0014-4754/95/070725-06$1.50 + 0.20 © Birkhäuser Verlag Basel, 1995

Leishmania donovani in hamsters: stimulation of non-specific resistance by novel lipopeptides and their effect in antileishmanial therapy*

K. Zehra[a], R. Pal[a], Anuradha[a], S. Y. Rizvi, W. Haq, B. Kundu, J. C. Katiyar[a] and K. B. Mathur

Divisions of Parasitology[a] and Biopolymers, Central Drug Research Institute, Chattar Manzil, P.O. Box 173, Lucknow 266001 (India)
Received 22 July 1994; accepted 15 December 1994

Abstract. Several novel type of lipopeptides were synthesized and evaluated for their ability to stimulate non-specific resistance against *Leishmania donovani* infection. Peritoneal macrophages isolated from young male hamsters treated with muramyl dipeptide (MDP) and various synthetic lipopeptides (6 mg/kg i.p.) 7 days earlier, were cultured in vitro and challenged 24 h later with *L. donovani* promastigotes. One lipopeptide, Central Drug Research Institute (CDRI) compound 86/450, exhibited significantly higher immunostimulatory activity than MDP. Its prophylactic activity was further confirmed in hamsters by giving 2 split doses of 3 mg/kg of the compound spaced at 2 weeks, i.e. on day −7 and +7 of challenge with *L. donovani* amastigotes. The prophylactic effect lasted for 7 days following the last treatment with compound 86/450. The antileishmanial action of sodium stibogluconate (SAG) was also found to be enhanced by 16% in hamsters primed with compound 86/450.
Key words. Hamsters; *L. donovani*; CDRI compound 86/450; muramyl dipeptide (MDP); sodium stibogluconate (SAG).

Existing drugs for visceral leishmaniasis are partially effective and potentially toxic[1]. Since *Leishmania*-infected patients are immunosuppressed[2,3], a combination therapy including immunostimulants has proved to be significantly more effective than immunotherapy or drug therapy alone[4–6].

Keeping the above strategy in view, we recently undertook the synthesis and bioevaluation of novel immunostimulating peptides with the objective of identifying potent compound(s) that would be suitable for incorporation in a regimen of combined immunostimulation and chemotherapy for the treatment of visceral leishmaniasis. In the first instance, a series of conformationally constrained analogues of muramyl dipeptide (MDP) were tested for their ability to stimulate non-specific resistance of hamsters against *Leishmania donovani* infection, with promising results[7]. A similar study was then carried out using compounds belonging to the class of lipopeptides synthesized in our laboratory. These peptide derivatives are unique in that they have neither the N-acetylmuramyl residue to MDP nor the meso-2, 6-diaminopimelic acid residue of the known immunopotentiating lipopeptides lauroyltetrapeptide (LTP) and FK-565[8]. In this communication, synthetic strategies for the novel lipopeptides N^{α}-Gly, N^{α}-($C_{11}H_{23}$, CO-Ala-D-Glu-NH_2)-Lys-NH_2 (85/374); N^{α}-Ala, N^{α}-($C_{11}H_{23}$, CO-Ala-D-Glu-NH_2)-Lys-NH_2 (85/91); N^{α}-Gly, N^{α}-(Ala-D-Glu-NH_2)-Lys-NH · $C_{12}H_{25}$(n) (84/201); N^{α}-Gly, N^{α}-(MeAla-D-Glu-NH_2)-Lys-NH · $C_{12}H_{25}$(n) (86/450); N^{α}-($C_{11}H_{23}$CO-Gly), N^{α}-(Ala-D-Glu-NH_2)-Lys-NH_2 (85/294); and N^{α}-($C_{11}H_{23}$CO-Ala), N^{α}-(Ala-D-Glu-NH_2)-Lys-NH_2 (85/90) and their immunopotentiating activity against *L. donovani* infection in vitro as well as in vivo, along with their effect on chemotherapeutic response of hamsters to sodium stibogluconate, are reported.

Materials and methods

Parasite and host. The strain of *L. donovani* (MHOM/IN/80/Dd8), procured from Imperial College, London in 1981, was maintained in male hamsters through serial passages (amastigote to amastigote). Promastigotes were cultured in NNN tubes with RPMI-1640 medium as an overlay. Promastigotes were harvested from the culture tubes, washed thrice in Locke's solution and counted in a haemocytometer. Appropriate inocula were prepared for experimentation.

