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STRAIN VARIATIONS IN MURINE MIF PRODUCTION[1]

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Stimulation of murine lymphocytes with antigen or mitogen *in vitro* can lead to the production of macrophage migration inhibition factor (MIF). In this study, variations in MIF production were examined in various inbred strains of mice. When BALB/c (H-2^d) and AKR (H-2^k) splenic lymphocytes were cultured with concanavalin A (Con A) in serum-free medium, good MIF production resulted within 24 to 48 hr. C3H/He (H-2^k) cells cultured under identical conditions produced low levels of MIF and DBA-2 (H-2^d) and C57BL/6 (H-2^b) cells made no MIF response. Interestingly, however, C57BL/6 cells could make a good MIF response when the cell cultures were supplemented with 2% fetal calf serum (FCS) or if FCS was added to the supernatants after incubation. A similar pattern was observed with the cultures stimulated with specific antigen. This pattern of reactivity demonstrates that variation in MIF production is not directly related to differences in the H-2 complex.

The ability of preformed MIF from one strain to react with target cells from different strains was also examined. It was found that MIF produced by Con A-stimulated lymphocytes of a given strain was capable of inhibiting the migration of allogeneic macrophages as effectively as macrophages from the MIF producing strain. Furthermore, immunoadsorption of MIF-active supernatants with alloantisera directed against H-2 regions failed to remove MIF activity. Taken together, these results suggest that MIF activity as well as production are not under direct H-2 control and that the MIF molecule does not share antigenic determinants coded by the murine major histocompatibility complex.

Extensive studies of migration inhibition factor (MIF)[3] have been performed in the human and in the guinea pig systems (1, 2). Only recently has the mouse been exploited as a model for studying MIF production (3–7), although a great number of reports are available documenting the capacity of murine cells to produce MIF (reviewed in 4). Availability of a suitable murine model provides the possibility of exploring genetic restrictions and control of MIF production and activity. To date, the only genetic restriction described involves a requirement for histocompatible macrophages in the production of MIF by stimulation of immune lymphocytes with soluble tumor antigen (8).

The present study was undertaken to examine strain variations in the MIF production in response to mitogen and specific antigen and to explore a possible relationship of MIF production and activity to the H-2 complex. The ability of target cells and MIF from different strains to interact was also explored. These findings define variations in the ability of different mouse strains to produce MIF that do not appear to be related to the H-2 histocompatibility complex.

MATERIALS AND METHODS

Animals. C57BL/6, DBA-2, BALB/c, and C3H/He mice were purchased from Health Research, Inc., Buffalo, N. Y., and AKR mice were obtained from Cumberland View Farms, Clinton, Tenn. All mice used in these experiments were males between 8 to 12 weeks of age.

Animal sensitization. Mice were immunized with 100 μg Bacillus Calmette Guerin (BCG) in PBS by footpad injections. Two weeks later the immunized mice were challenged i.v. through the tail vein with 50 μg purified protein derivative (PPD). Spleens were removed 48 hr post challenge for *in vitro* MIF production.

In vitro MIF production. Normal or immunized mice were sacrificed by cervical dislocation and their spleens were removed aseptically. The spleens were gently teased apart in Hanks' balanced salt solution (HBSS) and forced through a 100 mesh stainless steel screen to form a single cell suspension. RBC were lysed with 0.5% NH_4Cl and the cells were washed three times in HBSS and suspended at 1.5×10^7 cells/ml in RPMI 1640 supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. The viability of the suspensions, as determined by trypan blue exclusion, ranged between 75 to 90%. Half of the cell suspension was stimulated either with 2 μg/ml Con A (Grade IV, Sigma Chemical Co., St. Louis, Mo.) or 100 μg/ml PPD (a gift from the Department of Tuberculosis, NIH, Tokyo). The other half of the suspension was not stimulated and served as the control. The cultures were incubated in a 37°C humidified 5% CO_2 atmosphere for 48 hr. At the termination of the incubation, the control cultures were constituted with the appropriate antigen or mitogen and all supernatants were clarified by centrifugation at 2500 × G for 30 min. Con A was removed from the supernatants by batch treatment with Sephadex G-100. Completeness of Con A removal was assessed by direct hemagglutination of guinea pig RBC (9). The Con A supernatants were concentrated to their original volume by ultrafiltration with an Amicon PM-10 membrane. All supernatants were Millipore filtered (pore size: 0.45 μm) and stored at −70°C until use.

