Relation between Serum Complement and Plasma Electrophoretic Fraction Levels in Human Tuberculosis.* (19780)

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Complement is an important constituent of blood plasma and other body fluids as evidenced by its intimate association with antigen-antibody complexes, its participation in bacteriolysis, hemolysis, complement fixation, phagocytosis and other immunological reactions. It is a complex of plasma proteins associated for the most part with the globulins. As it occurs in serum as less than 1% of the total protein (0.03 to 0.05 mg C′ N per ml serum)(1), (0.15 to 0.20 mg C′ per ml serum)(2), electrophoretic measurements are not possible(3). Its marked activity in lysing sensitized erythrocytes, however, allows detection and measurement in small quantities.

Stavitsky, Stavitsky, and Ecker(6) have demonstrated marked and prompt reduction in complement after reinjection of a variety of antigens into previously immunized rabbits and give evidence that *in vivo* binding of complement by antigen-antibody complexes is responsible. Ecker and his associates(4) have summarized the numerous studies directed toward attempting to relate complement actvity to various infectious diseases and stages of disease as well as to serum sickness. In tuberculosis conflicting reports have appeared in the literature. Objectives of the present study were to determine 1) whether serum complement levels in human pulmonary tuberculosis differed from levels in normal, apparently healthy humans, 2) the effect of the administration of ACTH on complement levels in human pulmonary tuberculosis and 3) correlation of hemolytic complement activity with electrophoretic levels of plasma proteins in a group of patients receiving ACTH.

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Materials and methods. Complement titration. Complement titrations were performed on 3 groups of human subjects. Twenty normal, apparently healthy individuals, both tuberculin-positive and tuberculin-negative, on whom a total of 54 titrations were made, served as a control group. The second group consisted of 37 patients in various stages of pulmonary tuberculosis on whom a total of 59 titrations were performed. The third group consisted of 10 tuberculous patients who were to receive ACTH on whom a total of 65 titrations were done. Complement levels on most of these patients were obtained before ACTH was initiated and at intervals during several months of treatment.

Titrations were performed promptly on freshly drawn serum using the method of 50% hemolysis. Sheep blood was collected in Alsever's solution(7). The technic of Wadsworth, Maltaner, and Maltaner(7) was modified by the substitution of readings on the Coleman Universal spectrophotometer for visual color standards. Erythrocyte suspensions were standardized spectrophotometrically to correspond to 1,000,000 cells per ml. Hemolysin was used at its optimal concentration(7). Mg^{++} and Ca^{++} were not employed in the diluting fluid; it was felt that comparative figures of group analyses of the human sera of this study nevertheless have significance, inasmuch as optimal amounts of these ions raise 50% titers of guinea pig sera by a rather constant amount (78% to 88%), and of human serum by about 28%(19).

The method of calculating the 50% unit was based on the equation of von Krogh(8):

$$\text{Log } x = \log K - 1/n \log y/1 - y$$

where x is the amount of complement and y is the resulting degree of hemolysis. 1/n represents the slope of the line. When $y = 0.5$ (50% hemolysis), $y/1 - y = 1$, $\log x = \log K$ and $x = K$. Accordingly amounts of complement (x) were plotted against $y/1 - y$ on

TABLE I. Statistical Analysis of Complement Levels.

Group	Source of variation	D.F.	Variance	F	Required F 5%	Required F 1%
Normal subjects	Between patient	19	77.16	4.39*	1.90	2.50
	Within "	34	17.54			
Tuberculous patients	Between patient	36	52.75	1.79	1.95	2.62
	Within "	22	29.36			
Tuberculous ACTH patients	Between patient	9	74.77	3.10*	2.05	2.75
	Within "	35	24.08			

* F value highly significant.

TABLE II. Comparison of Mean 50% Units.

Group	Mean 50% unit in ml
Normal subjects	.0030
Tuberculous patients	.0026
Tuberculous ACTH patients	.0025

logarithmic paper and a straight line fitted through the points. The 50% unit is the ordinate of the point of intersection of the fitted line and the line $y/1 - y = 1$ where the degree of hemolysis is 0.5 (50%). The logarithmic method is advantageous in that it allows observed values which do not fall on the straight line to be discarded. Obviously inconsistent readings would therefore not erroneously influence the value of the 50% unit.

