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AGAR GEL PRECIPITIN ANALYSES IN LABORATORY-ACQUIRED TULAREMIA *

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Serologic analyses reported in the past of patients with laboratory-acquired (1) and naturally acquired (2–4) tularemia have revealed, at the onset of most of the laboratory-acquired infections, moderate to low agglutinin titers as a result of prior immunization. A diagnostic fourfold rise in titer usually did not take place until the third to fourth week of disease, with maximal responses during the second to third month. Agglutinins frequently persisted for years at levels somewhat below this maximum. In some patients a diagnostic rise in hemagglutinins was demonstrated a week earlier than with agglutinins (1). No importance has been attached to the presence of agglutinins or hemagglutinins as regards protection from infection, nor has the presence or absence of these antibodies offered an understanding of the clinical problems of relapse and reinfection (5–8).

The application of other techniques seemed indicated in a search for serologic tests that would detect a diagnostic change sooner and that would offer some immunologic explanation of relapse and reinfection. The present communication reports serologic measurements, including those obtained with the agar gel diffusion technique, on the sera of 29 patients with laboratory-acquired tularemia. Included in this group are five patients who experienced a clinical relapse in their disease and one who became reinfected.

MATERIALS AND METHODS

The 29 patients reported here represent proved cases of laboratory-acquired tularemia and are from the group reported by Overholt and colleagues (8). All but one subject had received prior immunization of a varying degree with the Foshay vaccine. Pulmonic or typhoidal tularemia occurred in 26 patients, and the other 3 had ulceroglandular disease. About one-half of the group was moderately to severely ill. Twenty-seven patients were treated with tetracycline, 2 g daily in divided doses, and two patients, in addition to tetracycline, received streptomycin, 1 g daily in divided doses. The variations in the duration of therapy are seen in Figure 4. Five patients experienced a relapse in disease from 4 to 13 days after therapy was discontinued. One of the patients represents a case of proved reinfection with tularemia.

Skin tests were performed intradermally with 0.1 ml of a 1:1,000 dilution in physiological saline of the standard phenolized vaccine on the flexor surface of the forearm. The test was considered positive if 10 mm or more of erythema or edema was present at 48 hours.

Baseline sera were available from the 3 months prior to the onset of disease in all but six patients. In this latter group sera taken not more than 6 months before the onset of disease were used. Samples of serum were collected at various times during the disease and convalescent periods. Serum samples, similarly handled, were available from five patients with brucellosis.

Agglutinin titers were determined before freezing and storage of the serum samples. Agglutination tests were performed by the Laboratory Section, Clinical Branch, Medical Investigation Division, with a technique that has been reported elsewhere (1). The reciprocal value of the last tube showing evidence of 1+ or greater flocculation was read as the titer. A fourfold rise was considered diagnostic.

Precipitins were determined by the double diffusion method of Ouchterlony (9). Eight ml of 1 per cent agar (Noble) in 0.9 per cent NaCl and 0.075 M phosphate buffer (pH 7.3), with 1:10,000 merthiolate as a preservative, was poured into flat-bottom Petri dishes (diameter 100 mm) and allowed to cool. A plastic pattern, metal cutter, and vacuum pipet for the removal of the plugs allowed preparation of the final plates. The chambers were 6-mm squares with a diffusion distance of 3 mm at the opposed corners. The chambers were arranged in three parallel rows so that eight antisera could be compared simultaneously on one plate against the central standard antigen. The antigen was prepared from an avirulent strain of *Pasteurella tularensis* (38A) grown for 48 hours in casein-hydrolysate broth. The whole cells were removed by centrifugation at 4,000 G and washed three times in phosphate-buffered saline. The cells were resuspended in this solution and subjected to sonic vibration for 1 hour at an energy of 9 kc. The resultant material was centrifuged at 18,000 G for 30 minutes with the final supernate used as the standard antigen. Micro-Kjeldahl determinations revealed 0.30 mg of nitrogen per ml in this solution.

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The central wells of freshly prepared plates were filled with antigen while antisera were placed in the peripheral wells. The plates were allowed to stand at room temperature. Daily readings were made by the same observer (K.V.) for 1 week using a colony counter modified to give indirect illumination. The 72-hour reading was found to be optimum in terms of the maximal number and clarity of lines. This reading was used in the final analysis. Fusion of lines of precipitation of adjacent sera was interpreted as representing reactions of closely related or identical antigen-antibody systems (9–11). Selected sera were titered by employing twofold dilutions in normal saline. The last dilution producing a visible line after 72 hours determined the titer.

