

The Methylene Blue Test

I. In Murine Leprosy and in Lesions Induced in Hamsters After Inoculation with Materials from Cases of Borderline and Lepromatous Leprosy^{1,2}

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It has been known for many years that the lesions of lepromatous leprosy patients retain methylene blue after injection of a 1 per cent solution of the dye. In 1956 Convit *et al.* (6) found that patients with the borderline type of leprosy retained the blue pigment in the lepromatous parts of their lesions, but not in those with a tuberculoid structure (4). Later Convit and Goihman (3) found that the methylene blue test was also positive in xanthomatosis, while skin diseases such as deep mycoses, leishmaniasis and trepanomatosis gave negative results.

Apart from the foregoing observations Convit *et al.* (5) succeeded in 1962 in producing granulomas in hamsters at the site of the inoculation of material from human borderline lesions. The hamster granulomas contained an abundance of acid-fast bacilli, in sharp contrast with the human lesions, but the pathogen resisted all attempts at cultivation in media used currently for mycobacteriaceae. It was likewise found possible to produce granulomas in hamsters by inoculating bacilli from lepromatous human lesions, but the bacilli that developed in the hamster from such precursors were found to be cultivable on numerous occasions (5).

It has seemed important to us to obtain

data on the behavior of leprotic lesions in animals in the presence of methylene blue. For this purpose we have carried out experiments with laboratory animals inoculated with bacilli from borderline as well as from lepromatous human lesions and also with animals inoculated with murine leprosy bacilli. The experiments with the latter were made in the laboratories of the Department of Dermatology of the Vargas Hospital in Caracas and those with the former took place in the Laboratory of Experimental Bacteriology of the Division of Sanitary Dermatology of the Ministry of Health, also in Caracas.

MATERIALS AND METHODS

The animals used in this study were the following:

1. Mice inoculated with *Mycobacterium lepraemurium* of a strain furnished us by Dr. Y. T. Chang of the National Institutes of Health, Bethesda, Maryland, U.S.A., from peritoneal mouse lesions of six months' development.
2. Uninoculated and apparently healthy control mice.
3. Rats with lesions of six months' development from inoculations with a strain of *M. lepraemurium* furnished us many years ago by the late Dr. J. D. Aronson of the Henry Phipps Institute of the University of Pennsylvania. These rats showed either ulcerating skin lesions about 1 cm. in diameter or nodules about 0.3 cm in diameter.
4. Uninoculated and apparently healthy control rats.
5. Golden hamsters (*Cricetus auratus*) inoculated intradermally on the back of

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their ears with bacilli of human borderline leprosy origin, obtained from lesions produced in hamster to hamster passages.

6. Golden and albino hamsters inoculated behind their ears with bacilli of human lepromatous origin, obtained from lesions produced in hamster to hamster passages. The experimental lesions on the ears of the hamsters inoculated with bacilli of borderline origin, as well as those from lepromatous precursors, were of six months' development, as in the case of the lesions in mice and rats from *M. lepraemurium*.

7. Uninoculated and apparently healthy hamsters, forming a control group.

The doses of methylene blue used and the manner of administering the dye are explained in Table I. At the end of all the experiments the animals were killed and autopsied immediately. The autopsy included a careful examination of all lesions, whether retroauricular, peritoneal, or visceral. In all cases the lesions were stained for acid- and alcohol-fast bacilli by the Ziehl-Neelsen method and examined microscopically.

RESULTS

In the animals inoculated with bacilli of borderline or lepromatous origin, and in those inoculated with *M. lepraemurium*,

the dermal and peritoneal lesions retained the methylene blue with an intensity proportional to the dose administered (Figs. 1 and 2.) The blue color became visible as soon as the peritoneal lesions were opened or the nodules of the ears were sliced. It was intensified as the tissues were exposed to the atmosphere.

A generalized, although somewhat faint blue color became visible in the viscera of the animals in the course of their exposure to the air. This color was presumably due to prior formation of the leucoderivative in all the tissues and subsequently reconversion by atmospheric oxidation into the original blue compound.

In the mice inoculated with murine leprosy it was found, as soon as the abdominal cavity was opened, that many lymphoid structures of the omentum were intensely blue. The presence of acid-alcohol-fast bacilli in these structures, and their absence in lymph glands that did not retain the dye, was proof of their infiltration from peritoneal lesions.

