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REPRINTS

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THE EXPERIMENTAL APPROACH TO THE CHEMOTHERAPY OF TUBERCULOSIS

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Thousands of failures[1] have attested to the inadequacy of the *in vitro* test as an experimental approach to the selection of agents potentially effective against *Mycobacterium tuberculosis in vivo*. Streptomycin, which combined high *in vitro* activity with dramatic *in vivo* effects in animals and man, was the first significant example of good correlation. Many theories have been proposed over the years to explain the failure *in vivo* of agents shown to be potent anti-tuberculars *in vitro*. The most notable of these stressed, (1) the lipophilicity of the mycobacterial cells and/or (2) the apparent inaccessibility of the tubercle bacillus, because of a limited blood supply to the lesion. These theories flourished despite contemporary evidence to the contrary. Youmans[2] in his review of the *in vitro* antitubercular activity of more than 3,000 substances showed quite clearly that there was no convincing correlation between *in vitro* activity and lipoid solubility. Jessen, as early as 1929, in his work on rosaniline dyes[3] and Florey in 1942, in his investigations with micrococcin,[4] showed that agents active in the test tube were indeed able to make contact with tubercle bacilli within the caseous mass of a tubercle.

Although it is important to stress that the weight of experimental information has gradually discredited the above theories as not being generally acceptable as an explanation as to why a compound active *in vitro* may not be active *in vivo*, no completely conclusive evidence is available today to account for the fact that many chemicals from diverse structure and solubility classes can inhibit *M. tuberculosis* in the test tube but not within a lesion. The example of cycloserine[5] also always reminds us that we can not simply bifurcate our thinking into test tube and animals, for all animals do not respond in the same way. As one may recall, cycloserine was active *in vitro*, did not affect experimental tuberculosis in the mouse, but turned out to be quite effective in the treatment of the disease entity in man.

The presence or absence of explanations notwithstanding, the fact remains that today, as it has been for the past 20 years, the experimental approach to the chemotherapy of tuberculosis revolves primarily around the infection and treatment of suitable animals hosts. The mouse, rat, guinea pig, hamster, rabbit, and monkey are available at present as experimental models. The choice of animal in many cases depends upon how many drugs the investigator wishes to test or what he desires to learn. Most would, however, agree that the mouse is the quickest and most reliable test animal for the primary screening of antitubercular agents. The antitubercular activity of today's most useful agent, isoniazid, was first discovered using the mouse as the exclusive experimental model.[6] Individual preferences do, however, exist in different laboratories as to the animals that should be used and the parameter that should be measured. It is the purpose of the paper to review the experimental techniques now available and to attempt to discriminate among them

with respect to the advantages they may offer and their success in generating useful drugs for the treatment of tuberculosis in man.

Prior to 1940, when practically all attempts at chemotherapy of experimental tuberculosis were disappointing, the problem of selecting adequate screening methods was of little more than academic interest. The use of the guinea pig was the accepted procedure primarily because many years of clinical diagnostic work had shown that this animal host was highly susceptible to the tubercle bacillus. The susceptibility of other animal species was not well defined.

Although from 1940 until 1950 the guinea pig retained its pre-eminent position in the experimental approach to the chemotherapy of tuberculosis,[7] an increasing number of publications began to appear that dealt with experimental tuberculosis and chemotherapy in the rabbit, rat, hamster, and mouse. Particular interest was generated for the use of the mouse as the experimental model as successful breakthroughs occurred with the discovery of para-aminosalicylic acid, sulfones, and streptomycin.[8] It would, therefore, now appear advantageous to discuss in somewhat greater detail the various experimental techniques available.