Indirect murine MIF agarose assay. A modification of Harrington and Stastny's (10) original technique was used. Peritoneal exudates from normal mice were induced by injecting 2 ml

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[3] Abbreviations used in this paper: MIF, migration inhibition factor; MI, migration index.

of a 12% sodium caseinate (Difco Labortories, Detroit, Mich.) solution i.p. 3 days before collection. The mice were exsanguinated and the peritoneal cavities opened through a midventral incision. Peritoneal exudate cells were lavaged out of cavity with a total volume of 15 ml HBSS. The cells were washed three times in HBSS and suspended at 1 to 2 × 10^8 cells/ml in RPMI 1640 containing 0.2% Sea Plaque Agarose (Marine Colloid, Rockland, Maine) and 10% heat-inactivated guinea pig serum. A 1-μl droplet of the cell suspension was placed in the center of each well of a Linbro FB-96 tissue culture plate by means of a 50 μl Hamilton syringe equipped with an automatic dispenser and a 22-gauge blunt-tipped needle. After the droplet solidified (15 to 20 min), 200 μl of chilled test medium were added to each well. Each sample was assayed at least in quadruplicate. The plates were incubated at 37°C for 18 hr in a humifidied 5% CO_2 atmosphere. The migration was quantitated on an inverted-light microscope equipped with a 0.5 mm^2 reticle (Edmund Scientific, Barrington, N. J.). Five measurements were made on each droplet. The diameter of the original droplet was determined and the distance from the edge of the droplet to the periphery of the migration was measured at four points 90° from each other. The four distance measurements were averaged and the total area of the droplet plus the migration were calculated. The area of the original droplet was subtracted from the total area yielding the migration area. A migration index (MI) based on the averaged migration of the four replicate sample was calculated as follows.

$$\text{MI} = \frac{\text{Migration area in test medium}}{\text{migration area in control}}$$

A migration index < 0.80 constituted significant MIF activity.

Serum MIF production. The procedure used was based on the protocol of Salvin *et al.* (7). Briefly, mice were injected through the tail vein with 100 μg BCG in 0.1 ml PBS. Three weeks later, the mice were challenged i.v. with 50 μg PPD. Four hours post PPD challenge, the mice were exsanguinated and the serum was obtained. In the MIF assays, the serum was diluted 4-fold and run as a 25% solution in RPMI 1640 culture medium.

Immunoadsorption. Potent alloantisera with H-2 specificities directed against D^d, $I^k + S^k$, $K^k + D^k$ and I^d regions were the generous gift of Drs. B. Benacerraf and M. Dorf (Harvard Medical School, Boston, Mass). Sepharose 2B agarose bead conjugates of alloantiserum were prepared by the method previously described (11). Briefly, 4 parts cyanogen bromide-activated Sepharose 2B beads were reacted with one part antiserum at 4°C for 18 to 24 hr. The conjugates were washed extensively and used immediately as immunoadsorbents. MIF-active supernatants and control supernatants were reacted with the antiserum conjugates for 1 hr at 25°C. The adsorbed supernatants were lyophilized, reconstituted to original volume with RPMI 1640 medium, and dialyzed extensively against the same medium. The samples were Millipore filtered and frozen at −70°C until assay.

RESULTS

Con A-induced MIF production in inbred stains of mice. By using the MIF-agarose assay, we studied the *in vitro* production of MIF by splenic lymphocytes from five inbred mouse strains. Every attempt was made to treat cells from each strain in an identical manner so that comparisons among strains could be made. Table I shows the average response of the spleen cells of five inbred strains to Con A stimulation. All MIF assays used target cells that were syngeneic to the cells producing the MIF. The MI represents the average of at least six experiments. Cell suspensions from BALB/c and AKR mice each produced good MIF activity. C3H/He gave a marginal response, and the DBA-2 and C57BL/6 cells made no detectable MIF. The results demonstrate that there is a variation from one strain to the other in ability to produce MIF, and that the H-2 complex alone cannot be responsible for this difference.

