Macrophage Migration Inhibitory Factor in Thymus[1] (37429)

JOHN C. HOUCK, CATHERINE M. CHANG, AND MARVIN PLATT
Biochemistry Research Laboratory, Research Foundation, Children's Hospital, Washington, D. C. 20009

For the past few years, it has been recognized that during the transformation of lymphocytes, a variety of biologically active molecules are released from these cells into the extracellular space (1). These mediators are important to the promulgation and prosecution of the inflammatory response characteristic of delayed type hypersensitivity and graft rejection (2). Although none of these mediators has been completely purified, a number of them have been extensively characterized biologically (3). Perhaps the best described of these extracellular mediators from lymphocyte transformation, or lymphokines, is macrophage migration inhibitory factor (MIF) (4).

Recently we have developed a simple rapid procedure for the determination of MIF *in vitro* which is at least 50-fold more sensitive than the preexisting capillary tube method when applied across species (5). Using this sensitive method, we have been able to demonstrate that saline extracts of the thymus of adult animals contain a small but significant amount of MIF activity when the thymus has been gathered from animals which have had immunologic experience. These results are reported below.

Materials and Methods. MIF activity was determined as described previously (5). Aliquots of peritoneal exudates from rabbits produced by preinjection with 0.1% oyster glycogen (ip) were placed in Leighton tubes and allowed to stand in Medium 199, containing 20% calf serum, for 1 hr at 37°. At this time, the Leighton tubes were rinsed 2 or 3× with medium, and the resulting mononuclear cells which had adhered to the bottom of the Leighton tube were covered with fresh medium. All these cells were phagocytic, as judged by their uptake of latex particles. After 18 hr incubation at 37°, these phagocytic mononuclear cells distributed themselves evenly across the bottom of the Leighton tube. At that time a notch was made in the monolayer of macrophages with a spatula. The cells detached by this procedure were rinsed away and the cell layer allowed to sit again undisturbed for another 15 min, when repeated rinsings were again necessary to remove any floating macrophages which had been displaced by the "wounding" of the monolayer. At that time, using an inverted microscope with a Whipple eyepiece, the straight portions of the wound edge were located and marked. The number of cells adjacent to this were then counted. Four hours later, the number of cells in this area adjacent to the edge of the wound increased considerably. This increase could be inhibited significantly when MIF-containing fractions had been introduced into the medium during the 4 hr of standing at 37° after initial counting. In this manner, a determination of the MIF activity of these fractions could be made (5). MIF activity of less than 20% was not found to be statistically significant and the values above this level were reproducible with an error of ± 10%.

Lymphoid tissue was removed and minced finely with scissors in the cold. This mince was then extracted with sonication into 0.15 *M* NaCl (5 ml/g). The macerated and sonicated tissue was allowed to stand in the cold for 18 hr, at which time the mixture was centrifuged at 12,000*g* in the cold for 30 min. The clear supernatant resulting from this procedure was removed and dialyzed against 200 volumes of pure water. After centrifugation, the contents of the dialysis bag were then lyophilized. This tissue extract is referred

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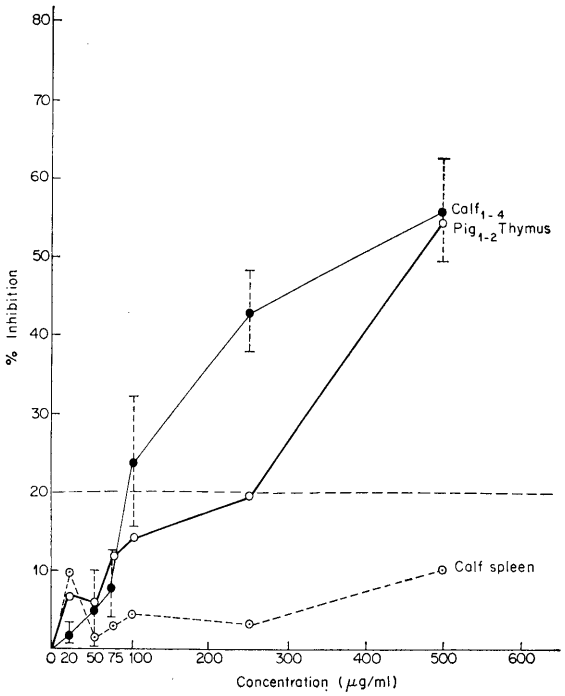

FIG. 1. The mean inhibition of macrophage migration demonstrated by various concentrations of dialyzed, lyophilized 0.15 *M* NaCl extracts of calf (4) and pig (2) thymus and calf spleen. Vertical lines are standard deviation.

to S_1.

Male Hartley strain guinea pigs, weighing 250–400 g, were obtained from Cann Laboratories (Wayne, New Jersey). One group of these animals received PPD-2 (0.0054 mg/0.2 ml) via ip injection daily for 4 days. These animals were rested for 6 days, and the 4-day cycle was repeated with further injections of PPD-2. Another group of 8 animals received PPD as described above and simultaneously but separately, 5 ml ip of 0.1% oyster glycogen. The untreated control, the PPD, and the PPD-plus-glycogen stimulated animals were sacrificed on the 15th day and their thymus and spleen removed. These tissues were treated as described above and S_1 prepared.