RESULTS

Agglutinins. The baseline sera of about one-half of the 27 patients treated only with a bacteriostatic agent had reciprocal agglutinin titers of 40 or more, and about one-fifth had titers greater than 160. Figure 1 reveals that there was a gradual rise in the concentration of agglutinins until maximal levels of 640 to 2,560 were reached by the fourth or fifth week. Thereafter a slight fall in concentration occurred. Thirteen patients who could be evaluated 1 to 3 years after their primary infection had titers ranging from 160 to 640. Patients who eventually relapsed seemed to lag in the development of agglutinins and reached peak levels later than did nonrelapsing patients.

Precipitins. Only one of the baseline sera from the 27 patients showed a faint line by the agar gel technique. This line occurred only with undiluted serum. All of these patients developed at least two lines during the course of their disease. Figure 2 reveals that the response with regard to the number of lines was maximal by the fourth or fifth week of disease; after this there was a gradual decrease. As in the case of agglutinins, precipitins tended to lag in relapsing patients with respect to the time of appearance and the number of lines formed during the first few weeks of disease. Two or three persisted in all the sera from 15 patients that could be evaluated from 1 to 3 years after their disease.

Precipitins were detected before a diagnostic rise in agglutinins in 13 of 23 patients. The rise in agglutinins and precipitins occurred together in 8 patients and agglutinins rose first in only 2 patients. Precipitins developed in 1 patient who had a high initial titer of agglutinins and who failed to show a diagnostic fourfold rise. Precipitins oc-

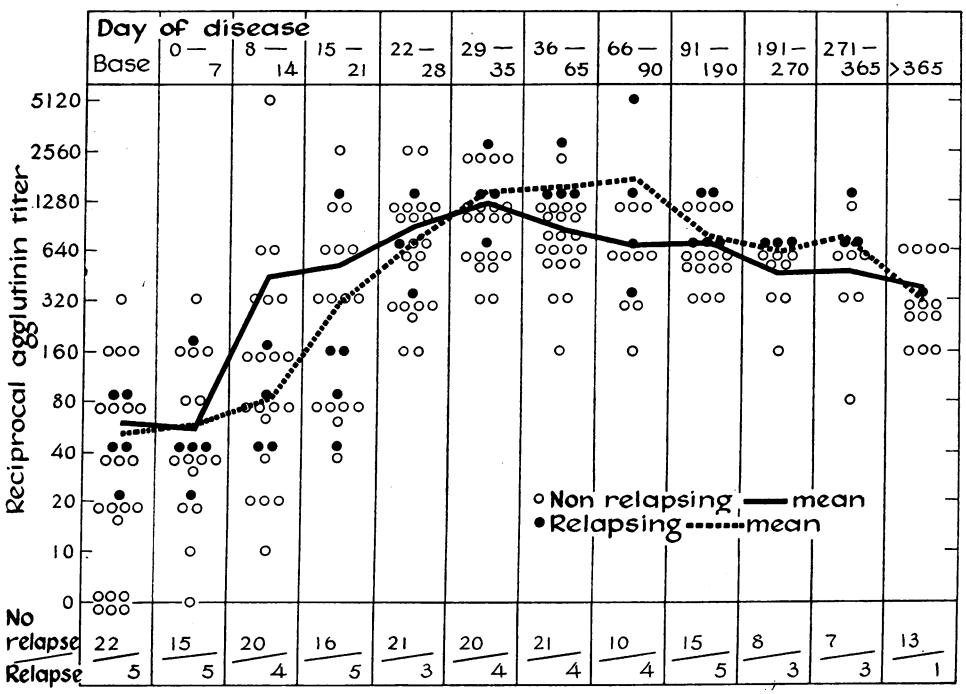

FIG. 1. SERIAL MEASUREMENTS OF SERUM AGGLUTININS IN 27 PATIENTS TREATED WITH A BACTERIOSTATIC AGENT.

