Human Macrophage Migration Inhibition Factor: Evidence for Subunit Structure

Abstract. *Macrophage migration inhibition factor (MIF) derived from human lymphoid cell lines was found to lose biologic activity on dialysis. Although activity was not recovered in the dialyzate, mixing experiments demonstrated that the components in the retentate and dialyzate could reassociate to restore activity. The fragment of larger molecular weight (> 10,000) could inhibit the activity of intact MIF, whereas the smaller molecular weight fragment (5,000 to 10,000) could not. These findings suggest that human MIF is composed of at least two noncovalently linked subunits. In analogy to the situation for certain bacterial toxins, one of these may represent an attachment piece for a target cell membrane receptor.*

Macrophage migration inhibition factor (MIF) is one of the nonantibody, lymphocyte-derived mediators known as lymphokines. It is a potent, reversible inhibitor of macrophage migration in vitro, is capable of altering macrophage adhesiveness in vivo, and may be responsible for the activation of macrophages for phagocytosis and target cell killing (*1*).

There is great variation in the observed molecular weight of MIF obtained from various species, and even within species (*2*). In the guinea pig, the usual range is from approximately 45,000 to 67,000 (*2, 3*), whereas in man, the lower limit is usually given as 22,000 to 25,000 (*2-4*). However, Yoshida and Reisfeld reported the existence of a guinea pig MIF with molecular weight of 12,000 (*5*) and, more recently, fractions of human preparations with molecular weights as low as 12,000 have been reported to have MIF activity as well (*6, 7*). These and similar observations have led to the hypothesis that MIF and possibly other lymphokines exist in a variety of states differing in the degree of polymerization of a low-molecular-weight "building block" unit which itself has biologic activity. Since the only available assays for MIF are bioassays, no information has been available as to the existence of functionally inactive subunits.

In this report, we present evidence that human MIF, of approximate molecular weight 22,000 to 25,000 by Sephadex chromatography and Amicon ultrafiltration, can be dissociated into two components, each of which is devoid of activity and which can be reassociated to restore activity.

For these studies, human long-term lymphoid cell culture lines were used as sources of MIF. Cell lines utilized were RPMI 1788, RPMI 8392, RAJI, RPMI 8402, and Molt 4F. The cells were maintained in suspension culture in RPMI 1640 medium supplemented with antibiotics and 10 percent fetal calf serum. They were washed twice in Hanks balanced salt solution. They were then resuspended in RPMI 1640 medium containing antibiotics but no serum, and incubated for 24 hours at 37°C in 5 percent CO_2 and 95 percent air. The cell-free supernatants from these preparations were used as lymphokine sources. Such culture fluids, which we have previously characterized, are known to contain lymphokines that are indistinguishable from those obtained from antigen- or mitogen-stimulated normal human lymphocytes (*7*). The MIF from all those sources behaved identically in the present study. The MIF activity was assayed against guinea pig macrophages as target cells by the capillary tube technique (*8*). Controls for lack of cytotoxicity and reversibility of effect on prolonged incubation of macrophages with mediator were performed in all experiments.

The first indication that human MIF in these preparations might be capable of dissociation into inactive fragments came from routine dialysis experiments. The MIF-containing supernatants were dialyzed against RPMI 1640 medium for 18 hours at 4°C at a ratio of one part of supernatant to 15 parts of medium (dialysis tubing obtained from Fischer, catalog No. 8-667). In a total of 12 out of 15 separate experiments, MIF activity was lost on dialysis (Table 1). In one experiment, activity fell to a borderline value, and in the other two experiments, there was significant but incomplete loss of activity.

These findings were surprising since the sizing data as well as previous reports (*2*) suggest that human MIF activity should be nondialyzable. We therefore attempted to determine whether the loss of activity was an artifact, and simply due to adsorption on the dialysis tubing. For this purpose, supernatants were placed in dialysis tubing and held, in the absence of external fluid for dialysis, in a sealed test tube to prevent dehydration. After 18 hours of incubation at 4°C, full MIF activity was retained. In an alternative procedure, other samples of MIF-containing supernatants were mixed with pieces of dialysis tubing and incubated in petri dishes for 18 hours at 4°C in order to increase the contact area between mediator and tubing. Again, no loss of activity was observed. These results demonstrate not only that adsorption could not explain our findings, but also that inactivation by degradation or denaturation does not occur under the conditions utilized.