Mouse. The mouse was first employed for the experimental approach to the chemotherapy of tuberculosis by Thomas and Dessau in 1939[9] who reported the ineffectiveness of a tubercular polysaccharide in this model. Present-day methods widely used throughout the world owe much of their success, however, to the fundamental studies of Youmans[10-12] and Donovick.[13-15] These investigators standardized the strains of mice to be used, the culture and the route of infection, and showed the response of this experimental animal under controlled conditions to available chemotherapeutic agents. In addition to the obvious advantages of the mouse with respect to size, cost and drug consumption, the standardization of the infection by using *M. tuberculosis* H37Rv made available to investigators a highly reproducible test system capable of preliminarily assessing the efficacy of an antitubercular agent in 21 days. The infection of mice by the intravenous route results primarily in pulmonary involvement with enlargement of the periaortic lymph nodes. The rate of death of the animals is a function of the concentration of tubercle bacilli in the inoculum. By the administration of approximately 0.5 ml. of a 10^{-1} dilution of a 7–10 day-old broth culture in Dubos medium practically all mice survive the full twenty-one days of the experiment.[16] Chemotherapeutic efficacy can be scored by grading the presence or absence of gross lung lesions of the miliary type and by culturing lung tissue for tubercle bacilli. This technique prepared the ground a few short years after it came into general use for the study of the antitubercular activity of nicotinamide,[17] para-aminosalicylic acid,[18] the thiosemicarbazones[16,19] and isoniazid.[6] The doubts existing as late as 1949 that the intravenous infection of mice was too severe a test for the screening of useful chemotherapeutic agents have not been borne out. Isoniazid, tested over the years in nearly 5,000 mice with this fulminating infection, has shown a range of CD_{50} values of 4.6–5.5 mg./kg. by diet, 1.9 to 3.8 mg./kg. subcutaneously, and 2.5 to 9.5 mg./kg. per os. The uniqueness of isoniazid in this infection with respect to (1) preventing the multiplication of bacilli after administration of low doses of the drug in the diet as shown by negative pathol-

ogy and negative cultures, (2) producing a lasting protective effect after withdrawal of the animal from the drug, and (3) exerting a chemotherapeutic effect if given to animals in which the disease had already progressed[20,21] presents a yardstick by which potential antitubercular chemotherapeutic agents must be measured.

Rat. The rat has not been extensively employed as a model for chemotherapeutic studies of experimental tuberculosis. The intraperitoneal or intravenous injection of large inocula of bovine or human tubercle bacilli results predominantly in pulmonary disease.[22-24] However, the rat has been found to be refractory to chemotherapeutic agents. According to the investigations of Smith *et al.*[25] the course of an infection in this experimental model could not be influenced by either streptomycin or the sulfones.

Guinea pig. The guinea pig is highly susceptible to human strains of *M. tuberculosis*. As has been mentioned earlier, it has been a useful tool in the confirmatory laboratory diagnosis of tuberculosis. It has also been an extremely useful model for the testing of chemotherapeutic agents and a standardized method for chemotherapeutic screening has been described in detail by Karlson and Feldman.[7] Whether inoculated by the subcutaneous or intramuscular routes, *Mycobacterium tuberculosis* H37Rv produces a progressive and predictable disease in the guinea pig. By either route 0.1 mg. moist weight of tubercle bacilli, which represents approximately 450×10^5 viable organisms, produces a palpable mass at the site of inoculation with enlargement of the regional lymph nodes. Ulceration will invariably occur at the site of inoculation and by the twenty-first to the twenty-eight day after infection one notes enlargement of the spleen and the presence of miliary type lesions in the lungs and liver. Untreated animals usually succumb about one hundred days after infection. At necropsy involvement of the axillary, superficial inguinal, and tracheobronchial lymph nodes, as well as nodular tuberculosis of the lungs and liver, and severe diffusive involvement of the spleen are found.[26] This experimental model has been used for the evaluation of practically every major antitubercular drug.[7,27-29] However, for the purpose of routine screening of a large number of compounds the guinea pig has certain definite disadvantages, namely the rather large amount of agent necessary for treatment and the predisposition of these animals, even under carefully controlled conditions, to spontaneously develop intercurrent pneumonic infections.