Male hamsters (40–45 g) served as the host. These were provided with standard rodent diet (Hindustan Lever, Bombay) and water ad libitum.

Synthesis of lipopeptides. The synthesis of all the lipopeptides was carried out in the solution phase. For compound 85/374 and 85/91, the common starting material was Boc-Lys(Z)[9]. It was converted into Boc-Lys(Z)-NH_2(I) via the mixed anhydride procedure (MA)[10]. Cleavage of the Z group of I by catalytic hydrogenation followed by coupling of the resulting amine with Z-D-Glu-NH_2[11] by the MA method yielded the dipeptide Boc-Lys(Z-D-Glu-NH_2)-NH_2(II). Catalytic hydrogenation of II and treatment of the product

* CDRI Communication No. 5034.

with Z-Ala-ONp[12] gave the tripeptide Boc-Lys(Z-Ala-D-Glu-NH_2)-NH_2(III) which after acidolytic cleavage of the Boc group and subsequent reaction with Boc-Gly-ONp[13] and Boc-Ala-ONp[14] afforded Boc-Gly-Lys(Z-Ala-D-Glu-NH_2)-NH_2(IV) and Boc-Ala-Lys(Z-Ala-D-Glu-NH_2)-NH_2(V) respectively. Removal of the Z group from IV and V by catalytic hydrogenation followed by reaction of the products with $C_{11}H_{23} \cdot COONp$[14] in the presence of HOBt gave the tetrapeptide derivatives Boc-Gly-Lys($C_{11}H_{23} \cdot CO$-Ala-D-Glu-NH_2)-NH_2(VI) and Boc-Ala-Lys($C_{11}H_{23} \cdot CO$-Ala-D-Glu-NH_2)-NH_2(VII) respectively. Finally, acidolytic cleavage of Boc groups from VI and VII yielded the lipopeptides 85/374 and 85/91 respectively as their hydrochloride salts.

The synthesis of compounds 84/201 and 86/450 started from the reaction of Boc-Gly[15] with Lys(Z)-OMe[16] in presence of DCC/HOBt[17] to get the dipeptide Boc-Gly-Lys(Z)-OMe (VIII). Saponification of VIII and subsequent treatment of the product with $C_{12}H_{25} \cdot NH_2$ by the DCC/HOBt method yielded Boc-Gly-Lys(Z)-$NH \cdot C_{12}H_{25}(n)$ (IX). Removal of the Z group from IX by catalytic hydrogenation followed by the treatment of the product with Z-D-Glu-NH_2[11] via the MA procedure afforded the tripeptide Boc-Gly-Lys(Z-D-Glu-NH_2) $\cdot NH \cdot C_{12}H_{25}(n)$ (X). Catalytic hydrogenation of X and reaction of the resulting amine with Z-Ala-ONsu[19] and Z-MeAla-DSU gave the protected tetrapeptides Boc-Gly-Lys(Z-Ala-D-Glu-NH_2)-$NH \cdot C_{12}H_{25}(n)$ (XI) and Boc-Gly-Lys(Z-MeAla-D-Glu-NH_2)-$NHC_{12}H_{25}(n)$ (XII) respectively. The final cleavage of Z and Boc groups from XI and XII by catalytic hydrogenation and subsequent treatment of the products with HCl/MeOH yielded the lipopeptides 84/201 and 86/450 respectively.

For the synthesis of compounds 85/294 and 85/90, the protected tetrapeptides IV and V were subjected to acidolysis for the cleavage of Boc group and the products allowed to react with $C_{11}H_{23} \cdot COONp$[14], in the presence of HOBt to get $C_{11}H_{23} \cdot CO$-Gly-Lys(Z-Ala-D-Glu-NH_2)-NH_2(XIII) and $C_{11}H_{23} \cdot CO$-Ala-Lys(Z-Ala-D-Glu-NH_2(XIV) respectively. Treatment of XIII and XIV with 2N HBr/AcOH afforded the lipopeptides 85/294 and 85/90 as their hydrobromides. The purity of all the new peptides was established by TLC and reverse phases HPLC. The characterisation of all the final peptides was done on the basis of their physicochemical data, including elemental analysis, optical rotation and ^{13}C NMR.

