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Inhibition of Macrophage Migration by Normal Guinea Pig Intestinal Secretions[1] (39279)

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Several studies over the last few years have shown that cell-mediated immunity (CMI) on mucosal surfaces may play an important role in protection against infection (1–7). This conclusion was drawn mainly from studies utilizing the respiratory tract wherein migration inhibitory factor (MIF) production was considered as evidence of CMI. Studies with the intestinal tract, on the other hand, offer no clear evidence as to the importance of CMI, although its presence would be expected considering the similar morphology and common endodermal origin of bronchial-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT) (8).

Several studies have shown that T-cells (thymus-derived cells) are present in GALT. Raff and Owen (9) and Chanana *et al.* (10) both found T-cells in mouse Peyers patches using the theta-antigen as a T-cell marker. Levin *et al.* (11) have shown that guinea pig Peyers patches contain T-cells killed by heterologous anti-T-cell serum and stimulated by Concanavalin A (Con A) and phytohemagglutinin. In a subsequent study, Levin *et al.* (12) further defined the functional capacity of guinea pig Peyers patch T-cells by demonstrating their ability to produce a lymphokine after mitogenic stimulation with Con A and their ability to induce a graft-vs-host (GVH) response. They demonstrated a GVH response using cells from rat Peyers patches as well.

It was with this background that a study was initiated to directly test the hypothesis that intestinal cultures from guinea pigs orally immunized with live and heat-killed *Vibrio cholera* organisms could produce MIF in response to *in vitro* stimulation with vibrio antigens. It quickly became apparent that culture fluids from both control and immunized animals produced MIF-like activity in the absence of antigenic stimulation. Thus, considering the gut to be a tissue constantly being exposed to antigens and since lymphokines (such as MIF) appear to be nonspecific in action, characterization of this normal gut MIF-like activity was undertaken.

Materials and Methods. Animals. Female Hartley guinea pigs weighing approximately 500–700 g were used as the source of intestinal tissue and peritoneal exudate cells for the migration inhibitory factor (MIF) assays.

Small-intestinal cultures. Guinea pigs were starved for 3 days but provided *ad libitum* with drinking water containing kanamycin sulfate (Kantrex, 0.5 g/2 ml) before sacrificing. Guinea pigs were killed by a lethal dose of 1 ml of sodium pentobarbitol (Pentosol). The entire small intestine was removed and washed by injecting 60 ml of Eagle's Minimal Essential Medium (Modified Auto Pow, Flow Lab) containing 270 mg/ml of glutamine, 5 units/ml of heparin, 300 units/ml of penicillin (referred to subsequently as MEM), into the intestinal lumen with a blunt needle. The intestine from one guinea pig was cut into approx 12-cm sections which were everted over glass rods and immediately placed into culture bottles containing 100 ml of RPMI 1640 (Grand Island Biological Company) with 100 units/ml of

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streptomycin and 100 μg/ml of penicillin. The bottles were incubated for 24 h in a stationary position at 37° in a 5% CO_2 incubator. Aliquots of the gut supernatant fluid were cultured for aerobic and anerobic organisms to check for contamination, and the remainder was treated as discussed under Results.

Sephadex G-100. The procedures of Remold *et al.* (13) were generally followed in the gel filtration experiments. A Sephadex G-100 column measuring 2.5 × 100 cm and equilibrated with phosphate buffered saline (PBS) 0.01 *M* PO_4, 0.15 *M* NaCl, pH 7.4, at 2.5° was used throughout. In all cases, 10 μg of [^{125}I]BSA was added to the concentrated supernatants as an internal marker before loading onto the column. Five-milliliter fractions were collected at a flow rate of approximately 15 ml/hr. The tubes were counted for ^{125}I, pooled in 30-ml fractions, dialyzed against three changes of 10 vol each of distilled water in the cold for 48 hr, and lyophilized in 15-ml aliquots. They were each reconstituted immediately before use in 5 to 7 ml of RPMI 1640 containing 10% fetal calf serum, penicillin, and streptomycin, and then tested for MIF activity.

