

The Antileprosy Agent B.663 (Clofazimine) and the Reticuloendothelial System

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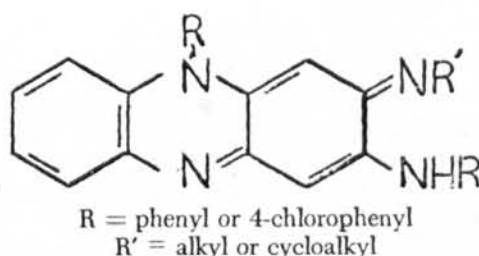
Our investigations into the chemotherapy of mycobacterial disease were begun in 1944. Four years later we announced⁽⁴⁾ the marked activity *in vitro* and *in vivo* of *Anilinoaposafranine* against *M. tuberculosis*. After years of explorative synthesis, a related series of phenazine derivatives (rimino-phenazines) showing remarkable antimycobacterial activity in experimental animals was discovered. One of the most active of these compounds -3-(p-chloroanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-(isopropylimino) phenazine (B.663)⁽³⁾ has been the subject of much investigation. Later we showed that B.663 and some analogous compounds on oral administration concentrated in cells of the reticuloendothelial system (RES) and their remarkable behavior in this regard suggested to us that they might "have an important role in the prophylaxis and treatment of tuberculosis and leprosy"⁽⁵⁾. The course of the above researches has recently been summarized⁽²⁾.

As members attending this symposium are well aware, B.663, or Clofazimine, as it is termed in the formulation Lamprene being marketed by Geigy of Basle, is, following the early work of Browne and Hogerzeil^(13, 14) and later others, now established as having an important role in the treatment of leprosy. It has also therapeutic significance in the treatment of Buruli ulcer due to *Mycobacterium ulcerans*⁽²⁷⁾.

It is not our purpose to-day to dwell on the clinical aspects of B.663—indeed we are not equipped to do so—but rather we wish to describe some experimental findings which we consider might be of interest, and of help to the clinicians working in the leprosy field and also to those workers

interested in the mycobacterial diseases generally and in certain facets of immunology.

Although in this paper we shall for the most part confine ourselves to B.663 because of its clinical importance reference will also be made to some others of the more than 200 related compounds which we have synthesized and examined, and which have features of particular interest. These are represented by the following general formula:-



EXPERIMENTAL RESULTS

Light microscope findings. A characteristic feature of these rimino-phenazines is that they are concentrated, in various degrees depending on their structure, within cells of the reticuloendothelial system and this occurs irrespective of the route of administration. The characteristic sequence following oral administration, as seen in the light microscope, has been published previously⁽¹⁹⁻²¹⁾. Briefly, these compounds appear initially in the macrophage cytoplasm in orange-red bodies 0.5-2 μ in diameter (Fig. 1), the time of appearance depending on the drug concentration in the diet and the chemical structure of the compound. Macrophages throughout the body are involved in this uptake but the actual time interval at which these inclusions or drug-containing phagosomes are first seen varies according to the organ. For example, the lung is one of the later tissues to show them. As administration of the more

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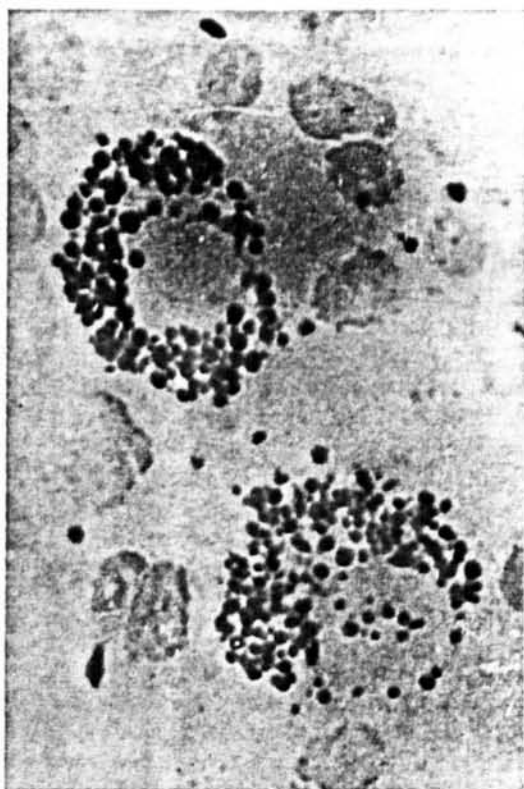


FIG. 1. Two macrophages in an unstained lung smear from a mouse treated with B.663 (0.05% in diet) for 14 days showing inclusions of B.663.



FIG. 2. Macrophage in an unstained lung smear from a mouse treated with B.663 (0.05% in diet) for 14 days showing gradual replacement of drug inclusions by crystals of B.663. (Reprinted from J. M. Dickinson, *Tubercle* 50 (1969) Supplement p. 23, with permission of E. and S. Livingstone, publishers of *Tubercle*).

