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The detection of normal and abnormal human sera by quantitative analyses of agar column diffusion patterns has been confirmed and amplified. Baseline precipitin patterns from 29 sera, assumed to be normal, were compared with diffusion spectra of 131 sera from hospitalized subjects. The same fast-moving faint zone, previously observed and believed to be associated with an increased concentration of mucoprotein, was evident. Diffusion data on this precipitin system, together with the data associated with albumin and globulins, were analyzed in order to detect certain serologic imbalances. These analyses showed serologic imbalances in hospitalized subjects in 36 percent more cases than was shown by the usual clinical C-reactive protein test. The results paralleled the physician's estimate of the stress produced by the particular disease state or condition.

A review by Wodehouse (1) indicated the wide variety of problems to which agar diffusion methods have been applied. Darcy (2), using a plate gel diffusion method (Ouchterlony type), showed that the "precipitin spectra" of normal and tumor-bearing rats are different. In subsequent investigations (3) he concentrated on a fast-diffusing glycoprotein of the mucoprotein variety. Increases in the concentration of this substance were believed to be associated with tissue growth.

In the Oudin type of agar diffusion column (4) external reactants (antigen) are layered on top of an appropriate antiserum-agar mixture (internal reactants) in a thin-bore glass tube. The disproportional concentrations and dissymmetries of the antigens cause them to migrate through the antiserum-agar at different rates. During this movement, precipitinogens and precipitins combine to form zones of various widths and densities at one or more levels. As a general rule, the external reactant concentrations govern the zonal diffusion, and the amount of antibody with which the antigen combines is reflected in the zone densities. The Oudin column was used in this and previous studies.

Since the reports by Darcy were not discovered in the foreign literature until this study and those preceding it were completed, the results reported here independently confirm his findings and extend such analyses to the detection of certain serologic imbalances in human sera. Our previous studies (5, 6), with 100 normal and 40 abnormal human sera samples, indicated that normal sera, when individually diffused into appropriate homologous rabbit antiserum, all showed a similar pattern of precipitin zones after 120 hours' reaction time at 24° C. Abnormal sera showed aberrant diffusion patterns and an additional zone. This zone is apparently associated with an increased concentration of a normal serum component(s) similar to mucoprotein or a glycoprotein complex (6, 7). It has also been possible to rely on certain marker zones in the diffusion patterns. By absorption technics it has been established that these zones are associated with beta and gamma globulin and with albumin. The relative mobilities and densities of these zones together with the occurrence of the 6' zone (fig. 1) are apparently sensitive criteria for detecting imbalances in human sera.

This study was concerned with the qualitative and quantitative differences between the agar diffusion patterns of 29 persons assumed to be normal and those of 131 persons hospitalized for various conditions. An individual assumed to be normal was one who claimed to feel well, evidenced no obvious external symptoms of a pathologic condition, and was working at his usual occupation. An abnormal person was one who was hospitalized and under the care of a medical staff.

MATERIALS AND METHODS

Antigens and antisera

A pool of normal human sera was used to prepare the rabbit antisera. Six rabbits were each given a 1-ml. subcutaneous presensitizing

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FIGURE 1

Serum agar columns (Oudin type), showing principal precipitin zones when abnormal human sera are diffused into standardized rabbit antiserum (120 hours). Zone 4 is associated with beta and gamma globulins and zones 5 and 6, with albumin; zone 6' is associated with glycoprotein. Human sera samples are (left to right): nephrotic syndrome; cirrhosis of liver; post - myocardial infarction (1½ months); cirrhosis of liver. Normal sera shows a pattern similar to the third column but without the 6' zone.

injection (about 11 mg. of nitrogen per milliliter). Three weeks later four similar 1-ml. subcutaneous injections were given on alternate days for a total of four injections. Seven days after the last injection the antisera were tested by the Oudin column method. Animals whose sera showed a good pattern and discriminatory possibilities were bled out by cardiac puncture the following day.