Methods of analysis. Evaluation of the results of the 50% hemolytic units were obtained by analysis of variance and correlation and regression technics which provide objective tests of the significance of observed differences(9,10). The analysis of variance is advantageous in that variation within each group is measured as well as variation between groups and estimates of variance are computed independently from these 2 sources.

Electrophoretic fractionation. Diagrams were obtained under very nearly the same experimental conditions in order to make comparisons possible. The plasma from fasting heparinized blood samples was diluted 1-6 with barbital buffer solution, pH 8.6 at 25°C, ionic strength 0.1; electrophoresis was carried out in the tall form of the standard 11 ml Tiselius cell in a water bath at 1°C. Ten milliamperes of current were passed for at least 3 hours. Areas of the peaks were obtained from enlarged tracings of the anode pattern photographs. Percent of total plasma proteins was determined according to the procedure of Tiselius and Kabat(11). Quantities as grams percent represent products of the percentages and the total proteins determined by the conventional Kjeldahl method, using a nitrogen factor of 6.25.

Results. Group comparison of complement levels of normal subjects, tuberculous patients and tuberculous patients receiving ACTH. To arrive at a basic measure of variation, 2 sources were considered; variation in complement levels from patient to patient (between patients), and fluctuation among repeated tests on individual patients (within patients). The variance from these 2 sources was separated and examined independently before being used as the basis of tests of significance of differences among average complement levels for the 3 test groups. The analysis of variance for each of the 3 groups is shown in Table I.

Variation in complement activity is seen to be greater from patient to patient than among repeated tests on individual patients. Conse-

TABLE III. Combined Analysis of Variance.

Source of variation	D.F.	Variance	F	Required F at 5%	Required F at 1%
Total	177				
Normal vs tuberculous	1	816.83	35.23*	3.93	6.88
Tuberculous vs ACTH tuberculous	1	57.85	2.50	3.93	6.88
Between patients	64	63.08	2.73*	1.45	1.67
Within "	111	23.13			

* F value highly significant.

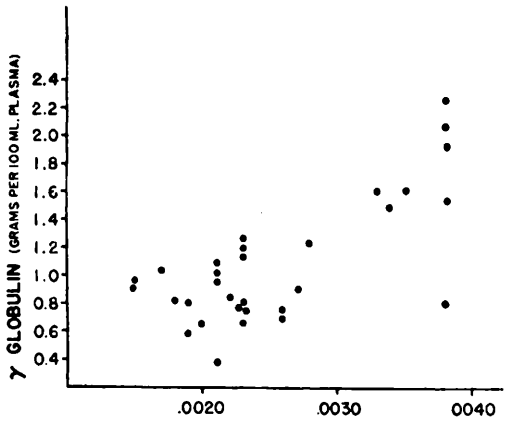

FIG. 1.

quently these 3 groups of data were combined to form an estimate of error against which differences in complement levels among the 3 groups could be tested for significance.

Means of the 3 groups of subjects are shown in Table II and the combined analysis of variance in Table III.

Table II shows that the highest complement activity occurred in tuberculous patients and the lowest in normal individuals (the larger the 50% unit, the less the complement activity). Analysis of variance shows this difference to be highly significant. The variance for the difference between the 2 groups of tuberculous patients, however, is not significant, being of about the same order as the variance between tuberculous patients, indicating that the difference between the tuberculous and tuberculous-hormone groups might be attributed to random sampling.

Comparison of complement levels on individual patients during course of ACTH administration. By plotting complement levels against time it was found that the trend was toward increased activity after long continued treatment. It is not marked nor is it immediate, a noticeable plateau being evident. A significant correlation of 0.32 was obtained between pairs of complement levels and time, indicating increased complement activity with continued ACTH administration beyond any variation attributable to random sampling.

Correlation of complement levels with electrophoretic components. Scatter diagrams prepared by plotting each electrophoretic component (albumin, alpha 1 globulin, alpha 2 globulin, β-globulin, gamma globulin and fibrinogen) against complement levels of identical blood samples showed that only in the case of gamma globulin was a trend evident. Fig. 1 illustrates the tendency for decreased gamma globulin to be associated with increased complement activity. The above sets of a complement-gamma globulin level give a highly significant coefficient of correlation (0.729, calculated F 34.32**, tabulated F at 1% level 7.56).