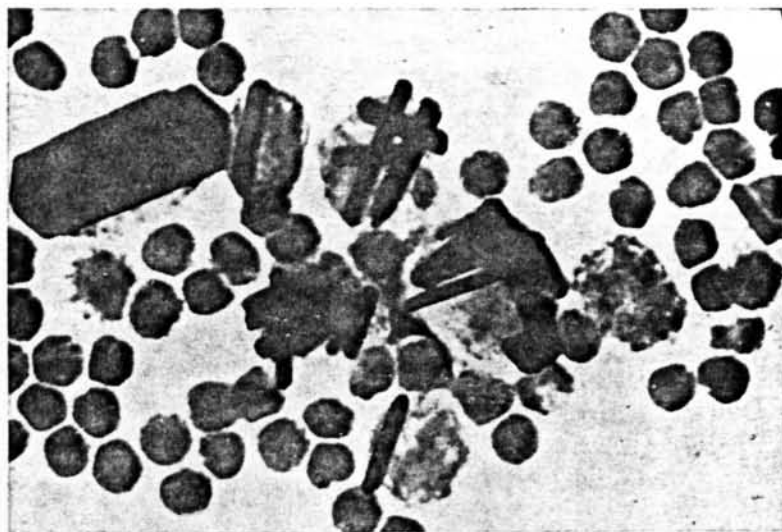


FIG. 3. Macrophage from mouse peritoneal fluid (heat-fixed, aqueous Giemsa stain) showing the variation in the size and number of crystals of B.663 per cell following 48 days' treatment with B.663 (0.05% in diet). Note absence of inclusions. (Reprinted from M. L. Conalty and R. D. Jackson, *British Journal of Experimental Pathology*, 43 (1962) following p. 652, Fig. 3, with permission of the Editor of that journal).

lipophilic rimino-phenazines is continued, the phagosomes darken in color and within many of them areas of greater density may be seen. This phagosomal stage is followed within days by the appearance of bright-red crystals together with the phagosomes (Fig. 2), and gradually such cells come to contain crystals only (Fig. 3). Methods of fixation or staining, employing alcohol, acids or lipid solvents, remove the drug, leaving the "ghosts" of the crystals behind (Fig. 4).

Electron microscope findings. In more recent work we have been able to follow this process of intracellular crystallization by means of the electron microscope. On using standard osmium tetroxide fixation it was found that the colored phagosomal contents did not dissolve out during fixation and subsequent ethanol dehydration, but that the actual drug crystals did so. The electron-dense bodies, corresponding to the colored inclusions seen by means of the light microscope, varied in size and in electron density (Fig. 5). Some of these smaller bodies appeared to fuse with other similar but larger bodies; in some instances fusion of electron-dense bodies of uniform opacity with other less dense bodies of more "granular" appearance was observed. The fusions would appear to correspond to the fusion of phagosome and lysosome as described by

de Duve (²⁴). These cytoplasmic inclusion bodies (phagosomes, lysosomes and phagolysosomes) increase in number, size ($0.2-1.5\mu$), diversity of shape and electron-density as treatment is continued (Fig. 6), and within some bodies electron-transparent areas of early crystallization where the B.663 has dissolved out are seen. Further treatment leads to larger crystals (Fig. 7).

Electrophoretic studies of blood serum of orally treated mice have shown almost complete binding of B.663 to the lipoproteins of the α and β globulin fractions(¹⁵). These lipoprotein-B.663 complexes are then taken up by the macrophages to form the electron-dense phagosomal bodies. Following fusion with lysosomes it would appear that the lipoprotein carrier is split off enzymically from the complex, with the resultant crystallization of the freed B.663.

Origin of lung macrophages. There has been considerable speculation as to the origin of lung macrophages. Nicol and his colleagues (^{22, 29, 30}) have reported that the liver and spleen were the main source of lung macrophages. They also postulated that a normal physiologic method for the elimination of particulate material was the mobilization of macrophages containing such material into the circulation, with subsequent excretion via the alveoli into the

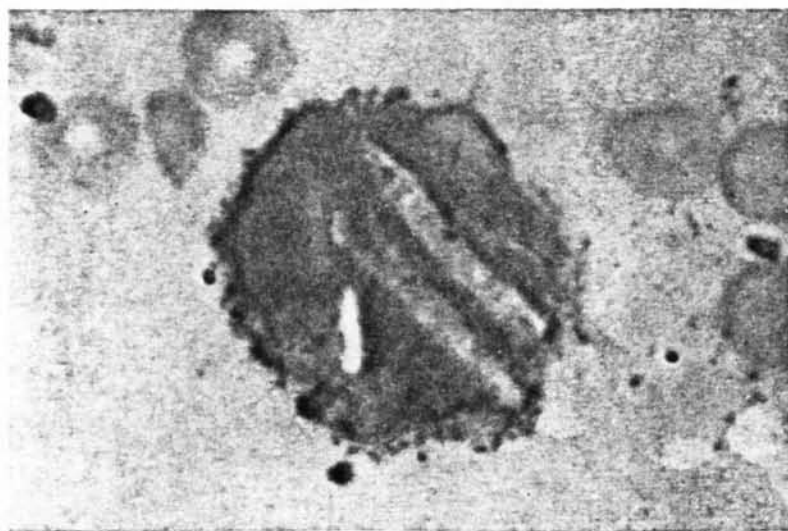


FIG. 4. Macrophage (Giemsa stain) from mouse peritoneal fluid showing the "ghosts" of crystals removed during fixation (48 days treatment, B.663 0.05% in diet). (Reprinted from M. L. Conalty and R. D. Jackson, *British Journal of Experimental Pathology* 43 (1962), following p. 652 with permission of the Editor of that journal).