Test compounds. The six novel lipopeptides and the standard immunopotentiator muramyldipeptide (MDP, Sigma Chemical Co., USA) were dissolved in distilled water at the desired concentrations. The aqueous solutions were administered to hamsters intraperitoneally within one hour of preparation.

Experimental protocol

Prophylactic efficacy

In vitro. Groups of 3–5 hamsters were treated only once with each test compound and MDP at 6 mg/kg intraperitoneally. Seven days later, macrophages of the hamsters were harvested[5]. The macrophages from untreated normal hamsters (injected with distilled water) were similarly collected.

The cells at the concentration of 1×10^6 in 1 ml culture medium (RPMI 1640 with 10% FCS) were incubated in triplicate (for each drug) in a CO_2 chamber at 37 °C for 24 h in Leighton tubes (Micro aid, Bangalore) with a glass cover slip in each tube, to allow cell adherence. Non-adherent cells were removed by washing with the medium. The infection of macrophages was accomplished by incubating them with promastigotes (2×10^6 in each tube) for 48 h. After the incubation period, the cover slips were removed, air dried, fixed in methanol and stained with Giemsa for microscopic examination. The infection was assessed by counting the percentage of infected macrophages and the average number of amastigotes in each macrophage. The assay was repeated 2–4 times with each test compound.

In vivo. In each experiment, 3–5 animals were allocated for each test compound. An additional similarly constituted group which received placebo (triple distilled water) served as the control. the initial protocol was the administration of test compound at a dose of 3 mg/kg on day −7 and the repeat dose of similar amount on day +7 of infection with 1×10^7 amastigotes per hamster intracardially (on day 0). The drug effect was assessed 25–30 days later by spleen biopsy[7,20,21]. The splenic dab smears were air dried, fixed in absolute methanol and stained with 10% Giemsa. The numerical assessment of parasites was made in treated and control groups of animals by counting the number of amastigotes in about 500 cell nuclei and calculating the number of parasites/100 cell nuclei. The efficacy was expressed in terms of percentage inhibition using the following formula:

$$PI = \frac{\left\{\begin{matrix}\text{No. of amastigotes} \\ \text{in control group}\end{matrix}\right\} - \left\{\begin{matrix}\text{No. of amastigotes} \\ \text{in experimental group}\end{matrix}\right\}}{\text{No. of amastigotes in control group}} \times 100$$

Two to five replicates were done.

One of the lipopeptides (86/450), which showed considerable immunopotentiation, was taken up for detailed investigation. MDP was used as a reference drug.

In one set of experiments, the extent of the immunostimulatory effect of compound 86/450 administered in a single dose of 6 mg/kg was examined in a group of 15 hamsters. These were divided into 3 groups of 5 each and infected with *L. donovani* amastigotes on day 7, 14

and 21 after drug administration. In a parallel run, naive hamsters with no treatment were also infected on the same days. The parasite burden was assessed on any day between 25 and 30 post infection (p.i.) as described above.

Combination therapy

In patent infection. Two groups (I and II) of 10 hamsters each were taken. Animals of group I were given compound 86/450 intraperitoneally at a dose of 3 mg/kg on days −7 and +7 of the infection (day 0) with *L. donovani* amastigotes. In half the animals, treatment with sodium stibogluconate (SAG, Stibanate, Gluconate India Ltd.) (10 mg/kg × 5 intraperitoneally) was initiated on any day between 25 and 35 p.i. and the other half served as untreated controls. The parasite assessment was done after autopsy on day 7 post treatment as described above. Group II, which received only SAG and no immunomodulator, served as the control for comparing the additive effect of the immunomodulator under test.

In prepatent infection. The grouping of animals and the administration of the compound 86/450 were the same as stated above. However, the treatment with SAG (10 mg/kg × 5) was initiated from the day of infection and continued up to day 5 p.i. The parasite assessment was done after autopsy on any day from 25–30 p.i. in both the sets of experiments. Two replicates were done.

Data analysis

Because of existence of heterogeneity of variables, the comparisons among experimental groups in vivo were made by the Fisher-Behran 'D' test and the Mann-Whitney 'U' test.