Discussion. Ecker(4) has pointed out that many apparent contradictions and inconsistencies in the literature can be attributed to lack of uniform technic and to inadequate definition of the limits of normal and abnormal complement titers. In this study an average 50% unit of 0.0030 ml was obtained for the normal group, which closely agrees with that found by Traub (0.0031-0.0032 ml)(12), who found no significant differences attributable to age, sex, blood groups or seasonal influence. Dulaney(5) found the median 50% unit in a group of normals to be 0.0045 ml, while Pohl and Rutstein(13) found a median 50% unit of 0.0049 ml. In this study, on the basis of group comparisons, the complement level of the tuberculous group was significantly higher than that of the normal group. References to complement levels of tuberculous patients expressed in 50% units were not found in the literature.

True complement titers of sera might conceivably be masked by the occurrence of natural heterophile antibody, known to influence the extent of hemolysis. Traub, however, found the 50% complement units of 10 sera which contained large amounts of heterophile antibody to be unaffected(12). Moreover in the present series, heterophile antibody was detected in only a few cases; in any case, dilutions of serum used for complement determinations would have obviated possible error from this source.

ACTH administration is known to result in alterations in serum proteins and gamma globulins may be significantly reduced in a variety of diseases. Some investigators have observed a lag period after ACTH before the decrease

in gamma globulin occurs(14). In the tuberculous group in the present study, elevated gamma globulin fractions tended to approach the normal after initiation of treatment. This decrease, coupled with the marked tendency for the association of decreased gamma globulin and increased complement activity, indirectly indicates increased complement activity after the hormone, as does the significant correlation between complement activity and time after treatment. Failure to detect a significant difference in the large group analyses between the means of the tuberculous group and the tuberculous-hormone group might be attributed to inclusion of results during a lag period in some of the treated patients, which would minimize the difference between the means of the 2 tuberculous groups and thus account for lack of agreement in the 2 methods of analysis.

It is known that serum globulin solutions are often anticomplementary(15,16). Davis and his coworkers(16) found that gamma globulin separated from human serum by electrophoresis was anticomplementary in high dilution but that recombination with other constituents, notably β-globulin, inhibited this property. Vaughan, Bayles, and Favour found decreased gamma globulin levels and raised complement titers in 4 cases of lupus erythematosus disseminatus treated with ACTH(17). Carey, Harvey, and Howard (18) confirmed these results in lupus erythematosus and also in periarteritis nodosa treated with ACTH. In the present study, also, a highly significant negative correlation between complement and gamma globulin levels was observed following ACTH treatment. There was complete lack of correlation, however, between complement content and β-globulin, as well as alpha 1 and 2 globulins, albumin and fibrinogen.

If complement activity were mediated through the gamma globulin content, then, in view of the consistent decrease in gamma globulin after ACTH administration in most of the patients in the present study, simultaneous elevation in complement levels would be expected. Results on the hormone group alone show an elevation, indicated by the significant negative correlation between complement levels and time. In the group comparisons, diminished complement activity was not noted in the untreated tuberculous group, which would be expected to have elevated gamma globulins. Unfortunately, electrophoretic patterns were not obtained on this particular group of subjects.

It would appear that a number of immunologically important factors are operative in influencing the amount of titratable complement, such as the binding of complement by "normal" gamma globulin, inhibition of the anticomplementary effect of gamma globulin by β-globulin and albumin, the observed reciprocal relationship of complement and gamma globulin levels in some diseases, the observed *in vivo* binding of complement by antigen-antibody complexes and probably others. Evaluation of results would depend upon knowledge of the interplay of these factors and the extent to which each is operative in any one individual.

