

Full Length Research Paper

In vitro* thalidomide does not interfere with the activation of complement by *Mycobacterium leprae

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One inflammatory event that may be involved in erythema nodosum leprosum (ENL) is the activation of complement, and thalidomide could suppress ENL by inhibiting its activation. To determine if thalidomide inhibits the activation of complement, we first incubated normal serum with *Mycobacterium leprae* or with zymosan in the presence of thalidomide. Residual functional complement was then determined by defining the dilution of the serum required to lyse 50% of the rabbit antibody sensitized sheep cells (CH50 Assay). Zymosan and *M. leprae* activated complement. The CH50 values in the serum incubated with *M. leprae* or with zymosan were equivalent to the CH50 values in the serum incubated with *M. leprae* or zymosan in the presence of thalidomide. Thalidomide did not disrupt the activation of complement by zymosan, a known initiator of complement activation by the alternative pathway, or by *M. leprae*.

Key words: ENL, thalidomide, complement.

INTRODUCTION

Thalidomide's mechanism of arresting ENL is not entirely known, nor is the factor(s) that precipitate ENL. Early ENL lesions show fragmented forms of *Mycobacterium leprae* (Job et al., 1964) with a preponderance of granular over solid staining acid fast bacteria compared to LL lesions without reaction Ridley (1960). There are also scattered accumulations of foamy macrophages in an advanced state of decay and varying numbers on neutrophils Ridley, MJ, and DS Ridley (1983). It has been proposed that the release of mycobacterium antigens from infected macrophages may be a factor that initiates ENL (Hastings, 1980; Naafs, 2000). The released antigen may then complex with antibodies initiating complement fixation by the classical pathway, or the released microbial constituents may activate complement by the alternative pathway. Regardless of which pathway of complement activation is involved both pathways lead to the activation of C3 with the subsequent formation of peptides that are chemotactic for neutrophils.

Direct experimental evidence for the involvement of complement in ENL was provided by Wemambu et al.

(1969). They described deposits of immunoglobulin and complement in acute cutaneous ENL lesions. *In vivo* activation of complement during ENL had also been described by Bjorvatn et al. (1976). They demonstrated significantly increased levels of C3b in the plasma of 70% of patients with ENL, but in only 18% in of patients not in reaction. Additionally, analysis of C1q precipitins in gel diffusion and C1`q binding have suggested that there are immune complexes and even immune complexes containing mycobacterium antigens in sera from patients with lepromatous leprosy. Saha et al. (1983) suggested that complement activation during ENL most likely occurs by the alternative pathway. Sera from 20 patients with ENL were collected at the first visit, and 4 weeks after successful therapy. After assaying the 20 paired samples they found that the C3 level after remission of ENL rose, ranging from 13 - 22% during therapy. Serum levels of C1q and C4 did not alter significantly during ENL and also showed no difference in patients on ENL therapy.

To activate complement we used zymosan, a known initiator of C3 activation by the alternative pathway, and *M. leprae*. The *M. leprae*, as prepared by the Laboratory Research Branch, National Hansen's Programs at LSU from nu/nu mice. They are very efficient in complement fixation, Lahari (2008) findings that support a hypothesis linking the killing and breakdown of intact leprosy bacilli

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and the release of breakdown mycobacteria products as a factor that initiates ENL. As the hemolytic CH50 assays provide insight into the integrity of the cascading events that culminate in the membrane attack complex, we investigated if thalidomide could interfere with the complement mediated lyses of hemolysin sensitized sheep erythrocytes. To do this, we incubated the thalidomide *M. leprae* or-thalidomide-zymosan-treated serum with sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibodies and assayed for release of hemoglobin. The dilution of the treated serum required to lyse 50% of hemolysin sensitized sheep cells (CH'50 Titer from the macro-assay or the CH50 Units from the micro-assay) were determined.

The complement remaining for the secondary hemolytic assay is proportional to the complement that was not consumed in the primary incubation.

We showed that zymosan and *M. leprae* can activate complement. The CH50 values in the *M. leprae* activated serum or zymosan activated serum were equivalent to the CH50 values in the serum treated with *M. leprae* or zymosan in the presence of thalidomide. Thalidomide does not appear to impede complement activation.