Results

Prophylactic efficacy

In vitro. Compounds 86/450 and 84/201 showed marked superiority over other lipopeptides as well as MDP (table 1). The activated macrophages from hamsters treated with these compounds acquired considerable resistance to the parasite, as only 54.6–57.4% of the macrophages became infected, as compared to the untreated controls. Besides, the number of parasites found was also very low (241 to 262 amastigotes/100 macrophages).

In vivo. The animal trials with compound 86/450 were in agreement with those of in vitro results. However, the trials failed to endorse the results obtained in the case of compound 84/201 (table 2). Compound 86/450, in a single dose of 6 mg/kg (on day −7) or a similar amount in 2 split doses (3 mg/kg on days −7 and +7) provided significant protection of about 75%.

However, the immunostimulatory effect was well marked only when the compound was administered once on day −7 of challenge (fig. 1).

MDP was found to be inferior in both the sets of experiments.

Combination therapy

The therapeutic efficacy of SAG in hamsters with patent infection and receiving compound 86/450 was found to be significantly enhanced (+16%; $p < 0.05$) as compared to the group which received no immunomodulator (fig. 2).

When the immunomodulator was administered along with the infective agent, the establishment of the parasite in hamsters was significantly less and consequently the therapeutic efficiency of the drug (SAG) was improved, giving 75.0% ($p < 0.001$) inhibition in comparison to the group treated with SAG alone. There was a 53% decrease in the parasite burden in the 86/450-treated group, and a 73% decrease in the 86/450 + SAG treated group, in relation to the group treated only with SAG (fig. 3).

Discussion

The therapeutic management of leishmaniasis requires intense and extensive treatment. The drugs in clinical use are variable in efficacy, and even in the recommended therapeutic dose they can be toxic. Since visceral leishmaniasis is characterised by marked immuno-

Table 1. In vitro susceptibility of peritoneal macrophages of hamsters infected with *L. donovani* and treated with different lipopeptides.

Grous/ compounds	Percent infected macrophages	Average number of amastigotes/100 macrophages	Percent inhibition of parasite multiplication (mean ± SE)
84/201	57.45 (2)*	262.50	53.3** ± 8.79
86/450	54.60 (4)	241.75	57.0** ± 6.8
85/90	62.00 (2)	581.00	NI
85/294	58.00 (2)	841.50	NI
85/91	66.00 (2)	568.50	NI
85/374	64.00 (2)	610.00	NI
MDP	72.00 (2)	424.00	24.5 ± 3.5
Control	74.90 (4)	562.25	

*Number of replicates.
**$p < 0.05$ (significant); NI = No inhibition.

Table 2. Prophylactic efficacy of lipopeptides in *L. donovani* infected hamsters.

Compound	Dose mg/kg	Groups	Parasitic burden[a] (mean with range)	Percent inhibition mean ± SE	Significance 'U' and 'D' test
84/201	3 × 2[b] (−7, +7)	Experimental	51.7 [7][c] (24–90)	NI	NS
		Control	42.9 [16] (14–100)		
88/450	6 × 1 (−7)	Experimental	27.0 [5] (10–50)	71.06 ± 8.6	*
		Control	93.3 [5] (75–109)		
	3 × 2 (−7, +7)	Experimental	19.00 [20] (2–50)	75.47 ± 2.1	*
		Control	77.47 [17] (20–250)		
	3 × 2 (0, +7)	Experimental	70.4 [6] (10–23)	NI	NS
		Control	52.5 [8] (20–85)		
	1.5 × 2 (−7, +7)	Experimental	33.65 [11] (12–55)	11.77 ± 2.3	NS
		Control	38.14 [7] (15–50)		
MDP	3 × 2 (−7, +7)	Experimental	40.00 [7] (29–54)	6.75 ± 1.98	NS
		Control	42.9 [16] (14–100)		

[a]Number of amastigotes/100 cell nuclei of splenic macrophages.
[b]Administration of immunomodulators on day before (−) or after (+) of challenge with Leishmania amastigotes.
[c]Number of animals in square parentheses.
NI, No inhibition. NS, Not significant, *$p < 0.001$.