These results suggested that we were losing a low-molecular-weight subunit necessary for the expression of MIF activity. If this were the case, then dialyzing repeated samples of MIF against the same dialysis medium should ultimately

Table 1. Loss of activity of MIF-containing supernatants after dialysis. The various cell sources are all of lymphoid origin. Migration indices (MI) (± standard error of the mean) are calculated as follows:

$$MI = \left(\frac{\text{area in experimental supernatants}}{\text{area in control supernatants}}\right) \times 100$$

An MI ≤ 80.0 represents significant MIF activity (*8*). Each MI value is the average of at least six determinations.

Supernatant source*	Migration index	
	Before dialysis	After dialysis
RPMI 1788	71.8 ± 2.0	91.5 ± 2.1
	59.4 ± 5.4	102.0 ± 11.2
	58.8 ± 3.3	93.1 ± 6.3
	65.8 ± 0.6	99.3 ± 2.0
RAJI	52.0 ± 4.0	83.0 ± 1.0
	41.5 ± 2.4	74.0 ± 1.1
Molt 4F	61.7 ± 0.8	90.6 ± 2.1
	75.0 ± 7.1	103.6 ± 11.9
	73.8 ± 3.7	102.4 ± 2.4
	47.1 ± 1.8	76.3 ± 0.8
	56.8 ± 3.0	145.2 ± 8.0
RPMI 8402	48.9 ± 2.3	80.5 ± 2.1
	66.4 ± 1.6	126.5 ± 2.2
RPMI 8392	69.5 ± 3.1	108.7 ± 3.0
	71.5 ± 3.9	104.7 ± 5.7

*Lymphoid cells were maintained in continuous culture. The supernatants were obtained from a 24-hour incubation in serum-free medium.

Table 2. Saturation of dialyzate by repeated dialysis of fresh samples of MIF-containing supernatants. Under the experimental conditions utilized, activity was retained in the retentate after the dialyzate had been used for two previous runs.

Sample No.*	Migration index (MI) for experiment		
	1	2	3
1	44.5 ± 1.1	32.8 ± 1.3	33.7 ± 1.4
2	97.0 ± 3.1	88.3 ± 2.1	101.7 ± 2.9
3	88.1 ± 2.0	93.4 ± 2.1	101.2 ± 2.4
4	77.6 ± 4.6	64.3 ± 4.2	61.2 ± 2.7
5	58.9 ± 2.7	61.8 ± 3.4	49.0 ± 0.9
6	69.7 ± 2.2	44.5 ± 2.0	52.1 ± 2.8

*For a given experiment, each sample represents a portion of the same MIF source. Sample 1 is undialyzed. The others are sequentially dialyzed against the same dialyzate. Each MI value is the average of at least six determinations.

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saturate the dialyzate and prevent further loss of activity. The results of three such experiments are shown in Table 2. Each sample is a portion of the same MIF preparation. Sample 1 is undialyzed; samples 3 through 6 are each dialyzed against the same dialyzate as that used for the previous samples. A point is reached (Table 2, sample 4) at which MIF activity is no longer lost from the retentate. Such a preparation dialyzed against fresh medium shows full loss of activity, as expected. This experiment demonstrates that the dialyzate can indeed be saturated. It should be noted that at no point in these experiments could MIF activity be found in the dialyzate. Parenthetically, this ability of dialyzate to allow macrophage migration to the same extent as the control medium shows that our observed results are not simply due to the removal by dialysis of a metabolite exerting a nonspecific toxic effect on macrophage movement.