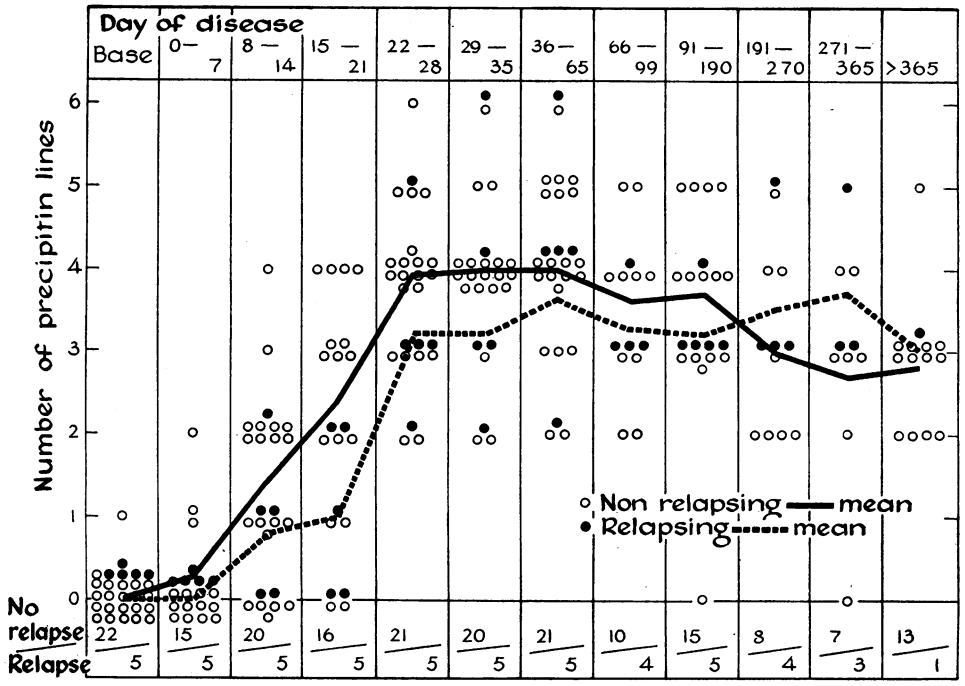

FIG. 2. SERIAL MEASUREMENTS OF SERUM PRECIPITINS IN 27 PATIENTS TREATED WITH A BACTERIOSTATIC AGENT.

curred before a positive skin test in 7 among 12 patients who could be compared.

Precipitin patterns. Starting with the line nearest the antigen well, the individual zones of precipitation were lettered A through F. A single serum from a patient was compared with sera obtained at other times, as well as with the sera of other patients. It was possible in this way to follow the course of individual antibody responses during the progress of disease in a single patient, and to show that these same responses occurred in other patients. Figure 3 is a diagrammatic presentation of the patterns seen and their relative positions.

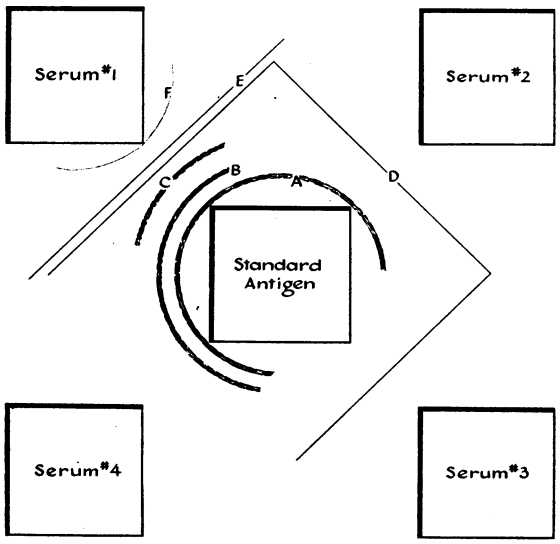

FIG. 3. DIAGRAMMATIC PRESENTATION OF AGAR GEL PRECIPITIN PATTERNS OBTAINED WITH THE STANDARD ANTIGEN AND VARIOUS HUMAN SERA.

A comparison of the earliest precipitin lines among the various patients revealed that D, DE, and DF occurred first in 16 of 22 nonrelapsing patients. Of the remainder, two patients initially developed lines AB, and four patients developed AD together or in association with other lines. The initial patterns of the five patients who relapsed did not differ from the others.

In patients without a relapse line D appeared sooner and reached maximal levels earlier than did line A. The latter first tended to appear in titers greater than D during the third week of disease. The maximal concentrations of the A precipitins were higher than the D precipitins. The occurrence of a relapse did not affect the maximal

levels of precipitins; however, there was a delay in the attainment of peak titers.

Precipitins persisted at appreciable concentrations. Little difference was seen in the titers among the sera of patients whose disease was 6 months to 1 year in the past as compared with those whose disease was 1 to 3 years in the past. Line A persisted in titers greater than line D. The D line was no longer detectable in the sera of 3 of 13 patients evaluated more than 1 year after their disease.