At the present time, however, the guinea pig still continues to be a useful tool for assessing the effects of a drug on progressive established disease once it has been shown to prevent tuberculosis in a test system where treatment is begun immediately after infection. As an example one might quote the work of Yanagisawa *et al.*[30] Animals are infected, as described earlier, subcutaneously with 0.1 mg. of moist organisms. Treatment, either orally by dietary admixture, or parenterally, is not administered until six weeks after infection. Therapy is continued for fifteen weeks. The therapeutic evaluation is based on the comparison of gross, histopathological as well as on cultural findings in the treated and untreated groups. Drugs that effectively prevent the development of tuberculosis in the mouse, such as streptomycin,[11] isoniazid,[6] and kanamycin,[31] are also effective in diminishing the intensity of established

disease in the guinea pig. It must, however, be pointed out that agents showing activity in the mouse will not necessarily show the same proportional ratio of activity in the guinea pig. For example, streptovaricin, which was one hundred times less active than isoniazid against the tubercular infection of the mouse when compared on a weight basis[32] was only ten times less effective when compared in advanced tuberculosis of the guinea pig.[26]

Before turning to the next experimental model it might be worthwhile mentioning that experimental methods are also available for treatment of guinea pigs infected by the intracardial route[33] and for the production of experimental tubercular meningitis by intracerebral inoculation.[34] The intracardial infection produces a rapidly lethal infection with an average survival time of thirty three days. The animals die with extensive involvement of the lungs, spleen, and lymph nodes. To a lesser degree the liver is involved. The known effective chemotherapeutics are capable of appreciably extending survival time of such animals if treatment is started immediately after the intracardial infection.

Syrian hamster. This animal has been used rather infrequently even though Dennis *et al.*[35] claimed that it had distinct advantages over the guinea pig. The Syrian hamster is cheaper to maintain and smaller in size, thus requiring less drug. It is also resistant to epizootic disease. The subcutaneous injection of 0.01 mg. moist *M. tuberculosis* H37Rv into a 50 gram hamster produces a slowly progressing disease similar to that seen in the guinea pig. Although the median survival time is usually about one hundred forty days, animals may survive for as long as two hundred sixty-five days. Even after a period of six weeks, macroscopic involvement of the lymph nodes, lungs and spleen can be seen in only 20, 5, and 0 per cent of the animals respectively. The slow development of the disease as compared to the guinea pig may, therefore, account for its infrequent use as an experimental model for the screening of antitubercular agents.

Rabbit. Although seldom used to assess the efficacy of chemotherapeutic agents, specialized and valuable information can be gained from experimental tuberculosis in the rabbit. From a historical point of view one should mention the intravenous infection of rabbits with avian strains of tuberculosis, which is rapidly lethal.[36] The first intimation of the chemotherapeutic activity of *p,p'*-diamino-diphenylsulfone came from the use of this method.[37]

The intratesticular infection[38] of large doses (5 mg.) of human tubercle bacilli produces characteristic localized lesions in two to four weeks. The progress of these lesions can be followed by inspection, by measurement of the lesion, or by examination of stained smears of the lesions. The localized nature of the lesions permits studies on the comparative distribution of chemotherapeutic agents in the tubercular and normal tests.

The technique of aerosol infection developed by Steenken and Wolinsky,[39] which produces a progressive confluent penumonic disease, allows studies in which the disease entity can be followed roentgenographically. To accomplish the infection, a rabbit is placed with nose protruding into a tightly fitted rubber sleeve. A small glass cylinder is then placed over the nose of the animal. Into the other end of the cylinder the suspension of *M. tuberculosis* var. *bovis*

is sprayed by means of a hand nebulizer. After infection the presence of the disease in the lung can be followed by serial chest roentgenograms. In a series of experiments where chemotherapy was started when advanced progressive lesions were present, favorable response was observed to streptomycin and isoniazid. Animals receiving PAS did not respond. The untreated controls showed progressive confluent pneumonic disease resulting in death two to seven months after infection.

