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TECHNICAL REPORT

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Interim Report on Work Unit (61245012)MR005.12-1200.4: Experimental occlusion of the nutrient pulmonary vessels in the horse and an investigation of their relationship to chronic pulmonary emphysema.

Report Title:

Immunologic studies in pulmonary emphysema.

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IMMUNOLOGIC STUDIES IN PULMONARY EMPHYSEMA

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During the course of the immunological work performed at the Clinical Investigation Center, U. S. Naval Hospital, Oakland, California from September 1962 - August 1964, a great amount of effort was devoted to the demonstration of antilung antibodies in the serum of horses presumably suffering from emphysema. This was attempted with the aid of a variety of *in vitro* tests including immunodiffusion, complement fixation, complement consumption, tanned red blood cell agglutination, and latex fixation. Of these, only the last two provided encouraging results. The antigen used for all of the studies was a crude saline extract of "normal" horse lung.

The tanned red blood cell agglutination work is considered significant not because it provided positive results with the horse sera, but because of the promising results obtained with the "known" test system - rabbit antihorse lung serum vs. tanned sheep red blood cells sensitized with horse lung extract. High antibody titers which were specific for the lung antigen were measured in the rabbit antiserum. The antiserum was considered to be specific for lung tissue because absorption of the serum with normal and horse serum and with lyophilized horse kidney failed to remove the agglutinating activity from the serum, but the agglutinating activity was readily removed from the antiserum by absorption with lyophilized horse lung. When a large number of sera from normal horses and from emphysematous horses was tested by the same method which provided good results in the known test system, no significant titers or differences between the group of normal horses and the group of emphysematous horses were observed.

Results somewhat different from the tanned red blood cell agglutination test results were obtained with the latex fixation test, which employed the same type of antigen preparation and comparable groups of normal and emphysematous horse sera. Results from these tests suggested that many horses, both normal and emphysematous, possess demonstrable circulating antilung antibody. The reliability of the latex fixation test was questionable, however, since it was difficult to repeat test results, even after extensive research to determine optimal test conditions. Such factors as pH, type of buffer, concentration of latex particles, and concentration of antigen were given individual consideration in the determination of "optimal" conditions; and extreme caution, including the use of scrupulously clean glassware, was excercised in the performance of the test. The test procedure (listed elsewhere) reflects what were considered to be the best conditions for performing the test.

It is believed that the antigen preparation was a very important factor contributing to the unreliability of the test. The concentration of the antigen preparation was difficult to regulate due to the manner in which it was prepared. This problem would have been averted to some extent if the actual antigen(s) which were responsible for the positive reaction could have been isolated, at least to some degree, from the lung extract and used in a purer form. Lung extracts which remained stored in the freezer for more than 30 days became increasingly less effective as test antigens. Therefore, no lung preparations were used which had been stored for more than 21 days.

It is not believed that the results of the latex fixation tests should be disregarded. The test appeared to be valid and reliable for distinguishing between "positive" and "negative" sera, but the extent to which a serum was positive was not always consistent, i.e., the titer measured for a serum on one occasion was not always found to be the same on subsequent occasions.

RESULTS

Using the latex fixation test, sera from horses with emphysema and from presumably normal horses were tested for their ability to react with a normal horse lung saline extract. In one group of ten emphysematous horses, six had positive sera. Of nine sera from normal horses tested on the same occasion, three were positive. In later tests of sera from 43 presumably normal horses, 13 of the sera were positive. In tests of another group of five emphysematous horses, three of the sera were found to be positive. Thus, the results of work performed in this laboratory indicate that about 60% of the sera from horses with emphysema have a measurable "antibody" titer against a saline extract of horse lung and that 30-35% of sera from normal horses have demonstrable antibody.

The titers were usually never greater than 1:80 and titers of less than 1:10 were not determined. The term "titer" as used here refers to the highest dilution of serum which gives a 2+ reaction with the antigen preparation. A serum was considered positive if it had a titer of at least 1:20.

The term "antibody" was used above for want, perhaps, of a better term because it is not believed that the positive reactions should be accepted unquestionably as being the results of an immunological reaction, i.e., and antilung antibody-lung antigen reaction. In order for the reaction to be properly classified as an immunological one, additional investigation would be necessary. It would be necessary for the sera to be tested against saline extracts of other normal horse organs and against normal horse serum to determine whether or not the reaction is specific to lung. It would further be necessary to determine whether or not the activity could be specifically removed by absorption with normal horse lung.