No correlations could be made of the character and quality of the precipitin response and of the age, sex, race, degree of immunization, time lapse since last immunization, and severity of disease of the patients. Among the 29 patients 3 had been infected via the skin rather than through the respiratory tract. The effect of the portal of entry on the precipitin response could not be accurately assessed because of the disproportionate numbers of respiratory infections, but no difference was discernible in the time of appearance or the evolution of precipitins in these two groups of patients.

Effect of treatment. The initiation of treatment with a bacteriostatic agent within the first week of the onset of disease was followed by a relapse in 5 of 14 patients. No relapses occurred among 13 patients whose treatment was initiated later.

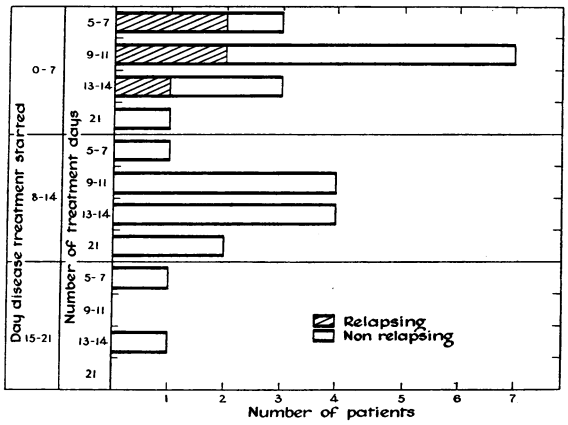

FIG. 4. THE RELATIONSHIP OF THE TIME OF INSTITUTION OF TREATMENT WITH A BACTERIOSTATIC AGENT TO THE OCCURRENCE OF A RELAPSE.

At the beginning of therapy, precipitins were not present in the sera of 17 of 22 nonrelapsing patients or in the sera of the 5 patients who experienced a later relapse. At the completion of therapy the D line, alone or in combination with other lines, was found in the sera of 20 of 24 non-

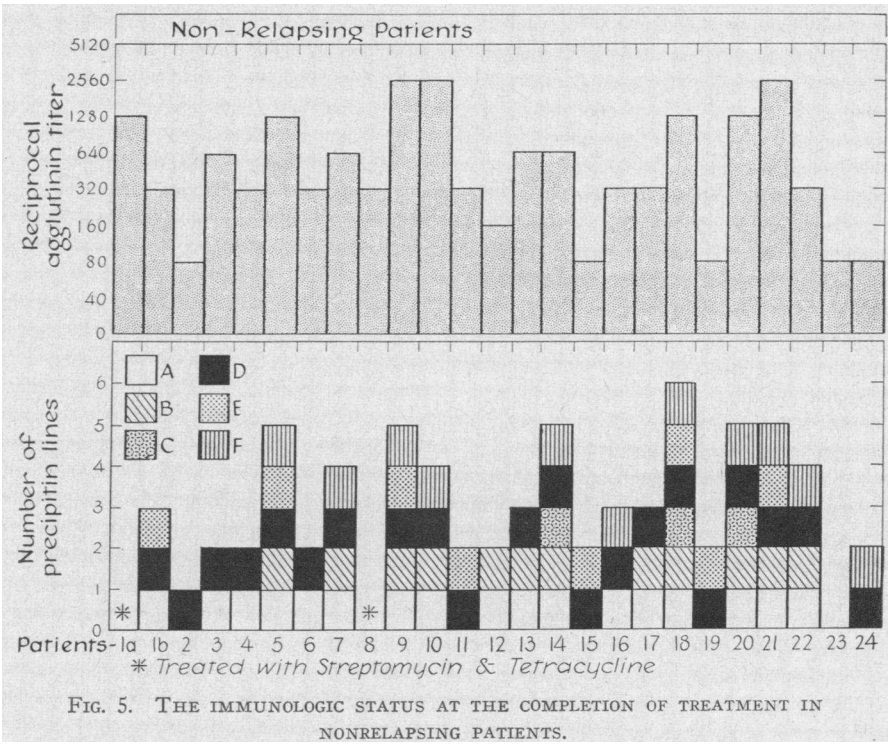

FIG. 5. THE IMMUNOLOGIC STATUS AT THE COMPLETION OF TREATMENT IN NONRELAPSING PATIENTS.

