGCT-reactive ascitic fluid, some evidence of tissue localization was obtained by immunofluorescence. No such localization occurred in the kidneys, but there was faint staining in the alveolar septa.

Heterologous lung reactive antibody. All guinea-pig antisera to mouse lung antigen proved reactive to washed antigen by the AGCT and pneumotoxic to unsensitized mice. AGCT activity usually remained high (5–6 units of consumption) throughout the entire 8-week collection period. Immunofluorescent studies of tissues from mice receiving immune serum demonstrated intense localization of globulin on kidney glomerular basement membrane and focal scattered localization along the alveolar septa. The lung localization was entirely random in its distribution.

Such sera were extremely pneumotoxic, producing marked cyanosis and dyspnoea or death within minutes of a 0·2 ml I.V. injection.

Effect of homologous lung CCT antibody on the pathogenesis of tuberculosis. The lung densities obtained from a trial assay of the bacterial suspension showed the tubercle bacilli to be highly infectious even at the highest dilution although there was some variation in individual responses. Mean densities ranged from 0·946 in mice receiving I.V. doses of 10^{-1} mg/mouse to 0·872 in those receiving 10^{-6} mg/mouse after 3 weeks of infection. Grossly, all lungs examined showed similar pathology, the majority of which was manifested as 1 mm nodular or haemorrhagic lesions and a general congestion throughout all lobes, the only exception being the group that received the fewest bacilli (10^{-6} mg) in which case the lungs demonstrated several small surface petechiae.

Fourteen groups of five mice each were given 0·2 ml injections of 10^{-5} mg of viable tubercle bacilli. The groups were then paired up, with mice from one group receiving a regimen of homologous lung antibody while those from the other group received a similar regimen of sham saline injections. The individual regimens consisted of from three to nine

injections spaced variously over the 21-day trial period. At the end of this period, all mice were killed and lung densities recorded. Selected lungs were preserved for histologic study. Table 1 presents the injection regimens and the mean densities from each group.

As can be seen from the data, the excessive tuberculous involvement of both the TEST and SHAM animals in Groups A and C obscured whatever earlier differences might have been seen between the two treatments. In group B there was a differential response between the lung reactive antibody treated mice and the saline treated animals although the densities obtained in the latter were so low it seemed questionable whether an infective dose had been given those mice. Group D showed a reasonably good overall indication of tuberculous

TABLE 1. Effect of homologous anti-lung CCT injections on lung densities of tuberculous mice

			Lung density	
	Group	Treatment regimen	Mean	Range
A	Test*	Three injections on days 1, 2 and	0·970	0·89–1·00
	Sham	3 after infection	0·998	0·99–1·00
B	Test	Three injections on days 1, 8	0·803	0·72–0·85
	Sham	and 15 after infection	0·723	0·68–0·85
C	Test	Six injections on days 1, 4, 8, 11,	0·978	0·91–1·00
	Sham	15 and 18 after infection	0·960	0·91–1·00
D	Test	Nine injections every 3	0·910	0·89–0·93
	Sham	days after infection	0·788	0·68–0·91
E	Test	Three injections on days 1, 3	0·915	0·91–0·93
	Sham	and 6 after infection	0·953	0·89–1·00
F	Test	Three injections on days 10, 12	0·875	0·84–0·90
	Sham	and 14 after infection	0·845	0·69–0·91
G	Test	Three injections on days 16, 18	0·798	0·68–0·91
	Sham	and 20 after infection	0·775	0·69–0·89

All animals received 10^{-5} mg *M. tuberculosis*.
* Test = anti-CCT fluid injections; Sham = Saline injections

involvement in addition to an apparently significant differential between the antibody-treated and the SHAM animals. The three remaining groups (E,F,G) each of which received three post-infection doses of lung reactive antibody (but on successive weeks respectively) demonstrated a progressive decrease in their average lung densities which was related to the time lapse between infection and antibody treatment.

Effect of heterologous lung CCT antibody on the pathogenesis of tuberculosis. Two experiments were conducted to assess the effects of injections of heterologous pneumotoxic guinea-pig anti-mouse-CCT antiserum in tuberculosis infected mice. In the first study (Trial A) sub-groups of mice were infected with two different quantities of *M. tuberculosis* (10^{-5} mg and 10^{-6} mg) followed by spaced injections of either immune or normal guinea-pig serum. Additionally, control groups of mice received either the tubercle bacilli alone or the serum regimen without being infected.