MIF test. Basically, a modification of David's (14) method was used. Nonimmunized guinea pigs were injected with 20 ml of light sterile mineral oil 1 month to 10 days before use. Two days before sacrificing, another injection of 15 ml of mineral oil was given. After sacrificing, peritoneal exudate cells were obtained by washing the peritoneal cavity with MEM. If bloody, the cells were placed in approximately 15 ml of 0.83% NH_4Cl and left for 10 min on ice. The unlysed cells were centrifuged and resuspended in RPMI 1640 (containing 100 μg/ml of streptomycin, 100 units/ml of penicillin, and 10% heat-inactivated fetal calf serum), and adjusted to 2.5 × 10^7 cells/ml. Cell viability was tested by trypan-blue dye exclusion; occasional cell suspensions with less than 95% viability were discarded. Fifty microliter capillary pipets were filled with the cell suspension and the ends sealed with a paraffin and oil mixture. The capillaries were then centrifuged at 110 *g* for 5 min at 4°, cut at the cell-liquid interface, and placed in Mackaness chambers (Berton Plastics, South Hackensack, New Jersey) with each chamber containing three capillaries. The chambers were then filled with medium (either with or without MIF material), incubated at 37° for 48 h, and the areas of migration determined using a photographic enlarger and a planimeter. The inhibition of migration was expressed as:

$$1 - \frac{\text{area of migration of experimental supernatant}}{\text{area of migration of control}} \times 100.$$

Two chambers were run for each sample tested, i.e., each experimental point represents the mean obtained from six capillaries.

Statistical tests. In order to determine if two values were significantly different, the Student's *t* test for two sets with homogeneous variances was used (15).

Results. Preliminary experiments with both normal and cholera-immunized guinea pigs indicated the presence of macrophage migration inhibitory activity in intestinal secretions. These fluids inhibited macrophage migration but did not effect cell viability as tested by trypan-blue dye exclusion. Representative data from one group of experiments are presented in Table I. Considerable variation existed between animals within a group. The addition of antigen (in the form of heat-killed organisms) had no effect on this inhibition. These observations therefore formed the basis for the experiments that followed.

In light of the reported mol wt of 25,000–

TABLE I. MACROPHAGE MIGRATION INHIBITION BY INTESTINAL SECRETIONS OBTAINED FROM NORMAL AND IMMUNIZED GUINEA PIGS IN THE ABSENCE OF ANTIGENIC STIMULATION.

Normal	Orally immunized[a] with live cholera	Orally immunized[a] with heat-killed cholera
76[b]	82	73
52	72	54
40	48	37
32	21	27

[a] Each of the four animals had received 7 mg (approx 10^{10} vibrios) of bacterial suspension 2 weeks prior to sacrificing.

[b] Percentage inhibition as compared to normal guinea pig macrophages in media.

55,000 for guinea pig MIF (13, 16) and the fact that endotoxin (mol wt > 100,000) was a likely inhibitory component of the guinea pig gut (17), it was decided that gel filtration would be the best initial approach for characterizing the MIF-like component(s) of intestinal culture fluid. Once the component(s) of the gut fluid was separated on the basis of molecular weight it could then be further characterized. Three different experiments were performed; one of which will be described in detail.

Gut culture fluid from four guinea pigs were pooled, clarified at 10,000 *g* for 15 min at 4° and then flash evaporated to approx a 30-fold concentration. An aliquot of the flash-evaporated fluid was diluted up to original concentration with water and tested directly in the MIF assay while the remainder was loaded directly on the Sephadex column. Subsequently, reconstituted lyophilized fractions were tested for MIF activity with and without various treatments as discussed below. A profile of the gel filtration column is shown in Fig. 1, where it can be seen that two major peaks of inhibitory activity are present. Fractions D and E showed 33 and 31% inhibition, respectively, while fractions A and B showed 25 and 29% inhibition. Fractions D and E contained materials with a mol wt approximating 25,000–50,000, well within the range for MIF reported by others (13, 16). Fractions A and B ranged from the excluded volume down to a molecular weight of approx 80,000. Assuming a linear dose-response of inhibition over an infinite amount of gut fluid, it was calculated that at least 90% of the original inhibitory activity was recovered in fractions A, B, D, and E.

