Tuberculosis control has attained to that stage of completion in which each individual case takes on a heightened status. So any laboratory test that speeds treatment assumes a new importance. Reported here is confirmation of the usefulness of a rapid virulence test for the offending bacillus.

A Study of the Neutral Red Reaction for Determining the Virulence of Mycobacteria*

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THE determination of bacterial virulence is of particular importance in the laboratory diagnosis of tuberculosis. Current practice consists of inoculating a suitable medium with suspect material and, if acid-fast forms develop, guinea pig inoculation is used to establish their pathogenicity. These procedures may require six weeks or even longer for completion. Because of this, the description by Dubos and Middlebrook in 1948 [1] of a rapid *in vitro* method of determining virulence of mycobacteria aroused considerable interest. This test consists of an alkaline buffered addition of aqueous neutral red to a suspension of methanol washed mycobacteria and observing the presence or absence of staining of the microorganisms. Virulent strains bind the dye and are stained pink to red; avirulent strains remain colorless.

Several workers have studied this reaction and have reported excellent agreement between the neutral red reaction of a strain and its animal pathogenicity. Hauduroy and Posternak,[2, 3] for example, found that 67 virulent strains gave positive neutral red reactions, whereas 67 avirulent strains were uniformly negative. On the other hand, Richmond and Cummings [4] in a study of human, bovine, avian and saprophytic mycobacteria encountered one strain from vegetable sources which was neutral red-positive but avirulent for the guinea pig.

For the past two years we have conducted a study of the neutral red test in our tuberculosis diagnostic sections. The virulence of all acid-fast organisms isolated from human clinical material was tested by both the dye method and by guinea pig inoculation. In addition a small number of stock strains of pathogenic and nonpathogenic mycobacteria were examined in this manner.

EXPERIMENTAL METHODS

Culture and Animal Inoculation—The neutral red test was run on cultured strains prior to animal inoculation and again on cultures reisolated from infected animal tissues. The acid-fast bacilli were isolated on Lowenstein's medium [5] and then subcultured to Du-

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bos's Tween-albumin medium[6] for preparation of animal inocula. Stock strains were cultured in Tween-albumin medium. Incubation in all instances was at 35° C. Animal pathogenicity was determined by inoculation of approximately 2–5 mgm. (wet weight) of bacilli into the groin of young 180–200 gm. guinea pigs. The injected animals were sacrificed and examined at six weeks. Homogenates of tissues showing gross evidence of infection were examined by stained smear and subcultured on Lowenstein's medium. Facilities for histopathological studies were not available.

Performance of the Neutral Red Cytochemical Test—The procedure employed was essentially that described by Dubos and Middlebrook. A loop of culture from a solid, or 1 ml. from a liquid, medium was transferred to a 15 ml. conical centrifuge tube and washed twice with 5 ml. volumes of 50 per cent methanol. The bacilli were then suspended in 5 ml. of Dubos's buffer solution and 0.2 ml. of 0.05 per cent aqueous neutral red added. The neutral red manufactured by the National Aniline and Chemical Company was used in this study. The reaction was read after standing for one hour at room temperature. Virulent strains bound the dye and stained from light pink to deep red; avirulent strains remained colorless. The color of the suspending buffer solution remained amber.

RESULTS

The neutral red reaction was read with the aid of a hand lens and using intense white light as the source of illumination. The intensity of staining varied in different strains from pale pink to deep red, and the time required for take-up of the strain varied from 5 to 60 minutes. It was noted that a considerable number of strains, if allowed to stand for several hours or longer, gave a red-brown color which could be erroneously considered a positive reaction. It was also noted that all of the bacilli of a given strain from solid medium did not bind the neutral red dye. However, the observation of dye-stained bacilli was considered a positive reaction.

A total of 195 acid-fast organisms were examined. One hundred and eighty of these were isolated from patients. Of the 180 recovered from patients, 168 were culturally consistent with *M. tuberculosis* and 12 were acid-fast chromogens. Thirteen of the remaining 15 acid-fast organisms were lyophilized stock mycobacteria, comprised of 4 human, 3 avian, 2 bovine strains, and 4 nonpathogens. The 4 nonpathogens included *M. laticola, M. stercoris, M. leprae,* and *M. phlei.* The human virulent strain, H37Rv, and its avirulent variant, H37Ra, were included in every series of tests as positive and negative controls. In these controls the results of the animal virulence test and neutral red reaction were in complete agreement in every case.