Another group of guinea pigs received daily for 5 days a suspension of attenuated Listeria monocytogenes ($> 1 \times 10^6$ organisms/ml). Each animal received 0.5 ml ip. Suspensions had been attenuated by heating at 60° for 30 min. Fresh suspensions of these killed organisms were prepared daily. All animals were sacrificed on the 6th day, thymus and spleen removed, and S_1 prepared as described above.

Thymus tissue was also obtained from 2 pigs and 4 calves. These animals had been vaccinated according to the requirements of the Food and Drug Administration prior to their being slaughtered. The thymus of 1 newborn calf, which had not been subjected to vaccination, was also obtained.

Experimental results. The effects of various concentrations of S_1 prepared from thymus of 4 different calves were studied in terms of their MIF activity. The results of these four studies were averaged and, along with the standard deviation of the mean, are shown in Fig. 1, where they are compared with the results obtained using the S_1 prepared separately from the thymus of 2 pigs. Statistically, inhibition of macrophage migration in this assay of less than 20% was usually not significant; therefore, the variability of MIF activity at the lowest doses of S_1 from animal thymus was probably not meaningful.

The extract of calf spleen gave no

significant MIF activity. Because this was not felt to be due to a lack of MIF activity of spleen versus thymus, recovery experiments of added thymus-derived S_1 to spleen S_1 extracts were performed. The thymus-derived S_1 was allowed to stand in the cold over two nights with the S_1 obtained from spleen. The next morning, the solutions were warmed to 37° and added to the macrophage culture and MIF activity assayed in the usual fashion (5). Essentially all of the MIF activity had been lost by exposure in the cold to spleen extracts.

Thymus-derived activity was found to be thermostable after exposure to 56° temperatures for 30 min; the activity was destroyed by preincubation with both chymotrypsin and neuraminidase, and all of the MIF activity in S_1 was found to be concentrated in the molecular weight range between 30,000 and 50,000 daltons, using the Amicon Diaflo system (6).

Extracts of newborn calf thymus demonstrated no apparent MIF activity. Extracts of control guinea pig thymus similarly did not demonstrate any MIF activity. The S_1 prepared from the thymus tissue derived from guinea pigs which had been pretreated with PPD also contained very little MIF activity. However, PPD plus glycogen (for the mobilization of guinea pig macrophages *in vivo*) administration resulted in a significant amount of MIF activity which could be demonstrated in the S_1 extracts of the thymus from these animals. An even more impressive amount of MIF activity could be demonstrated in the thymus S_1 from guinea pigs which had received injections of killed Listeria monocytogenes. These results are presented in Table I, which compares the MIF activity of various concentrations of the S_1 prepared from the thymus of control and stimulated guinea pigs.

Discussion. These data suggest firstly that while the thymus of immunologically sophisticated animals, *i.e.,* vaccinated calf and pig, contains an extractable MIF, the thymus from relatively immunologically inexperienced animals such as laboratory guinea pigs and newborn calf does not demonstrate such activity. In the case of the guinea pig, only after immunologic stimulation by either PPD, or better, PPD plus glycogen (to stimulate macrophage mobilization), or by injections of Listeria, was MIF activity found in thymus S_1. Secondly, the inability to demonstrate MIF activity in extracts of spleen was probably related to the unusually large concentration of proteases, to which MIF is extremely vulnerable, in these extracts. Thirdly, that this activity was similar to the MIF released from transforming lymphocytes was suggested by the fact that the thymus extract MIF was also thermostable, destroyed by neuraminidase or chymotrypsin treatment, and had a molecular weight between 30,000 and 50,000 daltons (4).

TABLE I. The Amount of MIF Activity Demonstrated by Various Concentrations of Thymus S_1 from Normal Control, PPD-, and Listeria-Treated Guinea Pigs.

	Percent MIF		
	500 μg/ml S_1	250 μg/ml S_1	100 μg/ml S_1
Control	0	0	0
PPD	24	0	0
PPD + glycogen	44	33	20
Listeria (killed)	65	38	22

Winkelstein (7) has demonstrated that the lymphocytes derived from thymus possess less than 1/10th of the MIF activity that circulating lymphocytes did. This is undoubtedly the reason why extracts of thymus did not demonstrate significant amounts of MIF activity in the capillary tube method. The sensitivity of our method for the assay of MIF across species is considerably greater than that of the technique of George and Vaughan (8) within species.

The importance of this observation, for us at least, lies in the fact that to isolate and purify MIF requires, among other things, a large and readily available amount of starting material. Although there is not much MIF activity in the thymus, a great deal of thymus can be obtained easily. Certainly this would be a better source material than having to collect supernatants of transformed lymphocytes from *in vitro* experiments.

Summary and Conclusion. Saline extracts of the thymus of immunologically experienced animals (pig, calf, and guinea

pig) have been shown by a new and very sensitive method to contain a macrophage migration inhibitory factor whose properties are similar to that of MIF obtained from the supernatant of transforming lymphocytes *in vitro*. This MIF activity could not be demonstrated in guinea pig or calf thymus until these animals had been immunologically stimulated by either soluble antigen or whole bacteria, however.

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