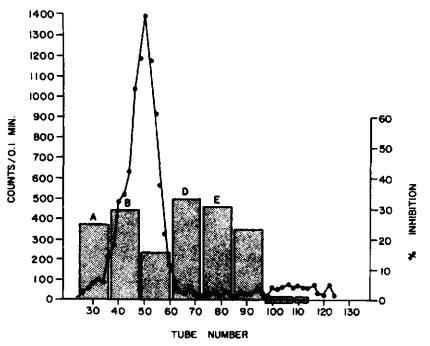

FIG. 1. [^{125}I]BSA marker profile (dotted line) and amount of macrophage-migration inhibition exhibited by fractions (bars) from normal guinea pig culture fluid flash evaporated and fractionated on a Sephadex G-100 column.

Several properties characteristic of antigen-stimulated MIF were then examined. The activity present in fractions D and E behaved as MIF for every property tested (Table II). It was stable to heating to 56° for 30 min but was destroyed after boiling for 1 h. It has previously been shown that epinephrine consistently inhibits MIF activity on macrophage migration (18). Addition of 10^{-4} *M* epinephrine significantly reversed the activity present in both D and E. D decreased from 33% inhibition to 14% inhibition ($P < 0.05$) after epinephrine was added while the original 31% inhibition in E was completely reversed (0% inhibition). Boiled control media or epinephrine had no effect on control macrophage migration.

In a subsequent MIF test, fractions A and B were pooled and tested for the same properties as D and E (Table II). AB gave 37% inhibition. It was stable to heating to 56° for 30 min (33%) but activity was partially destroyed after boiling for 1 h (15% inhibition). Instead of reversing activity, the addition of epinephrine had no effect on inhibitory activity (the difference between 37% inhibition and 42% inhibition was not significant). Therefore, AB differed from the low molecular weight activity in at least two respects.

Two additional culture supernatents were prepared and subjected to gel filtration examination. One of these was initially concentrated by lyophilization while the other was concentrated by positive-pressure dialysis prior to filtration. In each case, greater than 80% of the original activity was

TABLE II. EFFECTS OF VARIOUS TREATMENTS ON INHIBITORY FRACTIONS CONTAINING MIF-LIKE ACTIVITY.

Treatment	Fractions		
	D	E	AB
None	33 ± 8[a]	31 ± 11	37 ± 6
56° 30′	33 ± 2	27 ± 10	33 ± 8
100° 1 hr	0	0	15 ± 2
10^{-4} *M* epinephrine	14 ± 7	0	42 ± 3

[a] Percentage inhibition ± SD.

recovered after gel filtration. The major difference from the experiment described in detail above was that no activity was detected in the excluded column volume, i.e., all recovered activity was in the 25,000–50,000 mol wt range.