An additional selection of appropriate antisera was made by pooling sera. The pool used in this study is designated *Anti-Hu*.

The human sera[1] used as external reactants came from persons who had a diagnosed active condition or were convalescent. The samples were from the following disease categories: 22 vascular, 18 metabolic, 2 psychogenic, 3 allergic, 7 blood dyscrasia, 8 malignant, 6 skin, 8 gastrointestinal, 10 collagen, 12 liver, 25 infectious, and 10 miscellaneous.

Agar columns

Detailed descriptions for preparing the Oudin type of reaction column have been published (4, 8). Since the antiserum-agar must be kept at 46° C. for filling the columns and since it had been found in previous work (8) that this temperature may cause changes in the serum-agar, the antiserum-0.6 percent agar (1:1) mixture was prepared in 5-ml. quantities. To minimize errors in dilution, the filled columns were chosen at random. All reactions were prepared in duplicate and kept at 24° C. for 120 hours.

The external reactants were also overlaid on normal rabbit serum-agar mixtures. Antigens that showed definite precipitation under these conditions were discarded.

Quantitation of reactions

Instrumentation and procedures for the direct photometric graphing of agar column reactions by manual (10) and semiautomatic methods (11, 12) are published elsewhere.

In this study a recently developed automatic system involving the Serum Agar Scanner Instrumentation (SASI) (13) was used. With SASI, the zones in agar columns can be quantitated for both diffusion and density with high resolution. An adjustable chart drive, column speed, and recorder span permit the patterns to be spread out in both width and length.

For a more precise measurement of the diffusion of the faint 6' zone (*fig.* 1), a binocular microscope (20×) fitted with an optical micrometer was used to measure the relative diffusion of the leading edge of the 6' zone compared with the leading edge of the albumin or zone 6.

[1]Sera samples were supplied by Lackland Air Force Hospital, USAF, Lackland AFB, Tex.

C-reactive protein (CRP)

The sera were also layered above commercially prepared antisera to C-reactive protein. This was done by the prescribed clinical procedure (14). Since measuring the precipitates to obtain quantitative answers was considered unreliable (6), reactions were recorded as positive or negative after 2 hours' incubation at 37° C. followed by 46 hours' storage at 4° C

EXPERIMENTAL RESULTS

Results of the agar column analyses were divided into four groups: (1) qualitative evidence of a 6' or glycoprotein zone below the albumin or No. 6 zone (fig. 1); (2) quantitative comparison among sera of the diffusion distances of the 6' zone relative to albumin; (3) quantitative comparison of the mean density ratio between the Nos. 4 and 6 zones of each sample; (4) quantitative comparison of the mean diffusion distance of the Nos. 4 and 6 zones of each specimen.

Figure 2 shows the incidence of the 6' zone in all samples. As indicated previously the more rapid diffusion of this zone made it visible below the albumin or zone 6 and is believed to be due to an increased concentration of mucoprotein. In sera from severely ill patients, the 6' zone was often seen as early as the 6th hour of reaction time. Other details concerning this qualitative analysis are included in the legend of figure 2.

Table I shows the quantitative data obtained from measurements of zonal diffusion of the 6' zone, diffusion ratios of zones 6 and 4, and optical density ratios of zones 6 and 4. Among the normal samples with 6' zones (6' +), 12.5 percent were beyond the mean ±1 S.D. for diffusion of the 6' zone, whereas 44.0 percent of all samples from hospitalized subjects were beyond this "normal" range. Using the same "normal" range of diffusion for the 6' zone,