To this point the experimental diseases in animals which we have discussed are unlike those normally observed in humans. It is, therefore, not surprising that attempts were made to produce an entity which might closely simulate chronic cavitary tuberculosis in humans. Such an experimental model has been described by Lurie.[40] It consists of exposure of rabbits to aerosol inoculation in a closed chamber and results in the production of chronic ulcerative pulmonary phthisis. Because of the cumbersomeness of this technique, it seems not to have been accepted for the evaluation of chemotherapeutic agents.

Monkey. Schmidt and his co-workers[41] have extensively used the rhesus monkey as an experimental animal for assessing the value of chemotherapeutic agents in experimental tuberculosis, for determination of the toxicity of antitubercular agents,[42] and for the evaluation of the efficacy of vaccines in preventing the induction of experimental pulmonary tuberculosis.[43] The use of this species is not unreasonable because of its phylogenetic affinity to man. It would seem logical to believe that this system might better predict the possible activity of antitubercular agents in man. Animals infected intratracheally with human type bacilli, on autopsy, show extensive cavitary disease and involvement of regional nodes. Agents effective in humans such as isoniazid and PAS have been found to be effective in the rhesus monkey.[41] The thiourea derivative, thiocarbanidin, which is of limited efficacy in man[44,45] showed an appreciable effect in the mouse[46] and in the guinea pig[47] but only marginal activity in the rhesus monkey.[41]

Because of expense and care, the monkey is not a practical tool for the screening of large numbers of chemotherapeutic agents. It is, however, probably the most useful tool for more extensive and confirmatory studies of agents found in routine screening in mice and guinea pigs.

Summary

Techniques developed to test the *in vivo* antitubercular activity of chemotherapeutic agents have been discussed. The disease entity in mice would seem to be the simplest and most rapid method for screening multitudes of agents. After detection of an effective agent in this system and possibly confirmation in the guinea pig it would seem advisable to determine the effectiveness of the agent in the experimental tuberculosis of rhesus monkeys before clinical trial is instituted.

References

1. YOUMANS, G. P. 1947. Am. Rev. Tuberc. **56:** 376.
2. YOUMANS, G. P. 1953. Chem. Biol. Coord. Center. Natl. Research Council.
3. JESSEN, P. 1929. Beitr. Klin. Tuberk. **72:** 108.
4. FLOREY, H. 1952. Proc. Roy. Soc. Med. **45:** 71.