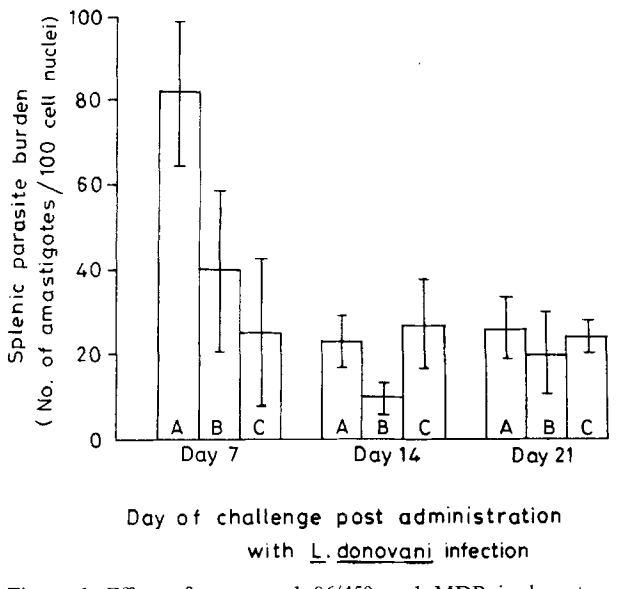

Figure 1. Effect of compound 86/450 and MDP in hamsters challenged with *L. donovani* infection at different time intervals. A) Control group. B) MDP treated group (dose 6 mg/kg × 1). C) Compound 86/450 treated group (dose 6 mg/kg). The data are expressed as the mean parasitic burden ± SE.

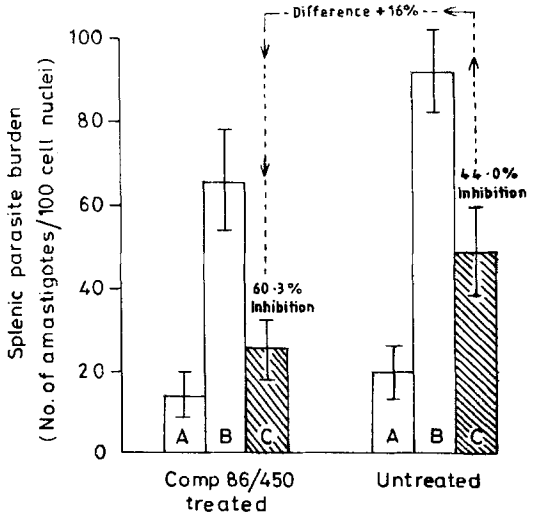

Figure 2. Effect of compound 86/450 on the chemotherapeutic response to sodium stibogluconate (SAG) in patent infection (Hamster infected with *L. donovani* amastigotes). A) Initial parasite burden (before treatment), i.e. on day 30–35 p.i. B) Final parasite burden (after treatment), i.e. on day 32–42 p.i. (control). C) Parasite burden after SAG treatment, i.e. on day 32–42 p.i. (experimental group). The data are expressed as the mean parasitic burden ± SE.

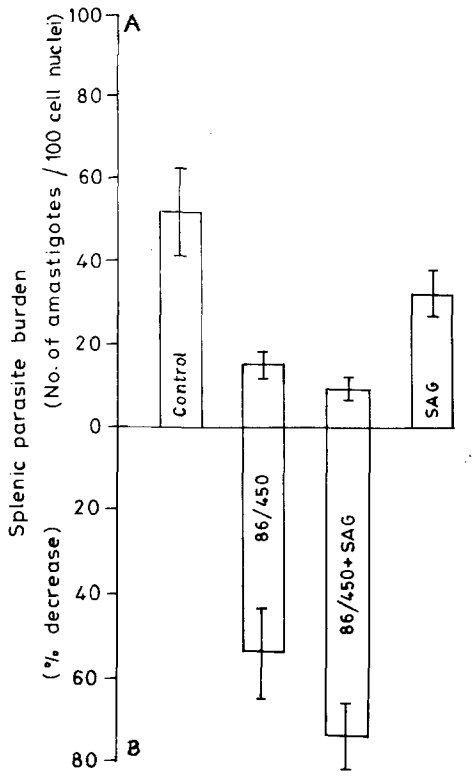

Figure 3. Effect of compound 86/450 on the chemotherapeutic response to SAG in prepatent infection. Hamsters infected with *L. donovani* amastigotes. A) The differences in parasite burden are shown in relation to the untreated control group. The data is also represented as percent inhibition of parasite multiplication in relation to the control group. B) % decrease in parasite burden is shown in relation to SAG treated group. The data are expressed as the mean ± SE.

suppression, and the host immunity is synergistic to drug efficacy, a rational approach to combat the parasitic scourge effectively would be to enhance the immune status of the host for the improvement of the therapeutic result. Under the circumstances, the use of immunomodulators in combination with leishmanicidal drugs would be a viable proposition.