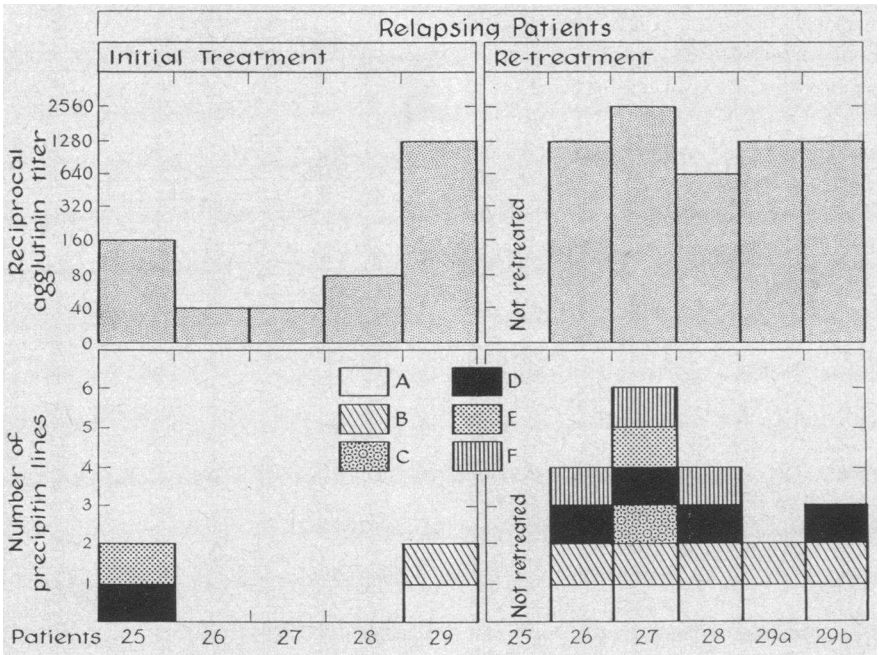

FIG. 6. THE IMMUNOLOGIC STATUS AT THE COMPLETION OF TREATMENT IN PATIENTS WITH A RELAPSE.

relapsing patients. The serum of Patient 12 had only the AB pattern at the discontinuance of therapy but the D line appeared some time within the subsequent 17 days. Two of three patients who did not have precipitins at the completion of therapy had received streptomycin in addition to tetracycline. One of these, Patient 8, never demonstrated precipitins. The other, Patient 1-a, developed a transient AB pattern and is of particular interest because he was reinfected 20 months later (1-b). The third, Patient 23, developed a transient AB pattern after 14 days of treatment with tetracycline which had been started on the second day of his disease. This patient was subsequently

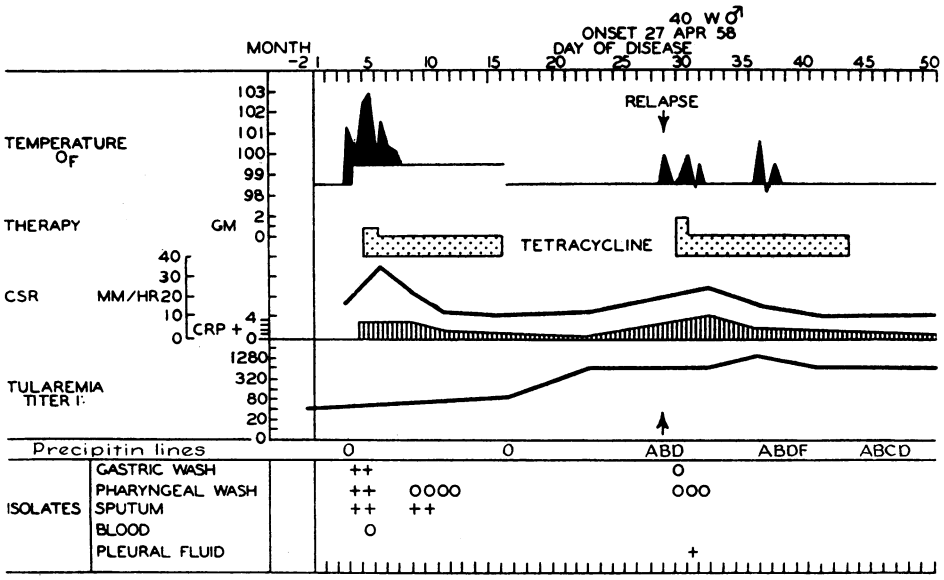

FIG. 7. CLINICAL COURSE OF A REPRESENTATIVE PATIENT WITH A RELAPSE IN DISEASE.

