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The Inhibition of Macrophage Migration Inhibitory Factor (38485)

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Recently we have described an extremely sensitive and rapid procedure for the determination of the migration inhibitory factor for macrophages (MIF) released by transformed lymphocytes *in vitro* (1). Using this procedure, we were able to demonstrate that aqueous extracts of thymus from immunologically sophisticated animals contained a significant amount of MIF activity which was like the activity obtained from the supernatant of transformed lymphocytes, i.e., thermostable, trypsin-labile, neuraminidase-labile, and having a molecular weight between 30,000 and 50,000 daltons (2).

Using this assay procedure and partially purified thymus-derived MIF activity, we have explored the effects of derivatives of epsilon-amino-caproic acid (EACA) upon MIF activity *in vitro* proceeding from the observation of Havemann *et al.* (3) that inhibitors of proteolytic activity such as soybean and lima bean trypsin inhibitors were capable of inhibiting the MIF activity obtained from the supernatant of transformed lymphocytes. The results of these studies, as reported below, indicate that derivatives of the protease inhibitor EACA are also capable of significantly inhibiting MIF at concentrations of the order of 10^{-5} *M* and that the ideal compound (active at 10^{-6} *M*) has four methyl groups between an acetylated amino group and a free carboxyl group.

Materials and Methods. MIF activity was assayed as indicated in the procedure according to Houck and Chang (1) in that peritoneal exudates from rabbits were obtained using ip injections of 0.1% oyster glycogen and these peritoneal cells were counted, diluted, and introduced into Leighton tubes. After 1 hr, these culture tubes were rinsed and the cells which had adhered to the glass were incubated overnight in Medium 199 containing 20% calf serum. After incubation, the cells spread themselves randomly and uniformly across the bottom of the Leighton tube. By using a sharp knife, a small scratch was administered to the adherent cell layer and the number of cells moving into this layer from the margin was counted, using an inverted microscope and a Whipple eyepiece (1).

Samples of partially purified MIF activity obtained from thymus were introduced into these cultures, as were samples of the various derivatives of EACA, either separately or together with MIF. The cultures were incubated for 4 hr, the number of migrating cells counted to determine the degree of inhibition of the normal migration rate of these macrophages *in vitro*, and then these cultures were incubated for 20 hr more. Inspection of the cultures which had been incubated for 24 hr with MIF alone indicated that migrating macrophages had essentially "repaired" or filled in the defect in the cell layer after this time. Cytotoxicity produced by some of the derivatives of EACA, however, were demonstrated to be essentially irreversible in that the scratch in the monolayer had not been "healed" by the migration of normal macrophages from the intact margin of the "wound."

MIF activity was obtained by extraction of acetone powders of calf thymus in the cold. These extracts were made up to 50% ethanol and the precipitate removed by centrifugation. This precipitate contained most of the proteolytic activity of this aqueous extract. The alcoholic supernatant remaining was then made up to 70% ethanol (v/v) and the resulting precipitate collected by centrifugation. All of the MIF activity from this extract was concentrated in this precipitate.

The precipitate from 70% ethanol was redissolved in water and subjected to ultrafiltration through Amicon Diaflo filters with the fraction between 30,000 and 50,000 daltons being collected, dialyzed, and lyophilized. This material contained sufficient MIF activity to inhibit approximately 50% of the macrophage migration *in vitro*,

as described above, at a concentration of 1.25 μg/ml. This extract was essentially free of proteolytic activity upon denatured hemoglobin, assayed in the Anson procedure (4) at pH between 3 and 8.

The various structural analogs of EACA were provided through the courtesy of Dr. John Ward, A. H. Robins Co., Richmond, VA.

Experimental results. A series of compounds at 10 and 1 μg/ml were dissolved in Medium 199, to which 1.25 μg/ml of thymus-derived and partially purified MIF extract was added. This served as incubation medium for rabbit peritoneal exudate macrophages, as described above. Control concentrations of each compound were similarly incubated without MIF in the medium in which the macrophages were being cultivated. Those compound analogs which were cytotoxic or effective upon macrophages in terms of inhibiting their migration were eliminated from this study. All the compounds which are described in Table I are materials which have no direct effect upon the migration of macrophages into the scratched notch, as described above.

The percent inhibition of MIF activity at 10 and 1 μg/ml for a number of these compounds is described in Table I. These compounds are organized in terms of the distance between free amino and carboxyl groups of the EACA derivatives involving both 3, 4, and 5 methyl groups and aromatic groups. The effects of acetylation of the amino groups for these compounds are indicated in this Table also. It was noticed that esterification of the free acid group completely eliminated any inhibitory effect of these compounds upon MIF activity *in vitro*.

From the data of Table I, it is clear that (a) the optimal distance between the amino and carboxyl groups is essentially equivalent to that of four methyl groups, and (b) that acetylation of the free amino group is enormously helpful in terms of increasing the inhibitory activity of these compounds. Therefore, we predicted that the compound *N*-acetyl-5-amino valeric acid would be the most active inhibitor of MIF activity in this series. The acetylated compound was synthesized and provided by Dr. John Ward of the A. H. Robins Company.