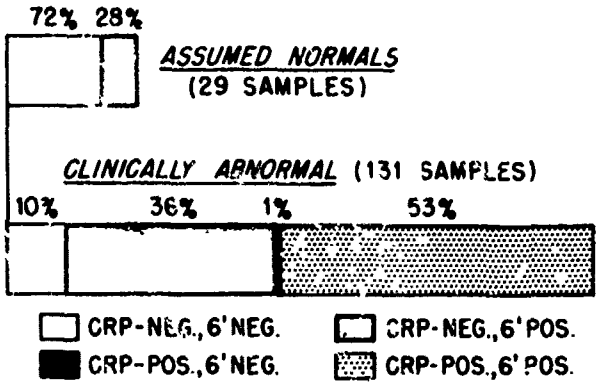

FIGURE 2

Comparison of C-reactive protein test with presence of 6' zone for human sera samples. Samples assumed to be normal were taken during winter months when subclinical sinusitis and related infections were prevalent. The 10 percent in the clinically abnormal group that were both CRP- and 6'- were from convalescent patients considered in good condition. Note that 36 percent of known abnormals showed serologic imbalances but a negative CRP. Additional information can be obtained by relative diffusion and optical density ratios of the precipitin zones shown in figure 3.

TABLE I

Means and standard deviations for the quantitative analyses of agar column diffusion patterns resulting from reactions between human sera and antihuman rabbit sera. Significant differences expressed in percent are indicated in the text

Category	Assumed normal				Clinically abnormal			
	Number of samples	Zonal diffusion		OD 6/4	Number of samples	Zonal diffusion		OD 6/4
		6 to 6'	6/4 ratio			6 to 6'	6/4 ratio	
CRP−, 6' −	21	0	1.40 ± .06	1.23 ± .05	13	0	1.38 ± .05	1.26 ± .08
CRP+, 6' −	0	0	0	0	1	0	1.42	1.26
CRP−, 6' +	8	1.15 ± .40	1.37 ± .07	1.23 ± .08	47	1.14 ± .50	1.36 ± .08	1.25 ± .08
CRP+, 6' +	0	0	0	0	70	2.08 ± .90	1.33 ± .13	1.21 ± .08
Total	29				131			

8.7 percent of the CRP negative (CRP−) and 67.1 percent of the CRP positive (CRP+) hospital samples were outside the diffusion range.

The density and diffusion data for marker zones 4 (beta and gamma globulin) and 6 (albumin) were obtained from the graphic diffusion patterns similar to those already published (6) (see also fig. 3). Considering only the ratios of the diffusion (D) distances of these zones (D 6/4), 17.2 percent of all assumed normal samples were beyond the mean ± 1 S.D., but 29.0 percent of the "clinically abnormal" samples exceeded this normal range. The largest percentage (41.4) of "abnormal" samples to exceed the "normal" range was in the CRP+, 6'+ group. Density ratios of zones 4 and 6 were also computed (OD 6/4) for both assumed normal and "clinically abnormal" samples. In the normal group, 24.1 percent were outside the mean ± 1 S.D. with the largest percentage (37.5) in the CRP− 6'+ group. With this "normal" range as a guide, 30.5 percent of all "abnormal" samples were outside the limits. The largest percentage (38.5) of "abnormal" samples that were outside the "normal" OD6/4 mean ± 1 S.D. was in the CRP+, 6'+ group. An agammaglobulinemic patient and a patient who was receiving repeated injections of gamma globulin both showed altered diffusion and density ratios for the albumin and globulin zones.

Except for the normal sera used to establish a baseline pattern, all other samples were coded by the physician (AIIK) prior to the laboratory analysis. In this way, neither the source nor the condition of the patient was known to the analysts. A later comparison of the agar column results indicated that in approximately 90 percent of the samples analyzed, the serologic results paralleled the physician's estimate of the stress produced by the illness.

Eleven coded samples not included in the data were assumed, when received, to be normal but were later serologically classified as "abnormal": 91 percent showed altered density ratios for albumin and globulin, and 73 percent had the abnormal 6' zone to a mild degree. In this group, 27 percent were CRP+. Further investigation established that all of these samples were taken from donors who had been recently immunized with influenza and typhoid antigens.