TABLE 2. Effect of heterologous anti-lung CCT serum on the lung densities and tuberculous involvement of lungs from tuberculous mice

Group	Treatment			Normal lung density	Gross lesions/* lung section	Presence of organisms	T.I.†	Adjusted‡ T.I.
	Tb (mg)	Immune serum (ml)	Normal serum (ml)					
H	10^{-4}	—	—	0·723	1	+	66·7	120·1
				(0·68–0·76)	1	+		
					0	...		
					2	++		
					1	+		
					2	00		
					1	0		
					1	+		
					0	...		
I	10^{-4}	0·1 (×3)§	—	0·710	1	0	199·9	314·4
				(0·68–0·84)	3	++0		
					6	+00++0		
					7	0+0++++		
					6	+++++0		
					3	00+		
					4	+++0		
					0	...		
					0	...		
					1	0		
					3	++0		

J	10^{-4}	—	0·1 (×3)§	0·727 (0·68–0·85)	1 2 4 3 1 0 3 2 1 1 2	0 +0 0000 +++ + ... 00+ 0+ 0 + +0	81·9	128·7
K	—	—	0·1 (×3)§	0·735 (0·69–0·81)	—	...	—	—
L	—	0·1 (×3)§	—	0·726 (0·68–0·79)	—	...	—	—

* As seen in single whole-lung H & E sections.

† T.I.—% tuberculous lesions × ave. number of lesions/whole lung section.

‡ Adjusted T.I.—% tuberculous lesions × ave. number of lesions/*infected* whole lung section (as determined by presence of acid fast micro-organisms).

§ All injections given every other day during first week of infection.

During the second week of Trial A several mice developed severe systemic anaphylaxis entirely independent of either the bacterial infection or of the antilung reactivity itself and was pathologically distinct from pneumotoxicity. The mice received 0·2 ml of a given serum per injection every 2 or 3 days. Anaphylaxis was most prevalent following the fourth injection.

Although the high mortality (as much as 80%) limits the usefulness of the data from Trial A, fluorescent antibody studies on representative lungs and kidneys of the serum-treated groups indicated definite globulin localization in the glomeruli and to a lesser extent the alveolar walls of those animals injected with the lung-reactive serum.

In the second study (Trial B) a single injection of 10^{-4} mg of live organisms was given to three groups of mice. The first group received three subsequent 0·1 ml injections of immune serum, the second was treated similarly with normal serum and the third received only the tubercle bacilli. Control groups of mice were treated with either immune or normal serum alone. During the course of the experiment, randomly selected mice were sacrificed on days 10, 14 and 20 and their lungs monitored for pathology by gross examination, lung densities and staining for acid-fast organisms. Upon termination of the trial at 4 weeks, lungs and selected tissues were submitted for density measurements, histological evaluation and immunofluorescence studies.

Table 2 presents the treatment protocol, lung densities, tubercle bacilli involvement, and tuberculous index of the lungs from mice in Trial B. It was disappointing to see that the measurement of lung density could not be used to assess any difference between immune serum or normal serum treated tuberculous mice. Histologic examination was much more rewarding in that the number of gross lesions per whole lung section was much greater in the immune serum treated animals than in the normal serum or untreated controls. The tuberculous index (T.I.) of the immune serum treated group was approximately 200 as opposed to values of 67 and 82 for the two control groups. When this value was adjusted to consider the infected lesions only (since not every lesion had demonstrable micro-organisms), the disparity between the immune serum treated animals and the controls was even more evident.

Non-infected control groups K and L were also examined histologically. Two out of ten lungs from normal serum treated mice showed single, tiny infiltrations of mononuclear cells. Three out of nine lungs from immune serum treated animals demonstrated similar reactions. Since the control mice were housed in separate quarters from the infected animals, no acid-fast staining was done on their lung sections.

DISCUSSION

Although large amounts of effective circulating antibody to homologous lung tissue have been shown to be readily obtainable in the rabbit, the inability to obtain sufficient quantities of high titred homologous mouse immune ascitic fluid necessitated switching to a heterologous system. Why such a small percentage of mouse ascitic fluid demonstrated AGCT activity was not understood although the capricious nature of the mouse to respond to immunization is well known. Moreover, the few samples of homologous lung-reactive antibody did not appear to be pneumotoxic.

The failure to correlate AGCT results with pneumotoxicity in either the homologous or heterologous system was consistent with the findings of Hagadorn *et al.* (1969) in that the

most reactive sera in terms of the AGCT did not prove to be the most actively pneumotoxic, although the reasons remained obscure. Of particular interest was that with the use of homologous ascitic fluid, a small amount of focal antibody localization was detected by immunofluorescence studies within the alveolar septa inspite of the fact that no acute pneumotoxicity was demonstrated. That homologous antibody was not detected in the kidneys may have reflected a greater specificity for lung tissue in the ascitic fluid than in the heterologous sera from which antibody readily and strongly localized primarily in the glomerular basement membrane. Finding that heterologous immune serum antibody preferentially localized in tissues other than the lung has been reported many times and was discussed by Willoughby & Dixon (1970) with regard to the relative inaccessibility of pulmonary vascular basement membrane antigens as opposed to those of the basement membrane of the glomeruli.