Our earlier investigations of such combinations using glycopeptide immunomodulators analogous to MDP along with SAG were quite encouraging[7]. The incentive for the synthesis of the present series of lipopeptides and assessment of their immunopotentiating activity came from the recent reports regarding the activity of certain desmuramyl peptides and their lipophilic derivatives[20]. Since compounds having no sugar moiety would be easier to synthesise, and therefore less costly, it was considered worthwhile to design peptides which would be devoid of N-acetyl muramic acid or N-acetyl-*nor*-muramic acid residues. It has been reported that the structural entity essential for the optimal adjuvant and anti-infectious activity of desmuramyl lipopeptides such as LTP, FK-156 and FK-565 is N^2-(γ-D-glutamyl)-meso-2(L), 2′(D)-diaminopimelic acid (A_2pm). However, the incorporation of the very expensive amino acid (A_2pm) in peptides involves tedious and circuitous synthetic procedures, so we decided to replace A_2pm by the common and easily available amino acid Lys and study the structure-function relationship of the resulting lipopeptides. A striking feature of these lipopeptides is that the lipophilic anchor, a lauroyl residue, has been shifted from the Ala-D-Glu-NH_2 part of the molecule to the Lys containing part of the tetrapeptide in the case of lipopeptides 84/201, 86/450, 85/294 and 85/90.

Of the various compounds evaluated, compounds 86/450 and 84/201, in which the lauroyl residue is located at the carboxy function of Lys, exhibited sufficient activity in vitro to warrant their assessment in animals. Of the two animal model available, hamsters were chosen for the present studies because, unlike the disease in BALB/c mice, visceral leishmaniasis in hamsters is progressive and uniformly fatal, and the chemotherapeutic response correlates very well with that in humans[6]. Because of the considerable individual variations in susceptibility and seasonal fluctuations in parasite intake[22], the data were analysed using the 'D' and 'U' tests.

The animal experiments confirmed the in vitro efficacy of compound 86/450, but failed to confirm that of compound 84/201. This may be due to the presence of a MeAla residue in place of the N-terminal Ala in the case of lipopeptide 86/450, which could result in the stabilisation of the peptide against enzymatic degradation from the N-terminal end in vitro.

The promise shown by compound 86/450 in both in vitro and in vivo experiments called for detailed investigations of its properties, including its potential use in combination therapy.

The administration of compound 86/450 in a single dose (day-7), or in the same dose split between 2 (days −7, +7) caused immunopotentiation in hamsters, resulting in a smaller parasite load. A lower dose (1.5 mg/kg × 2) was ineffective, so a total of 6 mg/kg in a single or two administrations appeared to be optimal to induce immunopotentiation.

MDP was found to have a weak action, and this was in accordance with earlier observations[6,7,23,24]. Besides, the short half life and potential toxicity restrict the use of MDP in humans.

The immunologically injured parasites are easily knocked out by drugs[25–27]. Accordingly, in the present study SAG in combination with immunopotentiator compound 86/450 yielded better results than when the drug was administered alone. However, in established infections, the therapeutic effect was not so marked, since the immunosuppression caused by the progressive infection was detrimental to drug action. The drug, when used in combination from the time of challenge, was found much more efficient than in the earlier protocol. The immunopotentiator used prophylactially has not only enhanced the immunity of the host but also lessened

the parasite burden. Misra et al.[28] demonstrated that a minimum amount of the drug is needed by each parasite if it is to be inhibited. The fewer the parasites, the higher is their share of drug. Hence, a low level of infection, together with sound immunity, produced better action of the antileishmanial compound.

The beneficial effects elicited by the compound 86/450, recorded as above, indicate that futher investigations should be done including a study of liposomised delivery, to establish its clinical usefulness in visceral leishmaniasis.