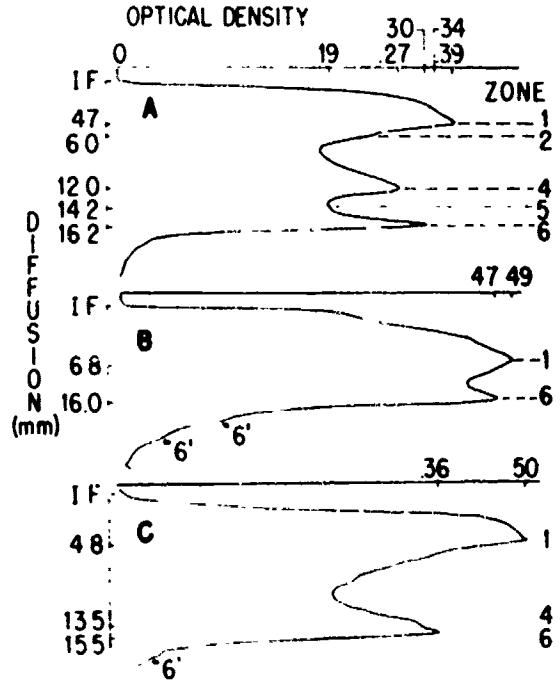

FIGURE 3

Normal and abnormal human-antihuman precipitin zone patterns in agar columns. These graphs were made with the Serum Agar Scanner Instrumentation (SASI) (13). Pattern A, typical normal pattern; pattern B, serum from a nephrotic syndrome; pattern C, serum from suspected malignancy. Reaction time, 120 hours at 24° C.

DISCUSSION

There are indications that latent conditions which do not show clinical evidence of disease or stress may be reflected in altered serum patterns (6). This might account for the 28 percent "assumed-normal" samples that were found mildly "abnormal" as indicated by the appearance of a 6' zone. These sera were CRP−.

Later studies with both human and rat sera have emphasized the necessity of standardizing the antiserum pattern by the analysis of a sufficient number of assumed normals. It appears possible, from these recent explorations and from the reports of Preer and Telfer (15) on diffusion rates relative to antiserum viscosity, that increasing the viscosity of the antiserum might prevent the appearance of the 6' zone below the albumin zone for a statistically significant number of normal sera. In this manner, laboratory standardization rather than rabbit selection could be utilized. It is obvious, of course, that the production of the correct

antibody spectrum is still subject to rabbit variability.

It seems plausible, from this and other studies, that the antigen fraction manifested as a 6' zone is a very sensitive but nonspecific indicator of internal stress. It appears to increase in a variety of conditions, some of which were not directly traceable to obvious pathologic symptoms. This substance, believed to be a mucoprotein, seems to appear in higher concentration before the appearance of a measurable amount of C-reactive protein. Although CRP and mucoprotein have different physical characteristics, they are both glycoproteins and may arise from the same or from similar sources within the body. Data reported here point to a lower stress threshold for the increased production of mucoprotein compared to CRP.

The possibility of nonspecific protein interaction and competition for antibodies by homologous and heterologous antigens in an agar diffusion column, indicates the necessity for caution in differentiating between normal and abnormal sera solely by means of the diffusion or density ratios of certain zones. Although altered diffusion ratios between albumin and globulin zones of whole sera have been noted to parallel the expected results in agammaglobulinemia and gamma globulin therapy, too few samples from patients with such conditions have been examined to rely only on this type of measurement. The cause of markedly altered density ratios (OD 6/4) for albumin and globulin zones in sera from recently immunized patients cannot at present be attributed to known alterations in A/G ratios or configurational changes within these fractions.

SUMMARY AND CONCLUSIONS

Previous studies indicated that normal human sera when diffused into an appropriate homologous antiserum-agar all showed similar diffusion patterns after 120 hours' reaction time at 24° C. Abnormal human sera showed altered diffusion patterns plus an additional zone. This fast-moving, faint zone was believed to be associated with an increased concentration of mucoprotein.