FIG. 5. Liver of mouse treated with B.663 (0.05% in diet for 7 days). Macrophage showing nucleus (N), mitochondria (M), Golgi apparatus (G), and endoplasmic reticulum (er). Micropinocytic vacuoles (mp), a multivesicular body (mv), dense inclusion bodies (D), with two showing fusion (y), are visible. Hepatic cell mitochondria (MH) and reticular fibers (rf) may also be seen.

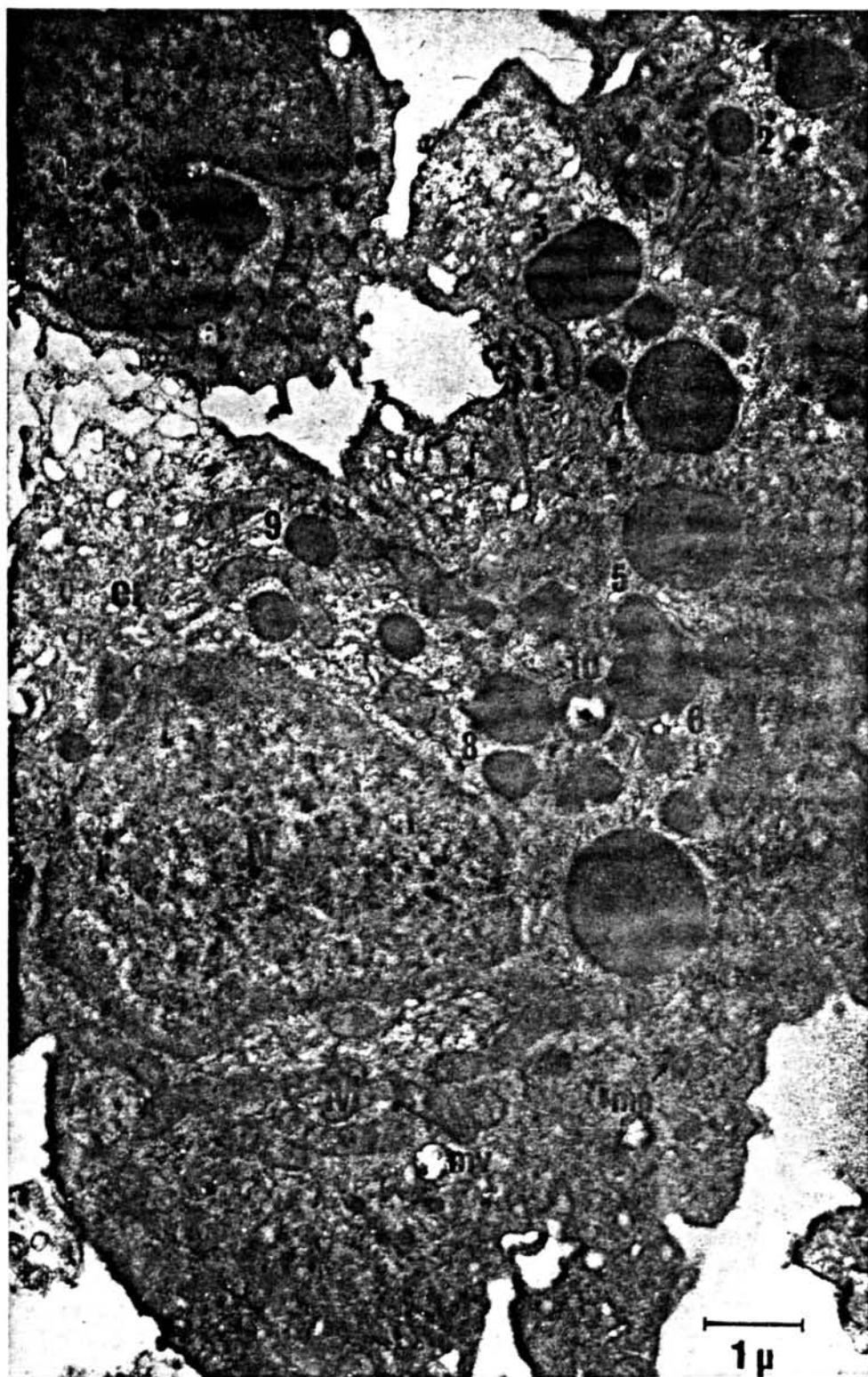


FIG. 6. Mesenteric lymph node of mouse treated with B.663 (0.05% in the diet) for 14 days. Macrophage showing nucleus (N), numerous mitochondria (M), endoplasmic reticulum (er), micropinocytic vacuoles (mp), and a multivesicular body (mv). Dense bodies (1-10) of various shapes, sizes and electron densities can be seen. Phagolysosomes (5,6) are present and near the center of the field there is early crystal formation within a phagolysosome (10). A lymphocyte (L) is also present.