One hundred and sixty-three acid-fast strains culturally consistent with *M. tuberculosis* were neutral red-positive and produced characteristic disease in the guinea pig (Table 1). Two additional acid-fast strains were neutral red-positive but failed to infect the guinea pig in repeated tests. Three were negative by both the neutral red and animal virulence tests. The 12 chromogenic strains were negative by both tests.

All the virulent stock strains, whether human, avian, or bovine, were positive by the cytochemical test (Table 2). One human, one bovine, and 2 avian strains failed to produce characteristic disease in the guinea pig, although the human and bovine strains were virulent for this animal when first isolated. Guinea pigs, of course, are not usually susceptible to avian strains. The 3 stock avian strains were found to be pathogenic for 16 week old chickens.

None of the known nonpathogens gave

TABLE 1

A Comparative Study of the Virulence of Acid-Fast Bacilli Isolated from Clinical Material Using the Dubos Neutral Red Reaction and Guinea Pig Inoculation

Strains	Number	Cytochemical Reaction		Guinea Pig Virulence	
		Positive	Negative	Positive	Negative
Culturally consistent with *M. tuberculosis*	163	163		163	
"	2	2			2
"	3		3		3
Total examined	168				
Acid-fast chromogens	12		12		12

TABLE 2

A Comparative Study of Virulence of Lyophilized Stock Cultures of Mycobacteria Using the Dubos Neutral Red Reaction and Guinea Pig Inoculation

Strains	Number	Cytochemical Reaction		Guinea Pig Virulence	
		Positive	Negative	Positive	Negative
M. tuberculosis var hominis	4	4		3	1
M. tuberculosis var bovis	2	2		1	1
M. tuberculosis var avian	3	3		1	2
Nonpathogens *	4		4		4

* The nonpathogenic group included *M. laticola, M. stercoris, M. leprae* and *M. phlei.*

a neutral red reaction nor induced detectable disease in the experimental animal.

DISCUSSION

It is significant to note that in no instance was a strain isolated which was culturally characteristic of *M. tuberculosis,* neutral red-negative, and yet pathogenic for the experimental animal, though two strains were isolated which gave a positive cytochemical reaction and were negative by the conventional guinea pig inoculation. The two neutral red-positive guinea pig negative strains culturally resembled *M. tuberculosis var hominis.* However, when they failed to infect the guinea pig, inoculations were made in 16 week old chickens and the results indicated that these strains were avian variants. It must be pointed out here that if the neutral red test had not been available, these two strains, on the basis of their cultural characteristics and guinea pig tests, would have been classified as nonpathogenic mycobacteria. It is significant that these two strains were the only acid-fast organisms recovered in repeated attempts from two patients with a tentative clinical diagnosis of tuberculosis.

Of interest was the fact that one bovine and one human lyophilized strain failed to infect the guinea pig though they were pathogenic for this animal when first isolated. This suggests that certain environmental conditions may modify virulence, as measured by animal inoculation, but does not impair the ability of the strain to bind neutral red. Such an attenuation of virulence is evidenced by BCG strains which give weak positive neutral red reactions but develop only self-limiting infections in the experimental animal. A more comprehensive study of the relationship of the neutral red factor to virulence is in progress in our laboratories.

CONCLUSIONS

The results of a study of 168 freshly isolated acid-fast strains culturally consistent with *M. tuberculosis* suggest that the neutral red test measures the viru-

dence of mycobacteria as satisfactorily as the conventional inoculation of experimental laboratory animals.

The use of the neutral red reaction effects an appreciable saving of time in the laboratory diagnosis of tuberculosis; animal virulence studies require from three to six weeks, the neutral red reaction two hours.

Furthermore, the use of this reaction reduces considerably the health hazard to laboratory personnel which is present when infected animals are maintained for pathogenicity studies.

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