Antigen-induced MIF production in inbred strains. Variations in MIF production from antigen-stimulated lymphocyte cultures were also explored. BCG-immunized C57BL/6, BALB/c, DBA-2, C3H/He, and AKR mice were used as the source of spleen cells. The results are shown in Table II. Cells from BCG-immunized BALB/c mice consistently made a good MIF response to PPD in culture. AKR cells and C3H/He cells were also effective, but less so than BALB/c. DBA-2 and C57BL/6 cells made no response. The strain variation in producing antigen-stimulated MIF demonstrates a similar pattern to that seen with Con A-stimulated MIF production. This again suggests that the level of MIF production is not governed solely by the H-2 complex nor is it the result of peculiar responses to Con A stimulation.

In this series of experiments, *in vivo* MIF production was also studied. Serum MIF activity was determined as described in *Materials and Methods.* Results here were difficult to interpret because of concomitant enhancing activity for migration. Nevertheless, BALB/c is still seen to be the most effective producer of MIF.

MIF activity on allogeneic target cells. The effect of Con A-induced MIF on allogeneic macrophage target cells was investigated. The results of these experiments are summarized in Table III. The data shows that there is only a small fluctuation from the migration index obtained with target macrophages from the MIF-producing strain to the migration index obtained with allogeneic macrophages in the assay. This demonstrates that Con A-induced MIF readily transcends the strain barrier and that murine target cells probably "see" MIF in an identical manner regardless of the source.

Lack of immunoadsorption with H-2-specific alloantisera.

TABLE I

Production of MIF by mouse spleen cells following CON A stimulation[a]

Strain	Migration Index ± S.E.
C57BL/6 (H-2^b)	0.98 ± 0.06
BALB/c (H-2^d)	0.51 ± 0.12
DBA/2 (H-2^d)	0.85 ± 0.09
C3H/He (H-2^k)	0.77 ± 0.06
AKR (H-2^k)	0.65 ± 0.11

[a] Data shown represent the average of at least six independent experiments for each strain of mice.

TABLE II

MIF production by stimulation with mycobacterial antigens

Strain	*In Vitro*[a]	*In Vivo*[b]
C57BL/6 (H-2^b)	0.92[c] ± 0.02	1.01 ± 0.04
BALB/c (H-2^d)	0.48 ± 0.07	0.66 ± 0.04
DBA-2 (H-2^d)	1.00 ± 0.04	0.87 ± 0.03
C3H/He (H-2^k)	0.74 ± 0.03	1.17 ± 0.05
AKR (H-2^k)	0.70 ± 0.03	1.73 ± 0.09

[a] MIF assay performed on supernatants from antigen-activated cultures. Data represent the average of three independent experiments.

[b] Serum activity as defined in text. Data represent the average of three independent experiments.

[c] Migration index (M.I.) ± S.E. activity.

TABLE III

Effect of CON A-stimulated MIF on unrelated murine PEC

MIF Source	Target Cells		
	AKR	BALB/c	C57BL/6
AKR	0.48[a] ± 0.05	0.38 ± 0.05	0.35 ± 0.04
BALB/c	0.48 ± 0.01	0.32 ± 0.03	0.35 ± 0.02
C57BL/6	1.01 ± 0.05	0.98 ± 0.02	1.03 ± 0.07

[a] Migration index ± S.E. Data represent the average of three independent experiments.

TABLE IV

Effect of FCS on C57BL/6 MIF production

Conditions of MIF Preparation	Migration Index[a]		
	Experiment 1	Experiment 2	Experiment 3
Con A stimulation	1.15 ± 0.01	0.90 ± 0.04	1.03 ± 0.01
Con A stimulation + 2% FCS during culture	0.54 ± 0.08		0.58 ± 0.04
Con A stimulation, 2% FCS added at termination of culture		0.61 ± 0.02	0.65 ± 0.02
2% FCS in RPMI compared	1.14 ± 0.01	0.87 ± 0.03	1.04 ± 0.02

[a] Migration index (MI) ± S.E. Data in each experiment represent the average of at least four independent assays.

MIF activity present in Con A-activated BALB/c and AKR supernatants could not be removed by treating these supernatants with immunoadsorbents of alloantisera directed against antigen coded by regions of H-2^d and H-2^k complexes. This indicates that MIF activity does not carry an H-2-specific antigen.