FIG. 7. Mesenteric lymph node of mouse treated with B.663 (0.05% in the diet) for 14 days. Macrophage showing nucleus (N), mitochondria (M), and assorted dense bodies (1-9), some showing fusion (3,8). The outline or "ghost" (C) of a fully formed B.663 crystal (the compound itself has dissolved out during the fixation process) is prominent. An earlier "precystal" stage is also to be seen (pc).

upper respiratory tract. Capell (17) and Pinkett *et al.* (31) considered that a considerable proportion of lung macrophages could be hemopoietic in origin. Bennett (7), using tissue culture technics, and Nelson (28), reviewing the results of metabolic studies, came to the conclusion that lung macrophages were different from others. Some of our studies in mice with the riminophenazines have provided supporting evidence for this.

We found (20) that some analogs of B.663 [Formula I, R = *parachlorophenyl* and R' = cycloalkyl (e.g., cyclohexyl, methyl or dimethyl substituted cyclohexyl, and cycloheptyl)—appeared as short, straight, needle-like crystals in the lung macrophages, whereas elsewhere the crystals were long and curved, short crystals being found, apart from the lung, only in the macrophages in peritoneal fluid.

Additional evidence to support the hypothesis that lung macrophages are "different" is provided by the observation of a different rate of accumulation of B.663 in the lung macrophages. Brief reference has been made to this earlier in this paper. It is found that the inclusion form of B.663 in lung macrophages is not observed until macrophages in other sites are already showing the stage of crystallization and that crystals are not seen in the lung until about five days after the appearance of inclusions, while in other tissues this interval varies from one to three days.

However, we have also noted where mice were treated with B.663 in the diet for six weeks, returned to ordinary diet and then killed, some at seven weeks and others at eleven weeks later, that there was a fall in the mean drug level in livers and spleens during these four weeks whereas the level in the lungs actually increased. This phenomenon would fit in with the migration of macrophages to the lungs as postulated by Nicol and his colleagues. We hope to deal with this in more detail in a future publication.

Chemotherapeutic and chemoprophylactic activity. It is hardly an exaggeration to regard leprosy as essentially a disease of

the reticuloendothelial system. Therefore the coincidence of intracellular leprosy bacilli and intracellular B.663 is indeed a happy one. All the evidence—chemoprophylactic activity in tuberculosis of mice (6, 16) and hamsters (34) (essentially an intracellular infection), *M. lepraemurium* infection in mice (also an intracellular infection) (18) and particularly the chemotherapeutic response in leprosy of humans—points to the availability of the intracellular drug for the intracellular bacilli. This is unlike the situation which has been observed in the case of *Brucella* infection, where intracellular bacilli are sheltered from likewise intracellular tetracycline (23).

One is encouraged to speculate that the peculiar affinity of the RES for B.663 is a property which could perhaps be exploited further. We see therapy as taking the form of loading the RES by a relatively short term of intensive therapy and thereafter as the provision of much lower doses to replace excreted drug and load newly emerged macrophages. Possibly these doses could be given at intervals. We would like to see some clinician exploring this possibility.

Phagocytic activity. In an attempt to determine the proportion of macrophages involved in B.663 uptake, a group of mice was injected intravenously with colloidal carbon following 13 days' treatment with B.663 at 0.05 percent in the diet, and liver, spleen and lung smears were examined. The results are shown in Table 1.

TABLE 1. Proportion of macrophages in B.663-treated mice showing carbon following intravenous injection.

Macrophage contents			
Organ	Carbon only	Carbon plus B.663	B.663 only
Liver	21%	79%	0%
Spleen	7	93	0
Lung	100		0

In the case of the lung it was not always possible to differentiate cells containing dust (the mice were fed a powdered diet) from those containing carbon, but, as in the case of the liver and spleen, none of the B.663-containing cells was free from carbon or dust. It seems legitimate to conclude also that the cells which contained carbon only, or in the case of the lungs carbon and/or dust, are younger macrophages which had been exposed to B. 663 for a time inadequate to permit the accumulation of visible quantities of B.663. (In other work we have reported that B.663-laden macrophages are capable of engulfing *Mycobacterium tuberculosis*)⁽²⁰⁾.

Clearly a quantitative assessment of phagocytic activity was indicated and this we have made, using the carbon-clearance technique of Biozzi, Benacerraf and Halpern⁽⁸⁾ in which mice are given a colloidal suspension of carbon intravenously (160 mgm. carbon per kgm. bodyweight) and the blood carbon levels are measured at intervals thereafter. The logs of the carbon concentrations plotted against time provide a linear regression coefficient which is taken as the *Global Phagocytic Index K*. This is a convenient measure of total phagocytic activity. A further index, the *Unit Phagocytic Index* α , where account is taken of splenic and hepatic hypertrophy, is derived from *K* as follows:-

$$\alpha = \frac{Wb}{Wls} \sqrt[3]{K}$$

Wb = weight of body

Wls = combined liver and spleen weights.