Acknowledgements. The authors are grateful to the Director, CDRI for his keen interest in this work. Thanks are also due to Shri S. K. Mandal for statistical analysis. The award of Senior Research Fellowship to K. Zehra, S. Y. Rizvi and K. Pal by Indian Council of Medical Research, New Delhi, is duly acknowledged. This work was partly supported by D. B. T., New Delhi.

1 Beck, J. L., and Davies, J. E. in: Medical Parasitology, 3rd edn, p. 60. Ed. C. V. Mosley Co., St. Louis 1981.
2 Bloom, B. R., Nature, Lond. *279* (1979) 21.
3 Ho, M., Koech, D. K., Iha, D. W., and Bryceson, D. M., Clin. expl Immun. *51* (1983) 207.
4 Haidaris, C. G., and Bonventre, P. F., Am. J. trop. Med. Hyg. *32* (1983) 286.
5 Adinolfi, L. E., and Bonventre, P. F., Am. J. trop. Med. Hyg. *34* (1985) 270.
6 Adinolfi, L. E., Peter, F., Bonventre, P. F., Marjorie, V. P., and Deborah, A. E., Infect. Immun. *48* (1985) 409.
7 Pal, R., Anuradha, Rizvi, S. Y., Kundu, B., Mathur, K. B., and Katiyar, J. C., Experientia *47* (1991) 486.
8 Werner, G. H., Floc'h, F., Migliore-Samour, D., and Jolles, P., Experientia *42* (1986) 521.
9 Scotchler, J., Lozier, R., and Robinson, A. B., J. org. Chem. *35* (1970) 3151.
10 Anderson, G. W., Zimmerman, J. E., and Callahan, F. M., J. Am. chem. Soc. *89* (1967) 5012.
11 Shealy, V. F., Oplinger, C. E., and Montgomery, J. A., J. pharm. Sci. *57* (1968) 757.
12 Bodanszky, M., Kondo, M., Lin, C. Y., and Sigler, F. F., J. org. Chem. *39* (1974) 444.
13 Bayer, E., Jung, G., and Hogermaier, A., Tetrahedron *24* (1968) 4853.
14 Bondaszky, M., Funk, K. W., and Fink, M. L., J. org. Chem. *38* (1973) 3565.
15 Brenner, M., and Huber, W., Helv. chim. Acta *36* (1953) 1109.
16 Iwai, M. I., Nakajima, K., Uno, A., Hase, S., Takeuchi, I., and Okawa, K., Bull. chem. Soc. Japan *43* (1970) 3246.
17 Konig, W., and Geiger, R., Chem. Ber. *103* (1970) 788.
18 Anderson, G. W., Zimmerman, J. E., and Callahan, F. M., J. Am. chem. Soc. *86* (1964) 1839.
19 Petit, G. R., in: Synthetic Peptides, vol. 2, p. 37–81. Van Nostrand Reinhold Co., New York 1971.
20 Beveridge, E., in: Experimental Chemotherapy, vol. 1, p. 257. Eds R. J. Schnitzer and F. Hawking. Academic Press, London 1963.
21 Pal, R., Anuradha, Guru, P. Y., and Katiyar, J. C., Med. Sci. Res. *17* (1989) 579.
22 Pal, R., Anuradha, Guru, P. Y., and Katiyar, J. C., Indian J. med. Res. *90* (1990) 59.
23 Parant, M., Parant, F., Chedia, L., Yapo, A., Petit, J. F., and Lederer, E., Int. J. Immunopharmac. *1* (1979) 35.
24 Lederer, E., in: Biochemistry of parasites, p. 206. Ed. C. M. Slutzky. Pergamon Press, Oxford 1981.
25 Hawking, F., in: Experimental Chemotherapy, vol. 1, p. 129. Eds R. J. Schnitzer and F. Hawking. Academic Press, London 1963.
26 Misra, A., Katiyar, J. C., and Sen, A. B., Inidan J. expl Biol. *18* (1980) 906.
27 Katiyar, J. C., Misra, A., Visen, P. K. S., Wiadomosci Parazytologiczne. TXXXII NHZ (1986) 163.
28 Misra, A., Visen, P. K. S., and Katiyar, J. C., J. Helminth. *55* (1981) 273.