Each of the sera which reacted with lung was also tested against normal horse serum as an antigen in the latex fixation test. About 15% of these sera were found to give a positive reaction with serum. When a saline extract of kidney was used as an antigen in a test of nine horse sera which had given positive reactions with horse lung, eight of the sera also reacted with the kidney preparation, giving titers approximately the same as those observed with the horse lung preparation. Of 11 horse sera included in the test which had not reacted with horse lung, three reacted with the horse kidney antigen preparation. Reactivity of these sera with antigen preparations from other tissues and organs was not investigated.

Several of the sera which gave a positive reaction with the lung extract were further tested to determine the effect which absorption with horse tissue preparations would have on their antibody titer against the horse lung extract. The results indicated that absorption of the serum with lyophilized lung prior to the test either eliminated or greatly reduced the titer. The antilung titer was not reduced by a similar absorption with normal horse serum nor by absorption with either lyophilized horse kidney or horse liver.

The effect of heat on the serum before testing was not extensively explored, but it was found that incubation of the serum in a 56° C waterbath for longer than 30 minutes resulted in decreased reactivity. The sera were routinely incubated for 15 minutes in a waterbath at 56° C before testing.

It was observed that positive sera did not always give positive reactions if lung extracts (the antigen preparation) from different horses were used. With this in mind, positive sera from four horses were tested against six lung extract preparations from six other horses. The four sera gave positive reactions against four of the six preparations, but neither of the sera was positive for the other two preparations. This experiment was the only test performed, so the validity of the results is certainly questionable. The results of the experiment suggest, however, that perhaps the antigen responsible for the positive reactions -- if the reaction is an immunological one -- is not generally specific for all horse lung tissue, but may be more narrowly specific for lung tissue only from horses of a particular "type".

SUMMARY

1. Of several standard immunological tests employed for determining the immunological affinity of horse sera for a saline extract of horse lung, only the latex fixation test was applicable.

2. About 30-35% of the sera tested from normal horses and about 60% of the sera tested from emphysematous horses reacted with a horse lung preparation in the latex fixation test. Most of these positive sera did not react with normal horse serum when tested in a similar manner, but nearly all of the positive sera which were tested for their reactivity with horse kidney showed positive reactions.

3. The affinity of the sera for the lung extract is removed by absorption with lyophilized horse lung, but not by absorption with normal horse serum nor with lyophilized horse liver or horse kidney.

4. The reactivity of positive sera is reduced by prolonged heating at 56° C.

5. There may be some individual tissue antigens which cause some horse sera to react with a lung preparation from a horse of one "type" but not of another.

TECHNIQUES USED

Absorption of Sera

Preparation of Tissue. Horse lung prepared as above through the homogenation step was lyophilized and stored in the refrigerator.

Absorption Procedure. It was found that 25 mg. of the lyophilized horse lung is as effective for removing activity from serum as is any greater amount. Therefore, this was the quantity used.

The lyophilized tissue was added to a centrifuge tube and washed with distilled water until the supernatant was no longer cloudy (about 3 times). The serum was then added to the washed tissue, mixed thoroughly and left overnight in the refrigerator. The following day the mixture was centrifuged and the serum collected. The titer of the absorbed serum was then determined in the usual manner.

The same procedure was used for absorption with horse kidney and liver.

For absorption with normal horse serum, an equal volume of whole normal serum diluted 1:5 in buffered saline was added to the test serum. This mixture was left overnight at 4° C., centrifuged and tested in the usual manner (with consideration being given to the fact that the serum was diluted 1:2).

PROCEDURE FOR LATEX FIXATION TEST

FRESH LUNG
↓
Weigh
|
Chop finely
↓
Wash, distilled H_2O (5-6x)
↓
Centrifuge
↓ Discard supernate
Adjust volume to 5x wt. with dH_2O
↓
Add NaCl & Merthiolate
↓
Homogenize (Waring blender)
↓
Stir overnight
↓
Centrifuge
↓ Discard residue
Freeze supernatant
↓
Thaw
↓
Centrifuge
↓ Discard residue
Mix with equal volume of particles suspension

BACTO-LATEX PARTICLES
↓
Shake & suspend
↓
Filter (#40 Whatman paper)
↓
Add to buffered saline to 14% T
↓
Incubate 1 hr. (37° C)
↓
Add 0.1 ml to each serum tube

TEST SERUM

Heat 15# at 56° C
Prepare serial dilutions using PO_4 buffer .0175M, pH 7.5 in 12 x 75 mm. tubes
0.5 ml/tube

↓
Shake
Incubate one hour (37° C)
Leave overnight at room temperature
Centrifuge 3 minutes at 2500 rpm
Read and record