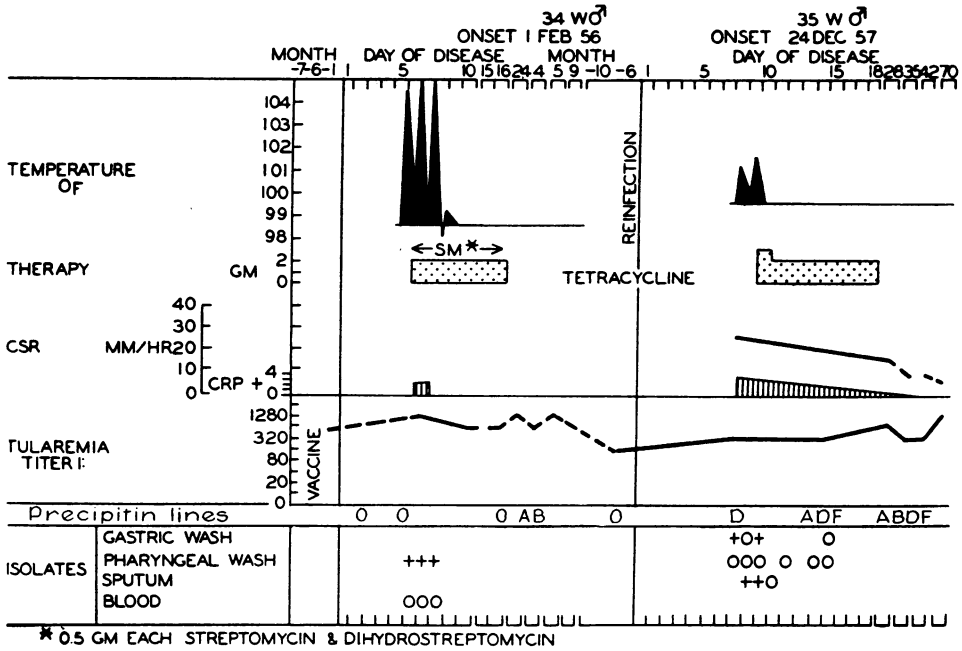

FIG. 8. CLINICAL COURSE OF A PATIENT WITH REINFECTION.

inoculated with a viable vaccine strain of *P. tularensis* and reacted as though he had had no previous experience with the organism.

Relapse. All relapsing subjects had moderate or severe primary clinical courses. Relapses occurred more frequently in patients with lesser degrees of immunization. One relapse occurred among 12 patients who had received at least a primary and two booster series. In contrast, 4 of 16 patients who had not received this degree of immunization had a relapse after the completion of therapy.

In relapsing patients this lag in antibody response probably reflects the effect of early therapy. However, the presence of significant agglutinin titers did not prevent relapses in these patients. The absence of precipitins at the completion of treatment in three of five patients seemed more important (Figure 6) in view of the fact that precipitins were present at this time in the sera of 20 of 22 similarly treated patients who did not experience relapses. Patient 25, with a low titer D line at the completion of therapy, was the only one of this group who did not receive retreatment. Three to six lines developed in all relapsing patients before or during retreatment; thereafter they experienced no further difficulty. The course of a representative patient (no. 28) is shown in Figure 7.

Patient 29 had two clinical relapses, with *P. tularensis* isolated on both occasions. He had not received prior immunization. At the conclusion of 14 days of treatment with tetracycline this patient's serum contained agglutinins in a titer of 1,280 and an AB pattern on agar gel. He relapsed 9 days after the completion of therapy. The agglutinins and precipitins were unchanged after re-treatment with tetracycline for 10 days; 39 days later he again relapsed and received an additional 14 days of tetracycline. The agglutinin titer was unchanged after this treatment period; however, in addition to the AB pattern, a D line was present. He has had no further difficulty.

Reinfection. Patient 1, a and b, represents a proved case of reinfection of tularemia. The infections were by different strains of *P. tularensis* and were separated by an interval of 2 years. On both occasions infection occurred in spite of high concentrations of agglutinins. Treatment with a combination of streptomycin and tetracycline was started during the first week of his initial infection. Although he had no precipitins at the completion of therapy, a relapse did not occur. During convalescence he developed tran-

sient AB lines in low concentrations. These were no longer present at the time of his reinfection. Only tetracycline was used in the treatment of his reinfection, and at the discontinuance of therapy, precipitins, including the D line, were present. Three lines are present more than 1 year later and he has experienced no further difficulty. His total course is shown in Figure 8.

Brucellosis. A cross agglutinin rise against brucella occurred in 3 of 29 patients. None gave a history of past brucellosis. In one patient the rise was associated with a relapse in disease rather than with his primary infection. Skin tests for brucella remained negative and agglutinins for brucella were gradually lost.