Although the amount of material was limited, it appeared that homologous antibody does predispose the animal to greater lesion production as measured by the increase in lung density in infected mice treated with nine doses of antiserum. Mice that received only three doses of antiserum showed little difference in lung density from those receiving sham injections of saline. Since tuberculosis is a chronic disease and since the production of such lung reactive antibodies only occurs in chronic diseases, if any such antibody is going to exert any effect, it could be supposed that the antibody must be continuously present. These conditions were more nearly met by antiserum injections every 3 days.

Such continued administration of heterologous immune serum was not possible due to the high incidence of anaphylaxis that developed in the animals in response to the foreign protein. In spite of the limited amount of heterologous immune serum that could be given the mice (three doses), the data obtained from Trial B clearly indicated that experimental *M. tuberculosis* infections in mice undergo enhanced and accelerated dissemination as a result of passive administration of heterologous lung-reactive antibody. The results were somewhat contradictory to those obtained earlier by Salomone & Coppola (1967) who, in a similarly conducted study, treated hamsters with rabbit antisera to hamster lung stroma and subsequently infected them with atypical mycobacteria. Examination of the lungs after a suitable incubation period showed identical lesions in both the treated and untreated animals. The authors concluded that experimental mycobacteriosis in hamsters by the strain used was not substantially modified by the immunologic induction of lung lesions. However, the results were consistent with the findings of Rheins & Burrell (1960) who passively administered homologous antilung serum to subsequently infected rabbits, and to Iliesco *et al.* (1966) in that our animals were not presensitized to tuberculin (as a result of active immunization with lung antigen in Freund's complete adjuvant) prior to infection, but rather received the lung antibody passively.

The specificity of the antilung serum is directed toward lung connective tissue and although this material is carefully washed free of serum proteins and other soluble antigenic matter, antibodies to such preparations occasionally contain blood cell and platelet reactivity. However, Hagadorn *et al.* (1969) demonstrated that adsorption of these antibodies did not inhibit pneumotoxicity. That the immunofluorescent reactions in the heterologous serum experiments were specific for guinea-pig globulin was shown by the fact that the anti-guinea-pig globulin conjugate was first adsorbed with the mouse lung antigen and that anti-mouse globulin conjugate reacted only minimally with the lung and not at all with the kidney. The localization of antibody in the kidney can be explained by the

presence of collagenous components in the glomerular basement membrane (Burrell *et al.*, 1966).

Still, the present results with heterologous antisera are artificial in that there is a definite difference in reactivity, if not specificity between heterologous and homologous antibody. In order to really ascertain whether autoimmune factors are participating in the pathogenesis of such infections, homologous antibody must be exclusively employed. Extended experiments of this nature are indicated and will depend on the availability of sufficient quantities of the appropriate sera. Hagadorn & Bloor (1971) have reported that intravenous injection of heterologous antilung serum into rats led to the release of lactase dehydrogenase isoenzyme-3 and resulted in alveolar and perivascular haemorrhage. Endothelial cells were torn from their basement membranes and accumulations of fibrin and other blood components entered the extravascular compartment.

A final contribution to come from the tuberculosis and heterologous antibody study was the development of a simple and valid means of characterizing and quantitating antibody effect as a function of lesion development, that correlated the enhanced spread of tubercle bacilli with the administration of immune serum. Attempts to simply quantitate organisms per lesion or total lesions *per se* do not tell the whole story. The T.I. on the other hand gives an indication as to the kind of lesion produced which in our hands was a more diffuse spread of the infection. This was especially helpful since lesions of unknown origin were seen in non-infected controls. The T.I. calculations seemed particularly useful in small animals from whom whole lung sections could be microscopically scanned with ease. The only criticism of the method (that the values were computed from relatively small areas of the lung) could be easily eliminated by using larger numbers of animals or, preferably, by examining serial sections from the same lung.

An area which still remained to be improved included a better correlation between the T.I. computations and lung density measurements. It was felt that quite possibly, maintaining the infected animals for longer periods or infecting them with larger numbers of organisms might have increased the lung densities. However, it is not known whether the increased tuberculous state might overwhelm the marked T.I. differential seen in the less heavily affected lungs.

ACKNOWLEDGMENTS

Supported by Research Grant EC00221-05 and Training Grant E00249-03 from the Environmental Health Service, Department of Health, Education and Welfare, U.S. Public Health Service and by gifts from the Charles McCamic Foundation, Wheeling, West Virginia through the American Thoracic Society.

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