PROCEDURE

Preparation of Lung Saline Extract for Use in Latex Fixation Studies

Fresh normal lung was obtained from an abattoir where horses are killed by a rifle bullet in the brain and exsanguinated immediately thereafter. The lung was transported to the laboratory in an insulated container cooled with ice. In the laboratory the lung was sliced into strips and areas containing large bronchioles or blood vessels were discarded. The strips of lung selected for use were weighed. The strips of lung were then finely chopped with a razor blade into pieces about 1 mm. in diameter. The finely-chopped lung was immediately dumped into a large beaker of cold (4° C) distilled water and stirred for a few minutes. The lung was removed from the distilled water by straining through a fine-meshed screen. The wash water was discarded and the lung added to fresh cold distilled water and the washing procedure repeated. The lung was washed in this manner until the wash water was either colorless or only slightly yellow in color. This usually required about 5 or 6 washes.

The thoroughly-washed chopped lung was then centrifuged in 50 ml. graduated centrifuge tubes in a refrigerated centrifuge at 3000 rpm for 30 minutes in an effort to remove as much distilled water as possible. Following centrifugation the supernatant was removed from the tubes and the volume of lung determined. The lung was then added to enough cold distilled water to give a final volume 5 times as great (in milliliters) as the original weight of lung used (in grams). NaCl was then added at a ratio of 0.9 gm/100 ml and merthiolate was added to effect a final dilution of 1:10,000.

The lung-saline mixture was then emptied into the Waring blender equipped with the tissue cell homogenizing attachment* and homogenized in the refrigerator at full speed for 5 minutes.

The lung homogenate was poured into a large beaker and stirred with a magnetic stirrer overnight in the refrigerator.

The following morning the lung homogenate was centrifuged in the refrigerated centrifuge for 1 hour at 3000 rpm. The supernatant was divided into 10 ml. aliquots and placed in the -25° C freezer for storage. The residue was discarded.

Preparation of Lung Saline Extract - Latex Particle Suspension for Measurement of Antilung Antibody in Serum

Lung homogenate previously described was thawed in 37° C water bath and centrifuged at 3000 rpm for 20 minutes.

A small aliquot (approximately 2 ml.) of Bacto-Latex particles†, .82μ (suspended well before use) was filtered through one thickness of Whatman #40 filter paper. Enough filtered Bacto-Latex particles were added to buffered saline to form a suspension which gave about 14% T on the Colemen Spectrophotometer at 650μ. This suspension was added to an equal volume of lung homogenate supernatant.

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* Scientific Products
† Difco Laboratories

Merthiolate was added to effect a 1:10,000 mixture.

The suspension was then incubated in a 37° waterbath for one hour at which time it was ready to be added to the test sera.

Preparation of Serum for Measurement of Antilung Antibody by Latex Fixation Method

Using freshly harvested serum (never plasma) or serum which had been frozen and thawed only once, and heated in a waterbath at 56° C for 15 minutes immediately preceding the test, serial two-fold serum dilutions were prepared in .0175 M Phosphate (Sorenson's) buffered normal saline, pH 7.5 (Merthiolated 1:10,000). The tubes used for this were 12x75 mm. "Kahn" tubes and dilutions were prepared so that each tube contained 0.5 ml. of a particular serum dilution. The range of dilutions varied from one test to another. In making the dilutions, a single 1.0 ml. pipette was used for each serum and after the serum had been added to a new tube of buffered saline it was mixed by pipetting up and down 8-10 times before it was transferred to the next tube.

Addition of Antigen Suspension to Serum

Lung extract-latex particle suspension in amounts of 0.1 ml. was added to each of the tubes containing 0.5 ml. of diluted test sera. Each tube was then mixed on the Vortex Junior mixer.

The tubes were next incubated for 1 hour in a 37° C water bath and then left overnight at room temperature.

The following morning the tubes were centrifuged for three minutes at 2500 rpm. (The time was measured from the moment the centrifuge speed reached 2500 rpm).

Tubes were read by gently tapping each and observing the manner in which the particles went back into suspension. In positive tubes a clumping of particles was observed; while in negative tubes the particles rose from the bottom of the tube in a manner similar to smoke rising from a fire. Each tube was graded on a minus to 4+ scale. To facilitate reading, tubes were read in diffused light (microscope light) against a black background. No tube was considered to be positive which gave less than a 2+ reading.

Tests Involving Other Antigens

This same procedure was followed when kidney was the antigen source. For latex fixation tests in which normal horse serum serves as the antigen, a 1:5 dilution of whole normal serum in buffered saline was added to the suspension of particles (adjusted to 14% T), and the usual procedure was followed thereafter.

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13. ABSTRACT

This report summarizes the attempts to devise an immunologic model in the horse which would simulate human chronic pulmonary emphysema, and it describes the immunologic techniques employed.

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