Five patients had measurable amounts of agglutinins for brucella, a positive skin test, or a history suggesting experience with brucella organisms. No change occurred in agglutinins as a result of their infection with *P. tularensis*. Agar gel precipitins were not detected with the *P. tularensis* antigen prior to infection with this organism. Neither a cross agglutinin rise nor agar gel precipitins were found in the sera of five patients with brucellosis who gave no history of tularemia.

DISCUSSION

The occurrence of multiple antigen-antibody systems in convalescent sera from patients with a variety of diseases and from hyperimmunized animals has been observed by other workers who used agar gel diffusion (12–19). Ormsbee and Larson (20) have shown no differences in soluble antigen preparations of two different virulent strains of *P. tularensis* by this method. Similarly, we have been unable to show any major differences when comparing the avirulent 38A strain with the virulent Schu strains. We have used an antigen preparation of the former in this study. The specificity of this antigen was confirmed by the nonreactivity of a number of sera from normal subjects and from patients with a variety of febrile illnesses. Patients with brucellosis were evaluated because of the known cross reactions that occur with the agglutination test (2, 3, 21, 22). Similar cross reactions were not observed in measurements with the agar gel technique. Several patients in our series with evidence of past experience with brucella were nonreactive before their infection with *P. tularensis*.

The data presented indicate that the number of precipitin lines paralleled measurements of antibodies by other serological methods. The agar gel technique had the advantage of giving a negative reaction in all but one baseline serum. Hence, the development of even one line during the early course of disease could be interpreted as having diagnostic significance. It proved particularly helpful in patients with high initial agglutinin titers who failed to show fourfold rises. Agar gel precipitins occurred before a diagnostic rise in agglutinins in 57 per cent of the patients. In only 9 per cent was the reverse true. In most instances this difference was less than 1 week. Skin test conversion, which Foshay (4) reports in most cases within the first week of disease, was compared with the onset of agar gel precipitins. No significant difference was noted.

Analysis of acute and convalescent sera by agar gel diffusion has been reported with few human diseases (23–25). It was therefore of interest to observe the serial changes that could be studied in our patients. Measurements of the concentration of the individual antigen-antibody reactions revealed that several of these systems had different cycles of onset, peak, and duration. An evaluation of these results was not within the scope of the present study, but it seems that such factors as the rate of release, physical state, and immunogenic capacity of the released antigens might play important roles. Studies designed to define the origin of these antigens on or within the microorganisms offer interesting additional avenues of research.

The modification of disease as a result of the timing of prior immunization, or chemotherapy, or both has been reported by a number of authors (6, 7, 26, 27). Correlations of serologic measurements with the clinical phenomena of relapse and reinfection have not been possible. The work of Smadel (26) with *Rickettsia tsutsugamushi* and of McCrumb and co-workers (6) and Saslaw (7) with *P. tularensis* seems pertinent to the observations reported in this communication. Smadel showed clearly, in studies on scrub typhus, that the frequency of relapse was related to the day of disease on which treatment with a rickettsiostatic agent was started. Relapses occurred with in-

creasing frequency as therapy was initiated earlier in the first week of disease. No differences in the maximal response of the OX-K antibodies were observed among the different groups; however, there was a suggestion that therapy within 4 days of the onset of disease might delay somewhat the appearance of agglutinins.

McCrumb and associates (6) have reported similar observations in comparing streptomycin and chloramphenicol in the prophylaxis of human ulceroglandular tularemia. Their study included control patients who were treated within 36 hours of the onset of their disease. Relapses were observed in two patients treated with chloramphenicol and in none of those treated with streptomycin.

Our experiences in the treatment of tularemia with a bacteriostatic agent are similar to those of Smadel with scrub typhus and of McCrumb with tularemia. These data support their observations that therapy within the first week of disease was associated with an increased frequency of relapse in clinical disease. A lag in the agglutinin response was evident, although the maximal titer attained was not impaired. Clinically, there was no way to predict which patients would relapse after the discontinuance of therapy. The degree of the agglutinin response was of no value in this regard.

For the first time, the measurement of agar gel precipitins has demonstrated an impairment in the immune response which may make it possible to predict which patients will later relapse. Additionally, the data suggest that the presence of one of these bands may closely parallel protection from reinfection and freedom from relapse. Among five patients experiencing a clinical relapse, no lines were present in the sera of three at the discontinuance of antimicrobial therapy for the primary attack. Line D was present in one patient's serum and he was the only patient who did not require re-treatment. An AB pattern was present in the fifth patient. The possible importance of the D line was particularly impressive in this man since he experienced two relapses before it could be detected in his serum. After the appearance of this line he experienced no further difficulty. A delay in reaching peak precipitin titers was evident in those patients with a relapse. On the other hand, the D line alone or in combination with other lines was present at the completion of therapy in the sera of 20 of 22 similarly treated patients who did not have a relapse.