Discussion. The data presented here indicate that the small intestine of normal Hartley guinea pigs produces *in vitro* an activity capable of inhibiting the migration of guinea pig peritoneal macrophages. When gut culture fluid was fractionated on Sephadex G-100 it was shown that two different peaks contained inhibitory activity. A low molecular-weight activity was found in all three gut cultures examined and resembled MIF for all characteristics tested. It was stable to heating at 56° for 30 min which has been shown to be characteristic of antigen-induced MIF by others (19–21). Its activity was destroyed after boiling for 1 h, which, although not previously reported, might be expected of a glycoprotein such as MIF. It was nondialyzable and approximated a mol wt of 25,000–50,000 on Sephadex G-100. This is comparable to the reported molecular weight of antigen-induced guinea pig MIF found by Remold *et al.* (13) and Bloom and Jimenez (16). Epinephrine completely inhibited the inhibitory effect of this material. This observation agrees with the findings of Koopman *et al.* (18) that β-adrenergic agents, such as epinephrine, can reverse both antigen- and mitogen-induced MIF activity. While the above studies certainly can be construed as supporting the concept that this low molecular weight activity is in fact MIF, an alternative possibility might also be considered. Namely, the low molecular weight component from normal guinea pig gut cultures could be some previously undiscovered factor which served to stimulate the production of MIF from the approximately 10% lymphocytes present in guinea pig peritoneal exudate cells. Future studies employing pure macrophage preparations would be required to eliminate this unlikely possibility.

The high molecular weight (>100,000) activity was most likely endotoxin-derived from the high numbers of bacteria normally present in the gut. This hypothesis is supported by the observation that the activity was relatively heat stable, i.e., a loss of only approximately 50% after boiling for 1 hr. The inhibitory effect of endotoxin on macrophage migration has been previously noted (17) and hence its presence (although not assayed directly here) would not be surprising. Finally, no evidence in the literature is available on the effects of epinephrine on the inhibition of macrophage migration by endotoxin. Therefore, while the study reported here does not prove this inhibitor to be endotoxin, it certainly does not appear to be a "conventional" MIF as has been suggested with respect to the low molecular weight inhibitor.

Although this study did not investigate the source of the MIF production there are several possibilities. T-cells may be specifically stimulated to produce MIF by bacterial antigens present in the gut. Yoshida *et al.* (22) have in fact shown directly that T-cells produce MIF in response to specific antigenic stimulation. B-cells present in GALT (11, 12, 23–25) may also be producing MIF since it has been shown (22) that endotoxin (a B-cell mitogen) can nonspecifically stimulate B-cells to produce MIF. A third possibility is that neither T-cells nor B-cells are producing MIF but that rapidly dividing mucosal cells are responsible for MIF production. Tubergen *et al.* (26) have shown that MIF is produced by nonstimulated continuously growing lymphoid and fibroblast cell lines. Since none of these explanations precludes the others, perhaps all three cell types are continuously producing MIF and thus each one partially contributes to total MIF production.

In spite of the difficulties in interpretation of certain aspects of this study, it is intriguing to postulate that lymphocytes in gut tissues are constantly being stimulated by bacterial antigens and/or endotoxin to produce MIF and other lymphokines normally associated with CMI. Levin *et al.* (12) did in fact have a higher background level of chemotactic activity in control Peyers patch cells than in control spleen cells. It would seem that additional antigens might not provide further stimulatory effect. Thus, could anyone ever find an efficacious vaccine for the production of local gut CMI?

Summary. The incubation fluid from 24-

hr cultures of normal guinea pig small intestines was found to contain activity capable of inhibiting the migration of normal guinea pig peritoneal macrophages. Each of three different culture fluids showed macrophage inhibitory activity in the 25,000–55,000 mol wt range when subjected to Sephadex G-100 gel filtration. One of these three preparations also contained inhibitory activity in the excluded volume.

The low molecular weight activity was nondialyzable and heat stable to 56° for 30 min. It was destroyed by boiling for 1 hr, and its activity was reversed by 10^{-4} *M* epinephrine. Based upon these properties it is suggested that this activity was due to migration inhibitory factor (MIF). The high molecular weight inhibitor was stable to heating and its activity was not reversed by epinephrine. This material was most likely endotoxin. The presence of MIF, which may be associated with T-cell activity in the intestine, suggests that cell-mediated immunity (CMI) could play a role in local protection against gut infections. Furthermore, its presence in normal intestinal secretions suggests that some MIF-producing cells are always being stimulated by normal flora. The question is thus raised whether further specific stimulation of local CMI in the gut could be successful.

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