MATERIALS AND METHODS

Thalidomide

Thalidomide, kindly provided by Celgene Corporation (Summit, NJ), was dissolved in DMSO or in an aqueous solution with a pH 3.0 (acid saline) [to maintain intact thalidomide] or an aqueous solution 0.85% NaCl with a pH 8.0 (alkali saline) [to induce hydrolysis of thalidomide] Shannon *et al* (1997). Aliquots of the aqueous preparations of thalidomide were frozen at -80° C and discarded after one freeze-thaw cycle. The final concentrations on thalidomide in the assays ranged from 4.0 - 0.25 µg/ml.

Metabolites of thalidomide were generated, as described previously, Shannon and Sandoval (2008). The reagents consisted of BD Gentest™ cytosol and microsomes from human liver (S9 fraction) and NADPH regenerating solutions (BD Biosciences Woburn, MA).

Activators of complement

Zymosan was purchased from Sigma. Fifty mg was added into 5 ml of Hanks Balanced Salt Solution and boiled in a 125 - 150°C mineral oil bath for 10 min. The suspension was centrifuged and the supernatant discarded.

Mycobacterium leprae

The Thai-53 isolate of *Mycobacterium leprae* was grown in the foot pads of athymic nu/nu mice, and harvested as described previously Truman and Krahenbuhl (2005). A suspension was prepared for staining and the acid fast bacteria (AFB) were enumerated. The suspension was adjusted to contain 1×10^9 AFB /ml of RPMI and labeled as crude-viable-intact *M. leprae*. One aliquot of the crude-viable-intact *M. leprae* was treated with 0.1 N NaOH as described by Lahiri *et al.* (2005). The crude-viable-intact *M. leprae* was also treated with alkali and disrupted by sonication and identified as

pure-alkali-treated-sonicated *M. leprae*.

Complement assays

The CH50 Titters from the serum of healthy donors was treated with thalidomide and zymosan as described by Mayer 1961 (see protocol 1). Zymosan was incubated with the test serum or with saline. For the macro-assay containing thalidomide the serum and zymosan were incubated with saline containing thalidomide. The final concentrations of thalidomide ranged from 0.25 µg/ml (10^{-7} M) to 2.5 µg/ml (10^{-5} M) in 5% v/v DMSO. The vessels were centrifuged and the supernatant diluted in complement buffer (Mayer, 1961). One milliliter with antibody sensitized sheep erythrocytes was incubated with various dilutions of the thalidomide-zymosan treated test serum. The reaction vessels were incubated for 90 min at 37°C, centrifuged and the optical density of the supernatant was determined at 542 nm.

In the assays that used various preparations of *M. leprae* to activate complement, the CH50 Units were determined in a micro-assay by a modification of the EZ Complement CH50 Test (Diamedix Corporation, Miami FL) (see protocol 2). The test kits and sera with a high CH50 value were purchased from Diamedix. To study the effects of alkali induced hydrolysis of thalidomide, sera was incubated with various preparations of *M. leprae*. Thalidomide treated with alkali was added for a final concentration of 4.0 µg/ml. The preparations were incubated for 1 h at 33°C. The supernatants were incubated with sheep erythrocytes sensitized with hemolysin, and the absorbance was determined at 415 nm.

Statistical analysis

The Mann Whitney or the Kruskal-Wallis tests were performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, and SanDiego CA. $P < 0.05$ was taken as significant.

RESULTS

Zymosan was used to activate complement in the serum collected from healthy lab personnel. Thalidomide, dissolved in DMSO, was in concentrations that are within the range of that achieved in the serum of healthy individuals following ingestion of thalidomide (Eriksson, 1997; Shannon *et al.*, 2007). The CH'50 Titer of the serum was determined and the data are illustrated in Figure 1. Compared to the serum treated with DMSO (solvent control) there was no difference in the CH'50 Titer of the serum treated with thalidomide prepared in DMSO.