In contrast to treatment with bacteriostatic agents, an adequate early course of treatment with a bactericidal agent has not been associated with relapse in tularemia. McCrumb and co-workers (6) showed that prophylaxis with streptomycin prevented the development of disease manifestations, while chloramphenicol seemed only to delay the incubation period. No agglutinin response was observed in the absence of constitutional symptoms. The consequences of this arrest of immune maturation by means of prophylactic therapy or by early use of a bactericidal agent are implied in the work of Saslaw (7), with intradermally challenged volunteers. He was able to reinfect volunteers with the same strain of *P. tularensis* 6 months after an initial infection which had been immediately treated with streptomycin. Moreover, in his experience the agglutinin titer was of no value in determining which volunteers could be reinfected.

The experiences of McCrumb and of Saslaw are similar to those observed in our two patients who received streptomycin in addition to tetracycline during the first week of their disease. Both patients demonstrated high agglutinin titers. Neither developed the D precipitin line, although one developed a transient AB pattern. Neither experienced a clinical relapse. One of the two patients was reinfected 1 year later, although a high concentration of serum agglutinins was present. At the time of reinfection he developed several precipitin systems including the D line and he has had no further difficulty in spite of continued risk through occupational exposure.

It is apparent that the primary difference between these two patients and those who received early treatment with a bacteriostatic agent was the almost complete failure to develop agar gel precipitins. The removal of the microorganisms with bactericidal therapy prevented the development of an adequate antigenic stimulation of the immune mechanisms in the course of the current infection. In view of the reinfection experienced by one of these two patients, it seems logical to assume that the relationship previously noted between the absence of agar gel precipitins and relapse can be extended to include susceptibility to

reinfection. Our limited experience suggests that patients who have failed to develop agar gel precipitins, particularly the D line, may possibly be reinfected under the appropriate conditions.

We have not observed reinfection among 26 patients who have developed a full complement of agar gel precipitins after treatment with a bacteriostatic agent, although many of them have had continued high risk through occupational exposure over several years. It is clear that bacteriostatic agents may also be associated with complete arrest of the immune response. Thus, two of seven volunteers treated prophylactically with a bacteriostatic agent by McCrumb and co-workers (6) developed neither constitutional symptoms nor an agglutinin response. Similarly, one of our patients treated with tetracycline on the second day of his illness responded as though he had been treated with a bactericidal agent. He did not experience a relapse after the completion of therapy, although no evidence of agar gel precipitins could be demonstrated. An agglutinin response was observed during the convalescent period, as was a transient AB pattern. This man was subsequently inoculated with a viable vaccine strain of *P. tularensis* and reacted as though he had had no previous experience with the organism.

Our experience indicates that the agar gel diffusion technique may be a useful method for measuring the effect of early antimicrobial therapy upon the host's immune response in tularemia. This technique has suggested a difference between the effect of bacteriostatic and bactericidal agents in this regard, and thus has offered a possible immunologic explanation for relapse and reinfection in tularemia. Further studies are indicated in this and other diseases to confirm and extend these observations. The measurement of agar gel precipitins in human beings immunized with killed and living attenuated organisms of *P. tularensis* will be reported in another publication.

SUMMARY

The agar gel diffusion technique has been employed for the study of antibody responses in 29 patients with laboratory-acquired tularemia. Five patients with subsequent relapses and one with proved reinfection are included in this group.

In spite of prior immunization, precipitin lines were absent at the onset of disease in most patients, as contrasted with the presence of low to high levels of agglutinins. The appearance of precipitin lines was detected somewhat earlier than was a diagnostic rise in agglutinins. The increase in number of lines paralleled the rise in agglutinins with a maximum of four to six lines attained by the fourth or fifth week in most patients.

The initiation of antimicrobial therapy during the first week of disease may profoundly suppress the development of precipitins. Withdrawal of the drug may be followed by a relapse, if the agent is bacteriostatic, or by cure and susceptibility to reinfection if the agent is bactericidal.

It is suggested that the absence of precipitin lines at the completion of treatment may possibly be associated with the occurrence of a later relapse or susceptibility to reinfection. The absence of the D line may be of particular importance in this regard.

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