In order to confirm our subunit hypothesis, it was necessary to demonstrate that the two putative subunits could reassociate to restore activity. For this purpose, we utilized sample 2 of the previous experiment as inactive retentate. As noted above, this dialyzed preparation was totally devoid of MIF activity. To obtain the smaller component, lost on dialysis, we utilized the dialyzate from the final run (sample 6) which was presumably saturated with that material. This was concentrated back to a concentration two times that of the original preparation by Amicon ultrafiltration on a DM5 membrane, which retains substances of molecular weight greater than 5,000. Retentate and dialyzate were combined in equal quantities and assayed in capillary tube preparations without prior incubation (Table 3). In each case, although retentate and dialyzate are each devoid of MIF activity, mixtures of both have significant activity.

We then attempted to determine if either the large MIF fragment in the retentate or the small fragment in the dialyzate could competitively inhibit the activity of intact MIF. As before, the retentate was obtained by dialyzing a sample of MIF against fresh medium. The dialyzate was one which had been used for repeated dialysis of samples of MIF and which, therefore, could be expected to contain large amounts of the small fragment. This dialyzate was concentrated to two times that of the original sample by Amicon ultrafiltration. Equal volumes of either retentate, concentrated dialyzate, or RPMI 1640 medium as control were mixed with fresh MIF, and the mixtures were assayed. In three separate experiments, we found that the presence of the retentate abolished detectable MIF activity. The dialyzate, under these conditions, had no effect. These results suggest that reassociation of subunits cannot occur in the environment of the cell surface. Otherwise, one might expect that the smaller subunit from intact MIF could associate with the added large subunit to create functional units. Another possibility is that the molar ratios of these components are critical for interaction with the target cell. This could come about, for example, if cooperative effects between adjacent MIF molecules are important for activity, in analogy to the situation for complement activation by membrane-bound immunoglobulin G.

Table 3. Restoration of MIF activity on mixing inactive retentate and inactive dialyzate. Although the original levels of activity of the untreated MIF were not attained, significant activity was achieved by simple mixing.

Preparation	Migration index (MI) in experiment		
	1	2	3
Untreated (intact MIF)	44.5 ± 1.1	32.8 ± 1.3	33.7 ± 1.4
Retentate*	91.3 ± 2.9	90.0 ± 2.3	95.1 ± 2.6
Dialyzate*	94.7 ± 2.6	87.7 ± 2.0	85.7 ± 2.5
Retentate + dialyzate	58.6 ± 1.3	62.5 ± 1.5	62.3 ± 1.1

*Procedures for preparing and concentrating these components are described in the text. Each MI value is the average of at least six determinations.

The initial observation that led to the above experiments was that, in our hands, human MIF lost activity upon dialysis. Although it is widely held that human MIF activity is nondialyzable [reviewed in (*3*)], in some instances this assertion appears to be based on size data from chromatographic and sedimentation techniques. In those cases where dialysis results are reported, substantial amounts of MIF activity may, in fact, disappear (*6, 9*). Nondialyzability of human MIF is also inferred from the fact that chromatographic separation procedures often involve a step of dialysis. This, however, is usually done under conditions of high salt and mediator concentration, since fractions are first concentrated by lyophilization. Under these conditions, we find that we can partially prevent loss of human MIF activity by dialysis. It may be that the stability of subunit association is critically dependent upon the ionic composition of the medium.

Although the experiments described above all utilized MIF from cultured lymphoid cell lines, we obtained results in two experiments with MIF prepared by antigen stimulation of human peripheral blood lymphocytes. This, in conjunction with the data on the correspondences between lymphokines from these sources, suggest the general validity of these observations for materials derived from human sources. In contrast to these, and in agreement with all reports in the literature, we find that guinea pig MIF does not lose activity on dialysis.

The model of human MIF structure that emerges from these studies appears to be similar to the situation for diphtheria toxin, in which two separable but linked fragments are involved in the expression of activity; one interacts with membrane receptors to facilitate the transport of the other (enzymatically active) component into the cell (*10*). It is tempting to speculate that the MIF subunits described here may prove to correspond to an attachment piece and to a component that confers the biological activity. Our finding that one, but not the other, subunit can inhibit the activity of intact MIF is consistent with this hypothesis.

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