For convenience we also include the $T(\frac{1}{2})^c = \frac{\log 2}{K}$, which is the time in minutes for the carbon concentration to be halved.

Our findings for mice treated via the diet at the rate of 50 mgm./kgm. B. 663 approximately daily, are set out graphically in Figs. 8 and 9. It will be seen (Fig. 8) that over the 29 weeks of treatment, while there is a progressive fall in the *K* value for the controls due to ageing⁽¹⁾, there is a very much greater fall in *K* value for the treated, the difference becoming significant at the 0.001 level as early as the fifth week

of treatment. This is also seen in the $T(\frac{1}{2})^c$ value. The profound depression in phagocytosis is likewise apparent when considered on the unit basis (Fig. 9), and at the same high degree of significance as early as the third week.

We have also examined for recovery in phagocytic activity, following its depression resulting from six weeks of treatment as above. These findings are set out in Figs. 10 and 11. It is clear (Fig. 10) that there was no substantial improvement during the first five weeks after stopping the administration of B.663, but that by the 11th week the *K* and $T(\frac{1}{2})^c$ values had returned to control levels but not so the α value (Fig. 11).

That there should be reduced phagocytic activity in B.663-"loaded" animals is perhaps not unexpected in view of the obvious curtailment of intra-macrophage space by the sequestered drug.

Immunological aspects. Naturally these findings of depressed phagocytic activity have intrigued us, particularly in view of the protective effect of B.663 against *erythema nodosum leprosum* (ENL) reactions in leprosy patients^(11, 12, 25, 26). These clinical findings suggest that antigens probably responsible for the ENL reaction belong to that group of antigens which require processing by macrophages in order to stimulate antigen-sensitive, thymic-dependent lymphocytes⁽³³⁾, and that the B.663 blocking effect prevents this processing. In this context also the possibility that B.663 might be of use in autoimmune diseases of the organ-specific type, such as Hashimoto's thyroiditis, must also be considered.

B.663 has also been shown to have anti-inflammatory activity in the experimental animal^(34, 37). It appears possible that the great diversion of lysosomes to deal with phagosomal-B.663 may be the key to such activity. Also, bearing in mind the effect of the intracellular antimycobacterial polyoxyethylene derivatives of tertiary octyl phenols on lysosomal activity⁽¹⁰⁾ the possibility of a direct effect of B.663 on the lysosomes cannot be discounted.

Incidentally, although one of these compounds, macrocyclon, failed in clinical tuberculosis⁽⁹⁾ and in leprosy⁽³⁸⁾, anti-