The preparation of *M. leprae* that was the most efficient in activating complement was the viable intact *M. leprae*. Using the sera with a high CH50 value purchased from Diamedix, we assayed for a dilution of the crude-viable intact *M. leprae* that would result in approximately 50% of its maximum capacity to activate complement. A dilution of 1:6 was determined (Figure 2). We used a 1:6 dilution of this preparation as a positive control for the activation of complement. We used pure-alkali-treated-sonicated *M. leprae* to determine the effects of disruption of integral *M. leprae* of the activation of complement. The effect of thalidomide hydrolyzed by treatment with alkali on

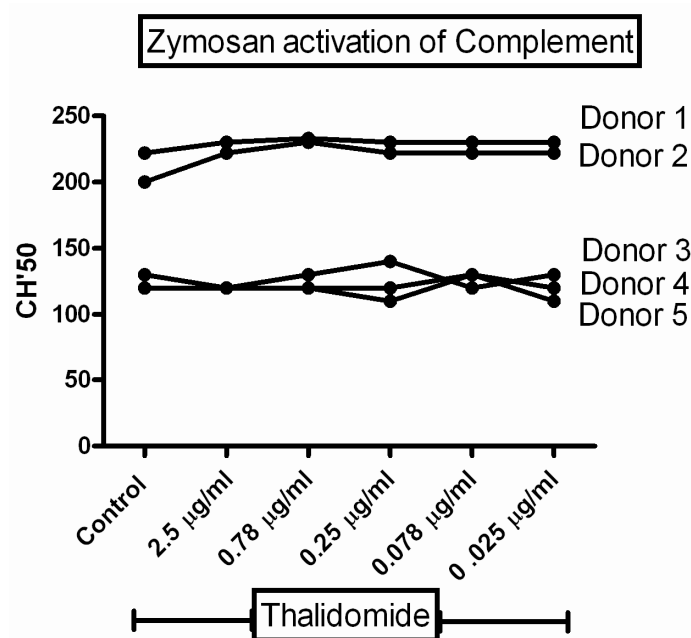


Figure 1. CH'50 titer after activating complement with zymosan in the presence of thalidomide.

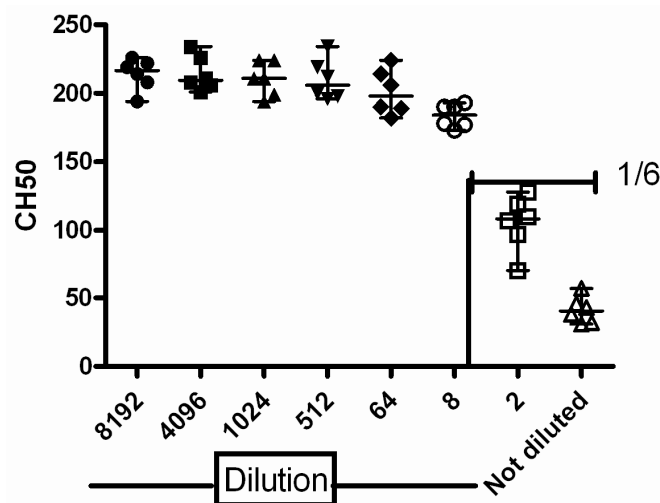


Figure 2. Titration of crude viable intact *M.leprae* for CH50 activity.

complement activated by these two preparations of *M. leprae* is illustrated in Figure 3. Comparing the solvent controls to the thalidomide treated samples; none of the *M. leprae* preparations altered the fixation of complement (Mann Whitney, test).

Thalidomide was incubated with activated human liver enzymes and after this treatment the metabolites were incubated with sera with a known CH50 value (Figure 4). After ranking the data from low to high and then analyzing the distribution by the Kruskal-Wallis test the

group that did not contain *M. leprae* was significantly different the other groups ($p = 0.012$).

DISCUSSION

One event that may initiate ENL is the discharge of *M. leprae* from infected macrophages into the extracellular environment. Thalidomide could interfere with the killing of *M. leprae*, or the release of *M. leprae* debris from