TABLE I. THE INHIBITION OF MIF ACTIVITY *IN VITRO* BY VARIOUS DERIVATIVES OF EPSILON AMINO CAPROIC ACID (EACA).

	Percent inhibition at:	
	10 μg/ml	1 μg/ml
A. Distance between NH_2 + COOH		
1. H_2N-$CH_2]_5$-COOH (EACA)	0	0
2. H_2N-$[CH_2]_4$-COOH	100	31
3. H_2N-$[CH_2]_3$-COOH	30	0
4. H_2N-$[C_6H_6]$-COOH	25	0
5. H_2N-$[C_6H_6]$-CH_2-COOH	0	0
B. Acetylation		
1. H_2N-$[CH_2]_5$-COOH (EACA)	0	0
2. C-C-N-$[CH_2]_5$-COOH	100	19
3. H_2N-$[C_6H_6]$-C-COOH	0	0
4. C-C-N-$[C_6H_6]$-C-COOH	94	0
C. Predicted compound (from A.2 and B.2)		
C-C-N-$[CH_2]_4$-COOH	100	100

The acetylation of 5-amino valeric (5-AVA) resulted in a compound which gives 100% inhibition of MIF activity at 1 μg/ml in this assay system, as shown in Table I. Actually, at a concentration of 0.1 μg/ml, or slightly less than 10^{-6} *M*, this acetylated 5-AVA compound effectively inhibited 100% of MIF activity *in vitro*.

The inhibition of trypsin by the three most active anti-MIF compounds described in Table I, namely acetylated EACA (epsilon amino caproic acid), acetylated 5-AVA, and 5-AVA itself was determined. All three compounds demonstrated no anti-tryptic activity when mixed with 90 μg/ml of purified bovine trypsin and incubated with denatured hemoglobin in accordance with the general procedure of Anson (4), even at doses slightly in excess of $10^{-3}M$ (200 μg/ml).

Partially purified thymus-derived MIF was mixed with 5-AVA at a concentration which would give 50% inhibition of the MIF activity *in vitro*. This mixture was then subjected to dialysis in the cold against 200 vol of Medium 199 and after 24 hr the material remaining inside the dialysis bag was assayed for its MIF activity *in vitro*. Results indicate that all of the inhibitory effect of

5-AVA upon the MIF preparation was removed by dialysis.

Discussion and conclusions. Our rationale for these sudies was based on the observation of Havemann *et al.*, that inhibitors of tryptic activity such as lima bean and soybean inhibitors as well as Trasylol were capable of inhibiting MIF activity in the George and Vaughan procedure (5). A well known clinically useful chemical inhibitor of proteolytic activity is EACA. Although EACA itself is not active against MIF below 10^{-3} *M*, acetylation of EACA resulted in a markedly more active inhibitor of MIF activity (B-2 in Table I). Removal of one methyl group in the chain separating the free amino and free carboxyl group of this compound also markedly increased its anti-MIF activity (A-2 in Table I). Esterification of the free acid group completely eliminated this anti-MIF activity and caused an increased amount of cytotoxic activity against macrophages *in vitro*. This data, as summarized in Table I, suggests that the ideal distance between the acetylated amino group and a free carboxyl group is four methyl groups as in 5-AVA, which is capable of inhibiting significantly MIF activity in vitro at 10^{-6} *M*.

Since the most active anti-MIF compounds possessed very little anti-tryptic activity while the most active anti-trypsin (EACA) had little anti-MIF activity, we do not believe that there is any correlation between protease inhibition and MIF inhibition. Further, partially purified thymus-derived MIF possessed no proteolytic activity upon denatured hemoglobin at pH between 3 and 8 at concentrations 10–50 times in excess of that required to demonstrate 50% inhibition of MIF activity *in vitro*. We therefore do not believe there is any meaningful coincidence between proteolytic and MIF activities.

Finally, there was no apparent high energy molecular interaction between partially purified thymus MIF and these EACA chemical analogs, suggesting that the mechanism of these analogs in inhibiting MIF activity *in vitro* probably involved an interaction at surface receptors for MIF on the macrophage itself.

Summary. Analogs of EACA were capable of inhibiting the *in vitro* activity of macrophage migration inhibitory factor (MIF). The most active analog of EACA in terms of MIF inhibition had four methyl groups separating an acetylated amino group from a free carboxyl group. This compound, acetylated 5-amino valeric acid, was capable of inhibiting completely MIF activity *in vitro* at a concentration of less than 10^{-6} *M* without demonstrating any appreciable anti-tryptic activity, even at 10^{-3} *M*. Anti-tryptically active EACA did not inhibit MIF except at very high concentrations (10^{-2} *M*). Further, the most purified preparations of MIF obtained from aqueous extracts of thymus that we have studied contained no significant proteolytic activity upon denatured hemoglobin at pH 3–8. Therefore, we do not correlate anti-tryptic with anti-MIF activity *in vitro*.

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