5. MULINOS, M. E. 1955/56. Antibiotics Ann. 131.
6. GRUNBERG, E. & R. J. SCHNITZER. 1952. Quart. Bull. Sea View Hosp. **13:** 3.
7. KARLSON, A. G. & W. H. FELDMAN. 1949. Ann. N.Y. Acad. Sci. **52**(5): 637.
8. SMITH, M. I. 1947. Amer. Rev. Tuberc. **56:** 377.
9. THOMAS, R. M. & F. I. DESSAU. 1939. Yale J. Biol. Med. **12:** 185.
10. YOUMANS, G. P. 1949. Ann. N.Y. Acad. Sci. **52**(5): 662.
11. YOUMANS, G. P. & J. C. MCCARTER. 1945. Quart. Bull. Northwestern Univ. Med. School. **19:** 210.
12. RALEIGH, G. W. & G. P. YOUMANS. 1948. J. Infectious Diseases. **82:** 197.
13. DONOVICK, R. 1949. Ann. N.Y. Acad. Sci. **52**(5): 671.
14. MCKEE, C. M., R. RAKE, R. DONOVICK & W. P. JAMBOR. 1949. Am. Rev. Tuberc. **60:** 90.
15. DONOVICK, R., C. M. MCKEE & W. P. JAMBOR. 1949. Am. Rev. Tuberc. **60:** 109.
16. GRUNBERG, E. & B. LEIWANT. 1951. Proc. Soc. Exptl. Biol. Med. **77:** 47.
17. MCKENZIE, D., L. MALONE, S. KUSHNER, J. OLESON & Y. SUBBA ROW. 1948. J. Lab. Clin. Med. **70:** 158.
18. YOUMANS, G. P., G. W. RALEIGH & A. S. YOUMANS. 1947. J. Bacteriol. **54:** 409.
19. LEVADITI, C., A. GIRARD, A. VAISMAN & A. RAY. 1950. Compt. rend. acad. sci. Roumanie. **231:** 1174.
20. GRUNBERG, E., B. LEIWANT, I. L. D'ASCENSIO & R. J. SCHNITZER. 1952. Diseases of Chest. **21**(4): 369.
21. GRUNBERG, E. 1953. Pediatrics. **11:** 82.
22. SMITH, M. I. 1926. J. Lab. Clin. Med. **11:** 712.
23. OSWALD, N. C. 1940. A.M.A. Arch. Pathol. **29:** 628.
24. WESSELS, C. C. 1941. Am. Rev. Tuberc. **43:** 449.
25. SMITH, M. I., W. T. MCCLOSKY & E. W. EMMART. 1946. Proc. Soc. Exptl. Biol. Med. **62:** 157.
26. STERN, K. F., J. E. GRAY & L. E. RHULAND. 1958. Am. Rev. Tuberc. **77:** 976.
27. STEENKEN, W. 1947. Am. Rev. Tuberc. **56:** 382.
28. KARLSON, A. G. & W. F. FELDMAN. 1953. Am. Rev. Tuberc. **68:** 75.
29. YANAGISAWA, K. K., K. KANAI & T. TACHIBARA. 1957. J. Antibiotics Japan Ser. A. **10:** 236.
30. YANAGISAWA, K. K., K. KANAI, N. SATO, T. HASHIMOTO & H. TAKAHASHI. 1958. Ann. N.Y. Acad. Sci. **76**(2): 88.
31. YANAGISAWA, K. K. & N. SATO. 1957. J. Antibiotics Japan Ser. A. **10:** 233.
32. RHULAND, L. E., K. F. STERN & H. R. REAMES. 1957. Am. Rev. Tuberc. **75:** 588.
33. STEENKEN, W., V. MONTALBINE & J. R. THURSTON. 1958. Ann. N.Y. Acad. Sci. **76**(2): 103.
34. SMITHBURN, K. C. 1936. J. Exptl. Med. **64:** 771.
35. DENNIS, E. W., F. C. GOBLE, D. A. BERBERIAN & E. J. FRELIH. 1949. Ann. N.Y. Acad. Sci. **52**(5): 646.
36. KARLSON, A. G. & W. H. FELDMAN. 1940. Am. Rev. Tuberc. **42:** 146.
37. RIST, N., F. BLOCH & V. HAMON. 1940. Ann. inst. Pasteur. **64:** 203.
38. SMITH, M. I. 1922. J. Med. Research. **43:** 45.
39. STEENKEN, W. & E. WOLINSKY. 1957. Am. Rev. Tuberc. **75:** 965.
40. LURIE, M. B. 1949. Ann. N.Y. Acad. Sci. **52**(5): 627.
41. SCHMIDT, L. H., A. A. GROVER & R. HOFFMANN. 1959. Trans. 18th Conf. Chemotherapy Tuberc. : 312.
42. SCHMIDT, L. H., R. HOFFMANN & H. B. HUGHES. 1953. Am. Rev. Tuberc. **67:** 798.
43. GOOD, R. C., R. HOFFMANN, P. N. JOLLY & L. H. SCHMIDT. 1961. Abstr. Ann. Meeting Natl. Tuberc. Assoc. Cincinnati, Ohio.: 34.
44. DONOHUE, R. E., S. KATZ, C. J. DUKE & M. J. ROMANSKY. 1959. Trans. 18th Conf. Chemotherapy Tuberc. : 317.
45. LARKIN, J. C. 1959. Trans. 18th Conf. Chemotherapy Tuberc. : 324.
46. YOUMANS, G. P., A. S. YOUMANS & L. DOUB. 1958. Trans. 17th Conf. Chemotherapy of Tuberc. : 365.
47. STEENKEN, W., V. MONTALBINE, M. M. SMITH & E. WOLINSKY. 1958. Trans. 17th Conf. Chemotherapy of Tuberc. : 368.