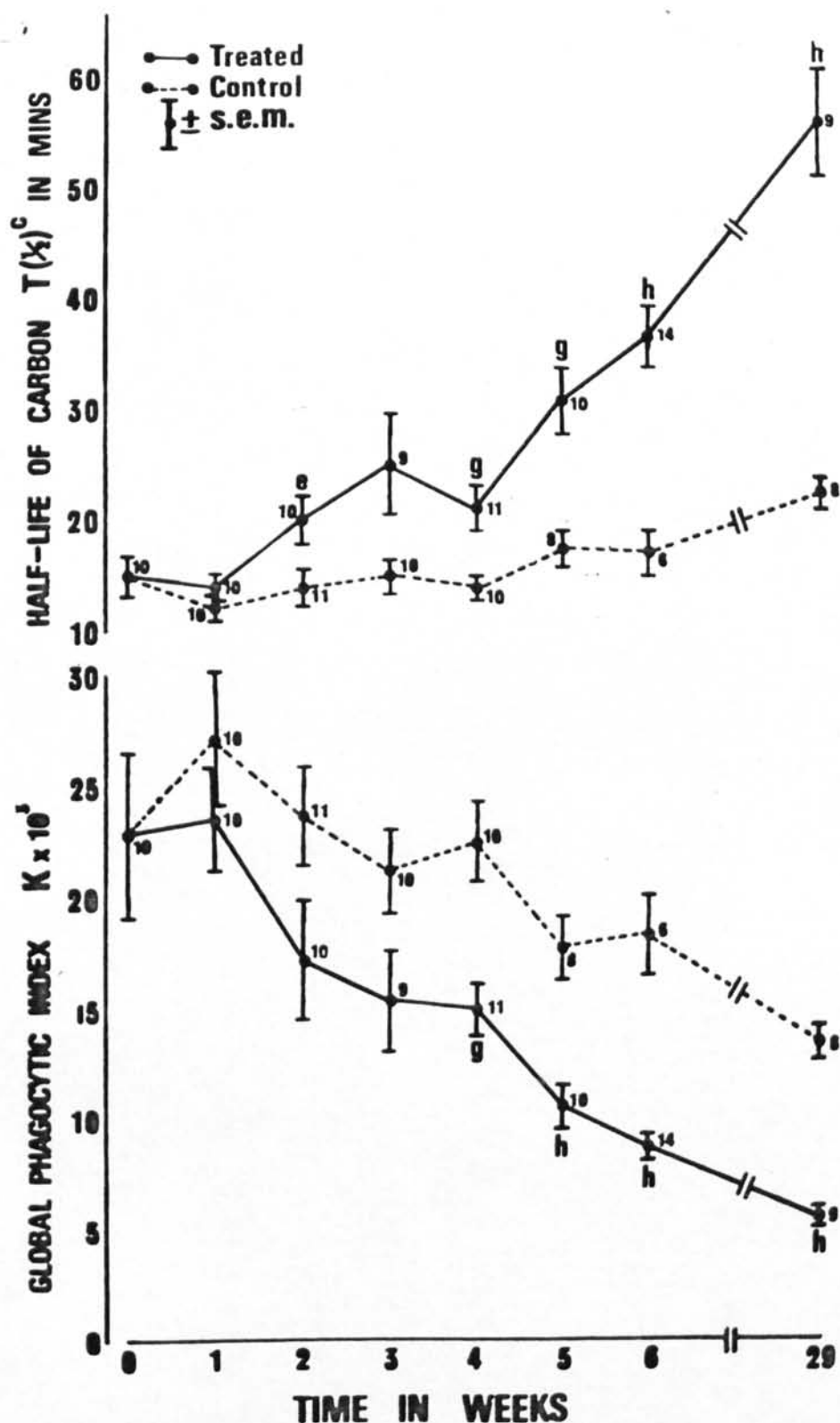


FIG. 8. K and T(1/2)^c indices of carbon clearance in mice during a 29 week course of B.663 (0.05% in the diet). The numerals indicate the number of mice on which each mean index is based and the letters indicate the degree of significance ("t" test) of the difference between the mean index of the test group and that of the corresponding control group (e = p < 0.05, f = p < 0.02, g = p < 0.01, h = p < 0.001) and the vertical line through each point denotes the limits of ± one standard error of that mean.

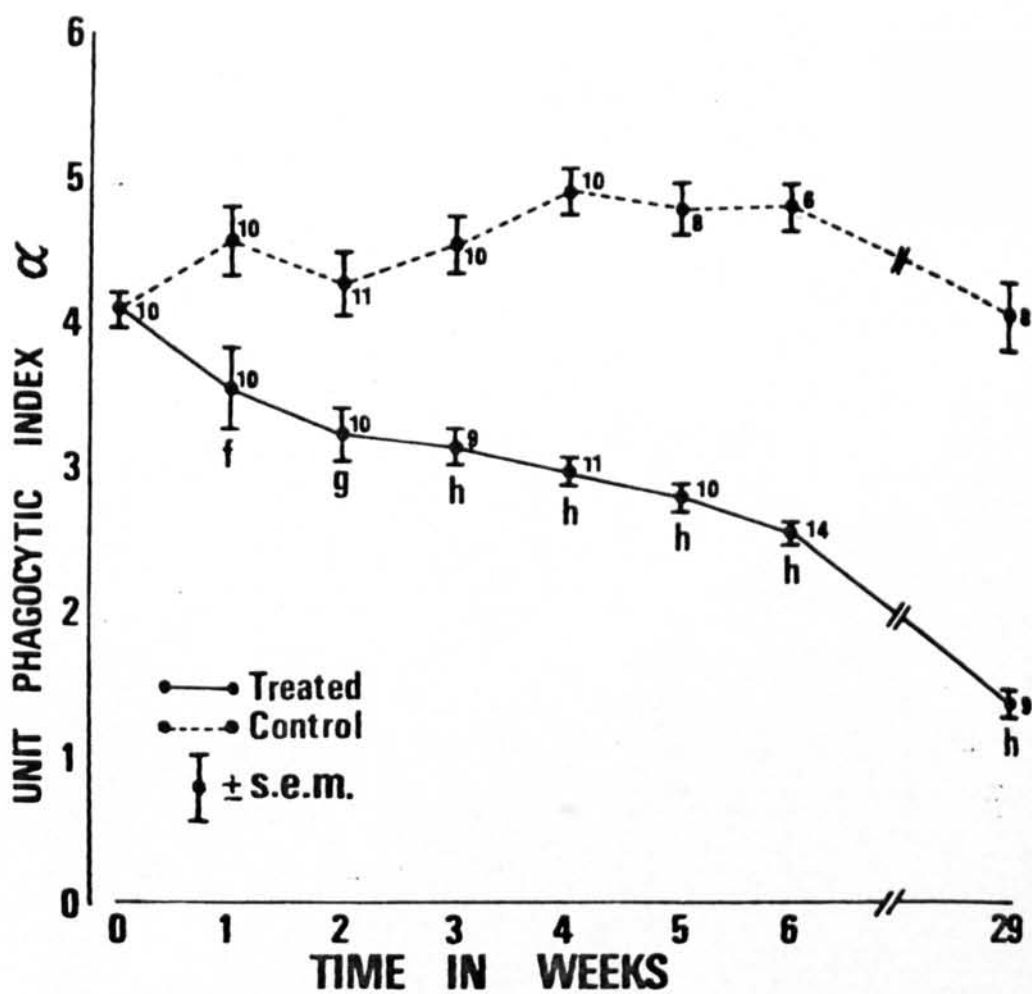


FIG. 9. Unit phagocytic index, α , of carbon clearance in the same mice as in Fig. 8. Legends as in Fig. 8.