In the autopsies made of the control animals no visceral tissues were found to be stained blue, but the presence of the leucoderivative was indicated by the blue tinge they assumed after being exposed to the atmosphere. As the animals had been killed a few hours after the administration

TABLE I. Doses, routes of administration and results of methylene blue test in animals with experimental leprotic lesions and with murine leprosy.

Type of disease in source material	Animals	Route	Dose in mgm./100 gm. body weight	Type of lesion	Results
Lepromatous	Hamster	Intraperitoneal	20 40	Dermal	0 +++
Borderline	Hamster	"	20 30 50	"	0 ++ +++
Murine leprosy	Rat	"	25 50 75	"	0 0 +
Murine leprosy	Mouse	Subcutaneous Intravenous	0 50 100	Visceral	0 ++ +++



FIG. 1. *Left side:* nodule in hamster inoculated with material from borderline leprosy. *Right side:* nodule similarly sectioned after repeated injection of hamster with methylene blue showing localization of the dye.

FIG. 2. Localization of methylene blue in the lepromatous mass in the peritoneal cavity of a mouse infected with *M. lepraemurium*, after injection of the dye.



of the last dose of the dye, evidently they had not had time to eliminate the leuco-derivative formed.

In the cutaneous lesions of the animals inoculated with murine leprosy, as well as in those produced by inoculation with bacilli of lepromatous or borderline origin, the autopsies revealed only a faint blue tinge in spite of repeated doses of the dye and the presence of acid- and alcohol-fast bacilli.

DISCUSSION

It is evident from the results of our experiments that the lesions that developed in the hamster after inoculation with bacilli

of lepromatous or borderline origin, fix methylene blue, as do also the lesions of murine leprosy.

In previous experiments (^{4,6}) it was shown that the only skin lesions of human patients so far known that reacted positively in the methylene blue test were those of lepromatous and borderline leprosy (in the latter the lepromatous components only) and xanthomas (³). These three diseases have one histopathologic feature in common, viz., the presence of "foamy cells" characterized by a high lipid content (⁸).

On the other hand, the experimental lesions produced in laboratory animals by inoculation with lepromatous, borderline

(⁵), or murine leprosy material (⁹), also show a "foamy" structure. The fact that such structures are found in all leprotic lesions that give a positive methylene blue test gives much strength to the hypothesis that the lipids characteristic of the "foamy" cells are somehow involved in the retention of the dye. At the same time, the fact that direct and prolonged exposure to the atmospheric oxygen produces a blue coloration in the viscera of healthy animals injected with methylene blue, might well indicate that all tissues are capable of retaining the dye and transforming it into its leucoderivative. Once so transformed, the leucoderivative might be temporarily combined with a lipid, but on exposure to air it is partly oxidized with regeneration of the blue color.

In human lepromatous and borderline leprosy and in the lesions produced in the hamster by their pathogens, as well as in murine leprosy and in xanthomatosis, it is possible that:

1. There is a decrease in the activity of an oxidation-reduction system of the lepra cell, which prevents the reduction of the dye to its leucoderivative, and/or
2. The leucoderivative is irreversibly reoxidized to the blue form and chemically or colloiddally combined with a cytoplasmic lipid.

There is an interesting observation that may have to do with the phenomenon of methylene blue retention. When tissue of laboratory animals that had been infected with *M. lepraemurium* and subsequently injected with the dye is homogenized in normal saline at 2°C the pigment is not dissolved in that medium, in which ordinarily it is highly soluble. This would indicate that it is firmly bound to some cytoplasmic substance present in infected but absent in healthy tissue.

The increase in intensity of the blue tinge on exposure to air would indicate the presence, jointly with the dye, of a leucoderivative labile to photooxidation with or without the intervention of a dehydrogenating enzyme.

The negativity of the methylene blue test in tuberculoid and indeterminate leprosy, as well as in deep mycoses, leishmaniasis

and trypanosomiasis, together with the weak positivity in animals with murine leprosy, may well indicate that one of the two factors that have been postulated as responsible for the retention of the dye is lacking in those lesions. In another investigation (²) we have found that there are lipids in normal tissues capable of fixing it strongly. We may suppose that wherever the dye is retained in the tissues there is a great modification of the oxidation-reduction activities in favor of oxidation. If that is so, it would explain the retention of the dye in lepromatous and borderline leprosy, although not necessarily its chemical or colloidal fixation to a lipid factor. Nevertheless, further investigations may reveal that the phenomenon of fixation is also connected with an aberrant oxidation process, although the exact aberration of the enzymatic functions may have to await determination. We can say with certainty, however, that lepromatous leprosy, as well as rat leprosy, and xanthomatosis also, have in common an enzymatic aberration that is intimately connected with the positive reaction in the methylene blue test.