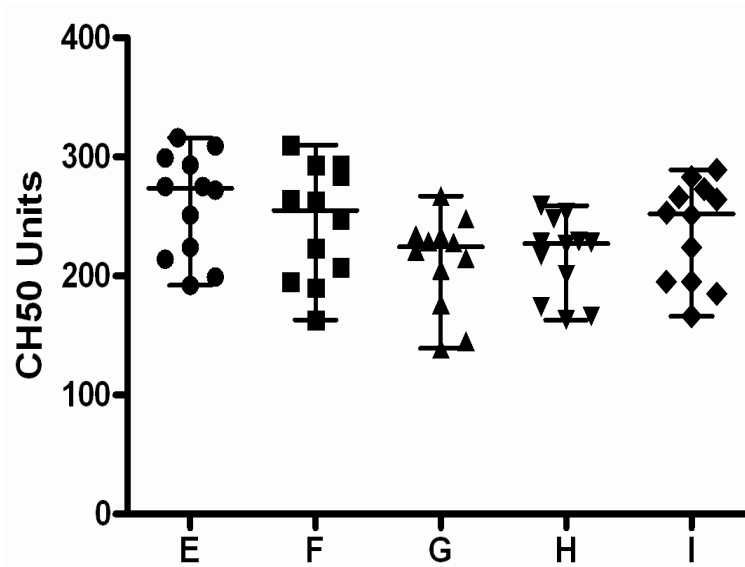


Figure 3. CH50 Units after incubating *M. leprae* with non-immune human sera and 4.0 µg/ml of thalidomide or alkali treated thalidomide; (E) Crude-viable-intact *M. leprae* + Hydrolysis products induced by treating thalidomide with alkali (Median and range); (F) Crude-viable-intact *M. leprae* + alkali (solvent control for E) E vs. F, Mann Whitney, $p = 0.37$; (G) Pure-alkali-treated-sonicated *M. leprae* + thalidomide; (H) Pure-alkali-treated-sonicated *leprae* + alkali (solvent control for G) G vs. H, Mann Whitney, $p = 0.97$. I. Pure-alkali-treated-sonicated *M. leprae*.

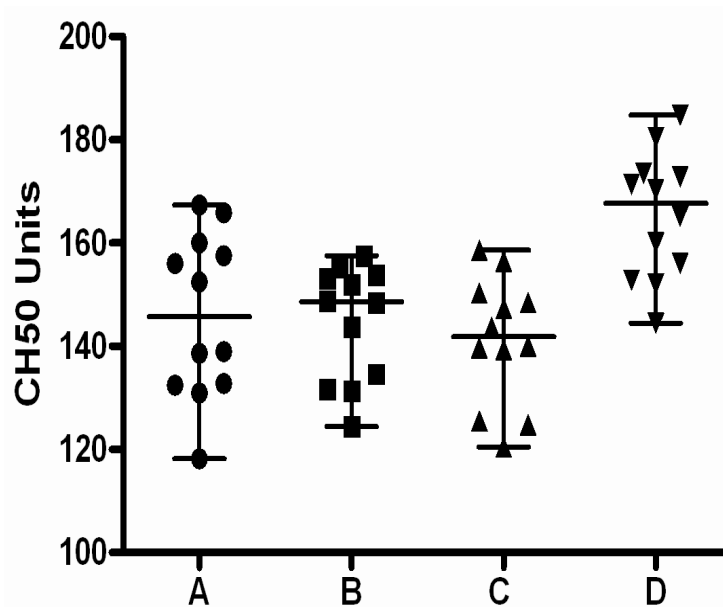


Figure 4. CH50 Units after incubating *M. leprae* with non-immune human sera and 4.0 µg/ml of thalidomide or thalidomide treated with activated liver enzymes; A) Crude-viable-intact *M. leprae* (Median and Range); B) Pure-alkali-treated-sonicated *M. leprae* + thalidomide; C) Pure-alkali-treated-sonicated *leprae* + metabolites of thalidomide derived by incubating thalidomide with liver activated enzymes. A vs. B vs. C Kruskal-Wallis test $p = 0.707$; D) *M. leprae* not present. Test sera treated with thalidomide and liver activated enzymes. A vs. B vs. C vs. D, Kruskal-Wallis test, $p = 0.012$.

tissue macrophages phagolysosomes. These actions would suppress the activation of complement by microbial antigens in immune complexes or microbial debris capable of activating complement by the alternate pathway.