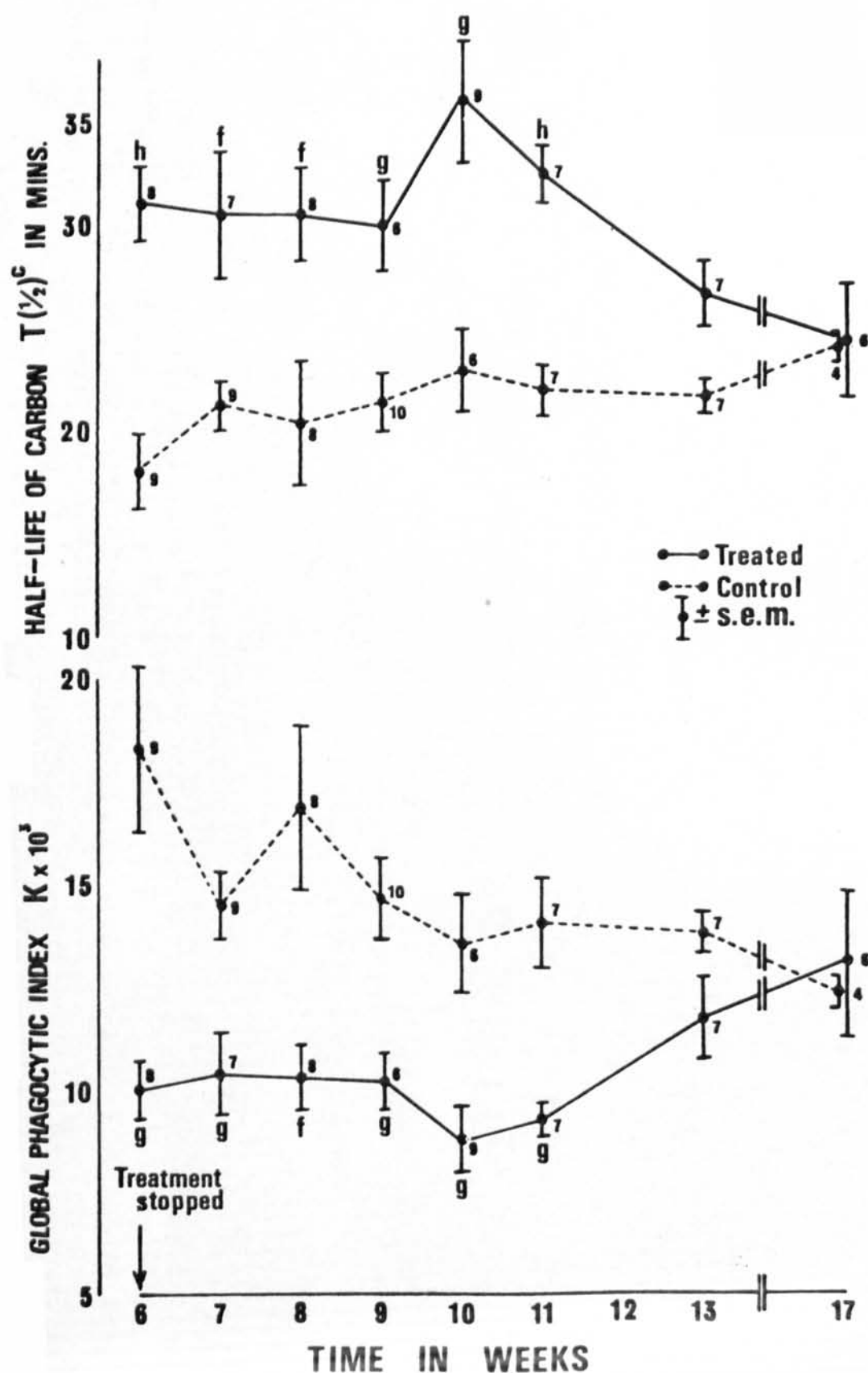


FIG. 10. K and $T(1/2)^c$ indices of mice examined at intervals following completion of a six weeks course of B.663 (0.05% in the diet). Legends as in Fig. 8.