Effect of fetal calf serum (FCS) on MIF production by C57BL/6 cells. The lack of MIF production by C57BL/6 splenic cells was rather surprising since several reports (3, 4, 12, 13) indicate that C57BL/6 lymphocytes responded well to *in vitro* stimulation with MIF production. In reviewing the experimental procedure, the main difference between their techniques and ours was serum supplementation of the culture medium in their studies. We therefore explored the effect of fetal calf serum (FCS) in our system. When 2% FCS was added to the spleen culture during the incubation period, good MIF activity resulted (Table IV). Further, if 2% FCS was added to the cultures only at the termination of the incubation, MIF activity could still be found in the stimulated culture supernatant (but not in FCS-treated control supernatants). Two percent FCS when added to fresh RPMI 1640 medium itself demonstrated no "MIF-like activity." Thus MIF activity was generated by interaction of FCS with the supernatant and not by an effect of FCS on the activated lymphocytes.

DISCUSSION

The present studies indicate that there is variation in the MIF level produced by spleen cells from different inbred mouse strains in response to Con A or specific antigen stimulation in serum-free medium. The demonstration that BALB/c (H-2^d) and AKR (H-2^k) cells consistently secrete high levels of MIF and that DBA-2 (H-2^d) and C3H/He (H-2^k) elaborate marginal levels or no MIF, suggests that MIF production is not directly linked to the H-2 complex. Thus, the H-2 complex alone cannot be responsible for the observed strain variation in MIF production. This conclusion obviously holds only for those activating agents tested, and further generalization awaits studies utilizing batteries of antigens. The similarities in responses between mitogen and antigen activation suggest that the strain differences in such responses is not the result of some peculiar activity of the Con A on the spleen cells. This is consistent with observations in other species on the identity of MIF induced by specific antigen or Con A (14).

The ability of the Con A-induced MIF produced in one strain to inhibit the migration of macrophages from unrelated strains agrees with results from other studies where MIF was produced by antigen stimulation *in vivo* (12, 13). These studies clearly show that the interaction of MIF with its target cell can transcend strain barriers. The inability to absorb MIF activity with alloantisera directed against H-2 regions suggests that the MIF molecule does not carry an H-2 coded antigenic determinant in contradistinction to the situation for lymphocyte-derived mediators involved in cell-cell collaboration (15–18). This lack of an H-2 antigen is consistent with the lack of strain specificity between the MIF source and the target cell.

The surprising aspect of this study was the apparent lack of a MIF response from antigen or mitogen-stimulated C57BL/6 mouse cells. This strain has been used in several investigations to produce MIF in response to antigen and mitogen (3–5, 12, 13). In reviewing those experiments, the main difference between the protocol used in this study and others is the absence of serum in our culture. We found that MIF could be generated by Con A-activated C57BL/6 lymphocytes, provided that the medium was supplemented with 2% heat-inactivated FCS. The function of the FCS was not due to enhanced viability of the lymphocytes during culture since incubation of cellfree supernatants from activated lymphocytes with FCS also led to the appearance of MIF activity. In the MIF agarose assay, 2% FCS itself in RPMI 1640 medium did not significantly inhibit migration compared to an RPMI 1640 medium without FCS. Furthermore, there was no significant difference between the migration in a conditioned (control cultured) medium containing 2% FCS. This demonstrates that FCS itself has no intrinsic MIF activity in the amount utilized, although Fox *et al.* (19) has demonstrated MIF activity in FCS in another system.

The precise role of FCS in these systems is unknown, however, several possibilities can be postulated: 1) enzymes in the FCS activate a MIF precusor molecule; 2) proteins in the FCS may complex with the MIF molecule rendering it active; 3) FCS may destroy or inactivate an enhancing activity in the activated supernatants, thereby unmasking the MIF activity; 4) FCS may inactivate an inhibitor of MIF; and 5) a product of the stimulated lymphocytes may activate a molecule in FCS conferring MIF-like activity on the FCS molecule. Experiments are now underway in our laboratory to test these possibilities. Another possibility is that FCS might have, in some way, enhanced pre-existing sub-detectable levels of MIF in the C57BL/6-derived supernatants. This possibility is difficult to exclude unequivocally. However, we performed experiments using BALB/c cells, which as stated above are MIF producers. Addition of FCS in this situation did not lead to enhanced MIF production.

The mechanisms underlying the strain variation of MIF production are still unclear. It may well be the result of interplay among various activated lymphocyte products in addition to MIF. Alternatively, it may be due to quantitative differences in the capacity for cell activation or to qualitative differences in the mediators themselves (more active MIF in certain strains). In any case, this variation in lymphokine production may be partially responsible for the strain differences observed in the

development of both delayed hypersensitivity and disease susceptibility in the mouse (20).

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