Summary. 1. Fifty per cent hemolytic units of complement determined on groups of normal individuals, tuberculous patients and tuberculous patients receiving ACTH showed highest activity in the tuberculous group and least activity in the normal group. Analysis of variance showed differences between the complement activity of normal and tuberculous individuals significant. 2. A significant correlation was observed between complement levels and time after hormone administration indicating a trend toward increased complement activity (decreased 50% unit) after long continued ACTH administration. 3. A highly significant correlation was seen between the concentration of gamma globulin and complement level, increased complement activity being associated with decreased gamma globulin concentration. No correlation between complement activity and other electrophoretic components (albumin, alpha 1 and alpha 2 globulins, β globulin and fibrinogen) was observed.

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1. Heidelberger, M., and Mayer, M., *J. Exp. Med.*, 1942, v75, 285.

2. Haurowitz, F., and Yenson, M. M., *J. Im-*

munol., 1943, v47, 309.

3. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry*, Ed. 1, Charles C. Thomas, Springfield, Ill., 1948.

4. Ecker, E. E., Seifter, S., Dozois, T. F., and Barr, L., *J. Clin. Invest.*, 1946, v25, 800.

5. Dulaney, A. D., *J. Clin. Invest.*, 1948, v27, 320.

6. Stavitsky, W. B., Stavitsky, R., and Ecker, E. E., *J. Immunol.*, 1949, v63, 389.

7. Wadsworth, A. B., *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, The Williams & Wilkins Co., Baltimore, 1947.

8. von Krogh, M., *J. Infect. Dis.*, 1916, v19, 452.

9. Snedecor, G. W., *Statistical Methods*. The Iowa State College Press, Ames, Iowa, 1950.

10. Fisher, R. A., *Contributions to Mathematical Statistics*, John Wiley & Sons, Inc., New York, 1950.

11. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, v69, 119.

12. Traub, B., *J. Path. and Bact.*, 1943, v55, 447.

13. Pohl, A. W., and Rutstein, D. D., *J. Clin. Invest.*, 1944, v23, 177.

14. Vaughan, J. H., Bayles, T. B., and Favour, C. B., *J. Clin. Invest.*, 1950, v29, 850.

15. Ecker, E. E., and Seifter, S., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 359.

16. Davis, B. D., Kabat, E. A., Harris, A., and Moore, D. H., *J. Immunol.*, 1944, v49, 223.

17. Vaughan, J. H., Bayles, T. B., and Favour, C. B., *J. Lab. and Clin. Med.*, 1951, v37, 698.

18. Carey, R. A., Harvey, A. M., and Howard, J. E., *Bull. Johns Hopkins Hosp.*, 1950, v87, 425.

19. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, v84, 835.

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Effect of Adrenal Hormone Overdosage on Bacterial Removal by the Splanchnic Viscera.* (19781)

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An adverse effect of adrenal hormone overdosage on the course of experimental infections has been reported(1,2). A higher incidence of positive blood cultures was noted in animals receiving cortisone who were infected with Group A streptococci and pneumococci(1,3). Some studies have shown a diminution in the inflammatory response(1,4,6). The cause of the increased incidence of bacteremia has not been shown but may be attributed to the failure of the inflammatory response to localize the pathogen at the site of injection or to the failure of the splanchnic vascular system to remove the bacteria after they have entered the blood stream. Gordon and Katsh(5) have demonstrated that adrenal insufficiency lowers endothelial phagocytosis and decreases the removal of particles in the liver and spleen. This report deals with the effects of overdosage of ACTH and cortisone on the ability of an animal to remove a non-encapsulated organism. There is no difference in the removal rate of such an organism before and after chronic adrenal stimulation.

Healthy 4 to 4.5 kg rabbits were maintained on a mixture of rabbit chow and oats with no periods of fasting. The animals receiving adrenocorticotrophic hormone (ACTH, Wilson's) were given 10 units every 4 hours for 24 hours before study. The animals receiving cortisone were divided into 4 groups. Two groups were studied at 4-5 hours after a single intramuscular or oral dose of 25 mg cortisone. The 2 remaining groups were studied after 4 days of therapy with 25 mg of cortisone a day given intramuscularly or orally, the last dose being given 4-5 hours before study. A bacteremia was maintained by constant infusion into a marginal ear vein of hemolytic *Micrococcus pyogenes var aureus*, coagulase positive. The splanchnic removal rate was determined by passing a catheter into the hepatic vein and comparing the number of bacteria found in cultures of the blood drawn simul-

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