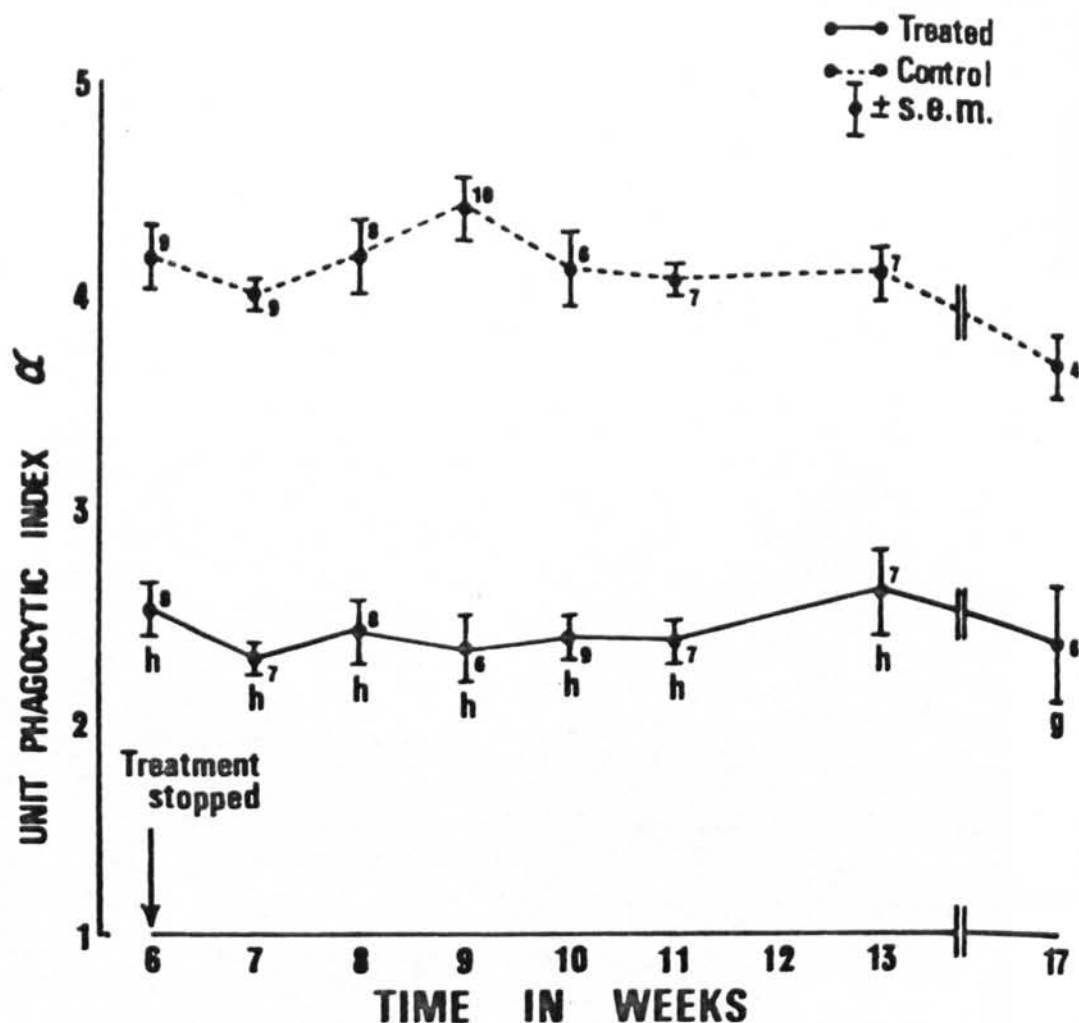


FIG. 11. Unit phagocytic index, α , in the same mice as in Fig. 10. Legends as in Fig. 8.

ENL activity in leprosy does not appear to have been thoroughly investigated, although there was a suggestion of such activity in one trial⁽³⁶⁾.

Recently antilymphocytic serum (ALS), administered intravenously in mice, has been shown by Sheagren, Barth, Edelin and Malmgren⁽³³⁾ to produce what they describe as a "profound" impairment of carbon clearance in mice, most marked at 48 hours, where the half-time for clearance was approximately doubled. These workers speculated whether the dose of concomi-

tantly administered drugs cleared by the RES might require reduction or if the blocking activity might help to explain potentiation of immunosuppressive drugs by ALS. Yet this blocking effect looks much less profound when compared with that which we have shown may be obtained with B.663. These very same points are, therefore, possibly even more relevant in the case of B.663.

All of these considerations have caused us to continue and expand our examination of B.663 and its analogs.

SUMMARY

The antimycobacterial agent B.663 (Clofazimine), particularly active in human leprosy and in Buruli ulcer due to *Mycobacterium ulcerans*, is selectively taken up by cells of the reticuloendothelial system (RES) to the extent that intracellular crystals of the drug are eventually formed. The stages in this process are illustrated by means of bright-field and phase-contrast photomicrographs and by electron photomicrographs, and the possible mechanism of intracellular concentration is discussed. The origin of lung macrophages is discussed in the light of the findings with B.663 and with related compounds.

The effect of the accumulated drug on phagocytic function has been investigated quantitatively in mice by means of carbon clearance technics. Notwithstanding the significant depression of both global and unit phagocytic function, which was observed with high doses of B.663, excellent results were obtained in mycobacterial infections. Prophylactic experiments showed that the intracellular drug was therapeutically significant. The possible mechanisms by which B.663 exerts its anti-ENL activity are discussed.

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