In an extension of the preliminary studies, results are presented of the analyses of 29 human sera, assumed to be normal, and 131 sera from hospitalized patients. The disease categories were vascular, metabolic, psychogenic, allergic, blood dyscrasia, malignant, skin, gastrointestinal, collagen, liver, infectious, and miscellaneous. Distance and density measurements of the diffusion patterns of zones associated with globulins, albumin, and mucoprotein were made from the records of an automatic scanning and recording device called the Serum Agar Scanner Instrumentation. C-reactive protein determinations by the usual clinical method were also done on the abnormal sera.

The results of the diffusion analyses paralleled the physician's estimate of the stress produced by the particular disease state or condition. Of the clinically abnormal samples, 36 percent were CRP- although the diffusion pattern was beyond normal range; 53 percent were CRP+ and had abnormal patterns; approximately 1 percent (1 sample) was CRP+ with a normal pattern; and 10 percent were CRP- with a normal pattern. The latter group (13 samples) came from patients who were convalescent or considered practically well. Of 29 normal sera, 28 percent were slightly beyond the usual normal range for the mucoprotein zone and were CRP-.

With adequate standardization of the antiserum, the agar column diffusion patterns of human sera appear to offer a nonspecific screening test for detecting serologic imbalances. This method, in addition to being considerably more sensitive than the C-reactive protein reaction, may provide some information relative to changes in other serum components.

REFERENCES

1. Wodehouse, R. P. Gel diffusion. A quasi-critical review of recent literature. Ann. Allergy 14: 96-113 (1956).

2. Darcy, D. A. Immunological discrimination between the blood of normal and tumor-bearing rats. Nature, London 176:643-644 (1955).

3. Darcy, D. A. Immunological demonstration of a substance in rat blood associated with tissue growth. Brit. J. Cancer 11:137-147 (1957).

4. Oudin, J. Specific precipitation in gels and its application to immunochemical analysis. *In* Methods in medical research, vol. 5, pp. 335-376. New York: The Year Book Publishers, Inc., 1952.

5. Glenn, W. G. Normal and abnormal human sera diffusion patterns in agar columns. Bact. Proc., p. 94 (1957).

6. Glenn, W. G., G. F. Lanchantin, R. B. Mitchell, and I. W. Marable. Normal and abnormal human-antihuman precipitin reactions in agar columns. School of Aviation Medicine, USAF, Report No. 58-32, Jan. 1958.

7. Glenn, W. G., G. F. Lanchantin, R. B. Mitchell, and I. W. Marable. Analyses of normal and abnormal human-antihuman precipitin reactions in agar columns. Texas Rep. Biol. & Med. 16, No. 3:320-332, Fall 1958.

8. Glenn, W. G. Serum agar methods and variables. School of Aviation Medicine, USAF, Report No. 56-116, Nov. 1956.

9. Glenn, W. G. Unpublished work at the School of Aviation Medicine, USAF.

10. Glenn, W. G. Serum agar measuring aid (SAMA). J. Immunol. 77:189-192 (1956).

11. Glenn, W. G. Direct photometry of diffusing precipitin systems for characterizing proteins. School of Aviation Medicine, USAF, Report No. 57-37, Jan. 1957.

12. Glenn, W. G., and A. C. Garner. Integration of human serum and serum fraction diffusing patterns. J. Immunol. 78:395-400 (1957).

13. Glenn, W. G. New instrumentation for the quantitation of agar precipitin systems. School of Aviation Medicine, USAF, Report No. 58-133. (*In press*)

14. Difco Laboratories. Bacto C protein antiserum, Bulletin No. 153, July 1956.

15. Preer, J. R., Jr., and W. H. Telfer. Some effects of gel diffusion techniques. J. Immunol. 79:288-293 (1957).