Studies are under way to determine the affinity of various lipids for methylene blue.

SUMMARY

The methylene blue test has been studied in laboratory animals with lesions produced experimentally by inoculation with material derived originally from lepromatous and borderline leprosy. A similar study has been made in rats with cutaneous lesions from *M. lepraemurium* and in mice with visceral lesions from the same pathogen. In all cases the study was made in comparison with control groups.

In all the inoculated animals a blue coloration appeared in the infiltrated tissue, but in the control groups no such retention of dye could be observed. The intensity of the blue tinge was proportional to the doses of dye injected.

The cutaneous lesions of murine leprosy (ulcers and nodules) gave weaker positive reactions than peritoneal lesions of the same origin or dermal lesions resulting from the experimental inoculation of leprotic material of human origin.

It is suggested that one or both of two

aberrant functions may be instrumental in bringing about the positive methylene blue test, viz., (a) an aberration in the oxidation-reduction favoring oxidation and inhibiting reduction, and/or (b) a combination of the dye with a lipid peculiar to lepromatous leprosy and xanthomatosis.

RESUMEN

La prueba de azul de metileno ha sido estudiada en animales de laboratorio con lesiones producidas experimentalmente por inoculaciones con material proveniente originalmente de lepra lepromatosa y borderline. Un estudio similar se ha hecho en ratones con lesiones cutáneas de *M. lepraemurium* y en ratones con lesiones viscerales con el mismo germen patógeno. En todos los casos el estudio se hizo en comparación con grupos de control.

En todos los animales inoculados una coloración azul apareció en el tejido infiltrado, pero en los grupos de control no se observó la retención del colorante. La intensidad del tinte azul fue proporcional a la dosis del colorante inyectado.

Las lesiones cutáneas de lepra murina (úlceras y nódulos) dieron reacciones positivas mas débiles que las lesiones peritoneales de igual origen o lesiones dérmicas resultantes de la inoculación experimental de material leprotico de origen humano.

Se sugiere que una o ambas de las dos funciones aberrantes pueden ser la causa de producir la reacción positiva del prueba de azul de metileno, ej., (a) una alteración en la función de óxido-reducción favoreciendo la oxidación e inhibiendo la reducción, y/o (b) una combinación del colorante con un lípido peculiar de la lepra lepromatosa y de la xantomatosis.

RÉSUMÉ

L'épreuve au bleu de méthylène a été étudiée chez des animaux de laboratoire présentant des lésions provoquées expérimentalement à la suite de l'inoculation de produits obtenus originellement chez des sujets atteints de lèpre lépromateuse ou borderline (dimorphe). Une étude similaire a été menée chez des rats atteints de lésions à *M. lepraemurium*, ainsi que chez des souris souffrant de lésions viscérales causées par le même agent pathogène. Chacune de ces études a été faite en se référant à des groupes témoins.

Chez tous les animaux inoculés, une coloration bleue est apparue dans le tissu infiltré. Par contre, dans les groupes témoins, aucune rétention semblable du produit colorant n'a pu être observée. L'intensité de la teinte bleue était proportionnelle aux doses de colorant qui avaient été injectées.

Les lésions cutanées de lèpre murine (ulcères et nodules) ont donné des réactions plus faiblement positives que les lésions péritonéales de même origine ou que les lésions dermiques résultant de l'inoculation expérimentale de matériel lépreux d'origine humaine.

On suggère que l'épreuve positive au bleu de méthylène pourrait dépendre d'une atteinte au niveau de l'un ou l'autre des systèmes suivants, ou bien des deux à la fois, qui fonctionneraient de façon aberrante: (a) le système d'oxydo-réduction, qui pourrait montrer un mal fonctionnement favorisant l'oxydation et inhibant la réduction, (b) une combinaison du colorant avec un lipide particulier à la lèpre lépromateuse et à la xanthomatose.

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