Thalidomide at concentrations as high as 10 µg/ml does not have an effect on the ability of highly viable nu/nu mouse derived *M. leprae* to oxidize 1-¹⁴C palmitic acid (unpublished observations), and it does not kill *M. leprae* in resting or INF-γ activated mouse macrophages harboring *M. leprae* (Tadesse and Shannon, 2005). These findings suggest that thalidomide does not affect the viability of *M. leprae*, a possible prelude to the release of mycobacterial antigens or debris from infected macrophages. It is possible that thalidomide could inhibit ENL by stabilizing macrophage phagolysosomes, and thereby prevent the liberation of microbial activators of complement into the extracellular environment. Hastings (1971) showed that thalidomide prevented the release of β-glycerophosphatase from lysosomes isolated from rat and human livers and incubated in an osmotic ally protected acid medium. Tadesse and Shannon (2004) showed that thalidomide protected red blood cells in plasma free blood from lysis by hypotonic solutions of saline. Thus, part of the mechanism of action of thalidomide in ENL may involve stabilizing the membranes of phagocytic cells, or retarding the release of antigens from phagolysosomes of macrophages.

Thalidomide is a low molecular weight, non-polar, pH labile molecule. Upon ingestion it undergoes hydrolysis by changes in the pH in the gastrointestinal tract and by enzymatic breakdown within cells. In its native state it can enter any cell in the body. Once inside a cell it undergoes hydrolysis by changes in pH and by metabolism by enzymes. Which of these molecules, arrest ENL is not known. To attempt to determine which state of thalidomide may be active in complement fixation, we treated the serum with thalidomide or thalidomide transformed by pH dependent hydrolysis or thalidomide bio-transformed by treatment with activated enzymes derived from human liver.

We reported that hydrolysis of thalidomide by exposure to pH > 8.0, abrogates its ability to enhance IL-2 as well as its ability to suppress TNF-α (Shannon et al, 1997). Others have shown that the teratogenic effects of thalidomide can be manifested only after the exposure of thalidomide to liver derived enzymes Fort et al. (2000). For thalidomide to inhibit angiogenesis, it requires metabolic activation by human or rabbit liver microsomes (Bauer et al., 1998). In this study we presented thalidomide to serum in its native state, as well as in a hydrolyzed state, or in an enzyme treated state. We show that complement activation by zymosan or by *M. leprae* was not impeded when the normal serum was treated with the various preparations of thalidomide.

When administered following proper safety guidelines, thalidomide is the treatment of choice for ENL. The anti-inflammatory and immunomodulatory properties ascribed

to thalidomide offer multiple sites for it to act in arresting ENL. It appears that ENL is a hypersensitivity reaction to *M. leprae* having humoral as well as cell-mediated immune components (Hastings, 1980; Naafs, 2000); and the reaction is likely to be the outcome of a complex interaction of immunologic and non-specific anti-inflammatory events. The roles played by antibody, complement, plasma cells, cytokines released by sensitized T-cells and antigen processing B-cells have yet to be elucidated.

In conclusion, racemic thalidomide or thalidomide transformed by hydrolysis or thalidomide bio-transformed by treatment with liver enzymes did not impede the activation of complement by zymosan or *M. leprae*.

PROTOCOL 1

Activation of complement by zymosan

1. Serum from 5 donors + thalidomide (0.25-2.5 µg/ml).
2. Incubate for 30 min at 37°C.
3. Add sheep erythrocytes sensitized with hemolysin (rabbit anti-sheep erythrocyte).
4. Incubate for 90 min at 37°C and collect supernatant.
5. Assay supernatant for release of hemoglobin and determine CH'50 Titer (Figure 1).

PROTOCOL 2

Activation of complement by acid fast bacteria

1. Thalidomide treated with alkali to induce hydrolysis (Figure 3)
2. Thalidomide treated with activated liver enzymes (Figure 4).
3. Add thalidomide treated with alkali or with liver enzymes to serum with a know very high CH 50 titer.
4. Incubate for 1 h at 37°C.
5. Add acid fast bacteria that are know to activate complement (Figure 2).
6. Assay supernatant for release of hemoglobin and determine CH50 Units (Figure 3 and 4).

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REFERENCES

- Bjorvatn B, Barnetson RS, Kronvall G, Zubler RH, Lamber PH (1976). Immune complexes and complement hypercatabolism in patient with leprosy. Clin. Exp. Immuno. 26: 388-396.

- Bauer KS, Dixon DC, Figg WD (1998). Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent. *Biochem. Pharmacol.* 55: 1827-1834.
- Eriksson T (1997). Pharmacokinetics of the enantiomers of thalidomide PhD Thesis. Department of Clinical Pharmacology, Lund University, Lund and Hospital Pharmacy, Malmö University Hospital, Sweden
- Fort D, Stover E, Bantle J, Finch R (2000). Evaluation of the Development Toxicity of Thalidomide using Frog Embryo Teratogenesis Assay-Xenopus (FETAX): *Biotransformation and Detoxification*. Teratogenesis, Carcinogenesis, and Mutagenesis 20: 35-47.
- Hastings RC (1980). Kellersbergerer Memorial Lecture 1979: Immunosuppressive/Anti-inflammatory Thalidomide Analogs. *Ethiop. Med. J.* 18: 65-71.
- Hastings RC (1971). Studies of the mechanism of action of thalidomide in inflammation Ph D Thesis, Tulane University New Orleans, LA.
- Job CK, Gide S, Macaden VP (1964). Erythema nodosum leprosum. A clinicopathologic study. *Int. J. Lepr. Other Mycobacterial Dis.* 32: 177-184.
- Lahari R, Sandoval FG, Krahenbuhl JL, Shannon EJ (2008). Activation of complement by *Mycobacterium leprae* requires disruption of the bacilli. *Lepr. Rev.* 79: 311-314.
- Mayer MM a(1961). Complement and complement fixation In: E. A. Kabat (ed.) *Kabat and Mayer's experimental immunochemistry*, 2nd ed. Charles C. Thomas Springfield ILL pp. 133-145.
- Mayer MM b (1961). Complement and complement fixation In: E. A. Kabat (ed.) *Kabat and Mayer's experimental immunochemistry*, 2nd ed. Charles C. Thomas Springfield ILL p. 146.
- Naafs B (2000). Current views on reaction in leprosy. *Indian J. Lepr.* 72: 97-122.
- Ridley DS (1960). A bacterologic study of erythema nodosum leprosum. *Int. J. Lepr. Other Mycobacterial Dis.* 28: 254-266.
- Ridley MJ, DS Ridley (1983). The immunopathology of erythema nodosum leprosum: the role of extra-vascular complexes. *Lepr. Rev.* 54: 95-107.
- Saha K, Sharma V, Chakrabarty AK, Sehgal VN (1983). Breakdown product of Factor B an an index to complement activation in lepromatous leprosy and it relation to bacillary load. *Scand. J. Immunol.* 17: 253-260.
- Shannon EJ, Sandoval FG, Krahenbuhl (1997). Thalidomide Hydrolysis of thalidomide abrogates its ability to enhance mononuclear cell synthesis of IL-2 as well as its ability to suppress the synthesis of TNF- α . *Immunopharmacol.* 36: 9-1.
- Shannon EJ, Sandoval FG (2008). Thalidomide and thalidomide transformed by pH-dependent hydrolysis or by liver enzyme treatment does not impede the proliferation of endothelial cells. *Immunopharmacol and Immunotoxicol.* 30: 307-16.
- Shannon EJ, Noveck R, Sandoval FG, Kamath B, Kearney M (2007). Thalidomide suppresses interleukin-6 but not tumor necrosis factor- α in volunteers with experimental endotoxemia. *Translational Res.* 150: 275-280.
- Truman RW, Krahenbuhl JL (2005). Viable *M. leprae* as a research reagent. *Int. J. Lepr. Other Mycobacterial Dis.* 69: 1-12.
- Tadesse A, Shannon EJ (2005). Effects of thalidomide on intracellular *Mycobacterium leprae* in normal and activated macrophages *Clin. Diagnostic Lab. Immunol.* 12:130-134.
- Tadesse A, Sandoval FG, Shannon EJ (2004). Stabilization of red blood cell membranes by thalidomide *in vitro*. *Immunopharm and Immunotox.* 26: 501-509.
- Wemambu SN, Turk JL, Waters MF, Rees RJ (1966). Erythema nodosum leprosum: A clinical manifestation of the arthralgia phenomenon. *Lancet.* 2: 933-935.