Cellular Immunity *in Vitro*: Elaboration of MIF by Thymic and Marrow Lymphoid Cells (36401)

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The thymus and bone marrow constitute two "central" lymphoid organs; participation of cellular elements from both appears to be required for establishing a functioning immune defense system (1). The activity of the thymus is particularly important in developing a class of lymphocytes primarily concerned with initiating cellular immunity (2). The resultant population has been termed "T" type lymphocytes, thereby indicating their dependence on the thymus (3).

However, the developmental events concerned with establishing a population of "T" cells are not well understood. By kinetic criteria, the majority of lymphoid cells populating either the thymus or marrow exhibits proliferative characteristics distinctly different from those peripheral lymphocytes which are capable of effecting cell-mediated immune responses (4). Further, in many *in vivo* assays, cells from these central lymphoid organs have proven to be weak immune effectors (5).

Recently, it has been shown that sensitized, immunologically competent lymphocytes, when incubated with antigen, elaborate a spectrum of chemical mediators; one function of these mediators is to expand the number of cells responding to the immunologic challenge (6). One important mediator, macrophage migration inhibition factor (MIF) is presumed to inhibit the random movement of phagocytic cells *in vivo*, thereby localizing them at the site of antigen deposition. *In vitro* assays for the elaboration of this factor have proven to be a sensitive and highly specific measure of cell-mediated immunity (7, 8).

To further evaluate the activity of different precursor populations of lymphoid cells, studies were undertaken to examine their ability to elaborate MIF. The results show that lymph node cells are highly effective in initiating this type of immune response; in contrast, neither thymic nor marrow lymphoid cells proved effective as mediators of cellular immunity.

Methods. Male, Hartley strain guinea pigs were used in these studies; animals were immunized by 1 ml injections of H37Ra mycobacteria in Freund's adjuvant into foot pads and subcutaneous sites. Two to three weeks after immunization, animals were skin tested with 25 μg of purified tuberculin protein (PPD). Cutaneous reactivity was evaluated at 24 hr; only those animals showing an excess of 5 mm palpable induration were employed.

Animals were killed by ether asphyxia and, using aseptic techniques, lymph node, thymus and bone marrow cell suspensions were prepared as previously described (9). Cell counts were performed using standard hemocytometer techniques. For each assay, controls consisted of a similar cell suspension prepared from nonimmunized guinea pigs.

Peritoneal exudate cells (PEC) were obtained from nonimmunized guinea pigs by the intraperitoneal injection of 30 ml of sterile paraffin oil. Four days later, the animals were killed and the peritoneal cavities extensively washed with Hanks' balanced salt solution. Exudate cells were enumerated and mixed with graded concentrations of lymphoid cells from either sensitized or nonsensitized animals. Differential cell counts on peritoneal exudate fluids showed that 80 to 85% of the cells were macrophages. Sterile hemato-

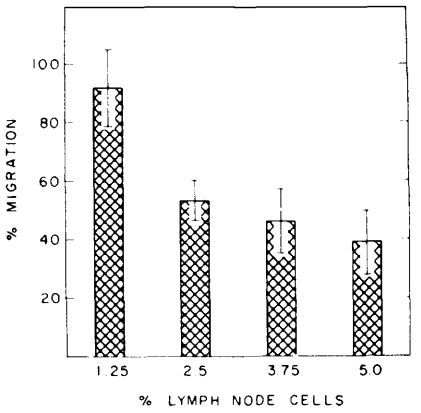

FIG. 1. The ability of graded concentrations of lymph node cells from tuberculin positive animals to inhibit the migration of unsensitized peritoneal exudate cells. Values are mean ± one SE.

crit tubes, heat sealed at one end, were filled with 10×10^6 mixed cells in Eagle's MEM. Tubes were centrifuged for 5 min and cut at the interface between cells and supernate. The cell-containing fractions were attached to Sykes–Moore cover slips using commercial airplane glue and chambers were filled with Eagle's MEM supplemented with 20% guinea pig serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and PPD (25 μg/ml). All chambers were incubated at 37° for 48 hr. At 24 and 48 hr, the area of migration was evaluated by projecting the capillary tubes onto a microscope viewing screen and measuring the area by planimetry. The following formula was used to calculate the extent of migration:

$$\% \text{ migration} = \frac{\text{Area of migration in tubes containing lymphoid cells from sensitized animals}}{\text{area of migration in tubes containing lymphoid cells from nonsensitized animals}} \times 100.$$

For each animal, individual points consisted of the average migration observed with a minimum of four replicate capillary tubes. The area of migration in individual tubes rarely differed from the mean by more than 15%. The percentage of migration for each cell concentration represents the average of 6–12 animals. As an additional control, in each experiment, the migration of PEC from sensitized animals was compared to that observed with cells from nonsensitized donors.

Cell transfer experiments were performed by the method of Chase (10). Sensitized lymph node, thymus or marrow cells were administered by intraperitoneal injection into nonsensitized recipients. These latter animals were skin tested with 25 μg of PPD two days after transfer. Lymph node cells were tested in concentrations ranging from 10 to 50×10^6 cells; marrow and thymus cells in concentrations of $100–500 \times 10^6$ cells. Each point represents the average of 5–10 recipients.

Results. The migration of peritoneal exudate cells (PEC) from animals sensitized to mycobacteria was markedly inhibited in the presence of tuberculin protein. In 25 experiments, the average area of migration was reduced to 55 ± 4% (SE) when evaluated at 24 hr and 51 ± 5% after 48 hr. In these experiments, as in all others reported in this study, the migration measured at 24 hr was virtually identical to the 48 hr point. Thus, all data presented constitute that determined after 24 hr incubation. Further, in 48/50 determinations using PEC from sensitized donors, the area of migration was decreased to 80% or less when compared to cells from nonsensitized animals. Thus, reduction of 20% in the average area was considered the minimum criterion for significant inhibition. These results are in accord with previously accepted criteria of significant response (7, 8).

Lymph node cells from positively reacting animals proved highly effective in inhibiting the migration of unsensitized peritoneal cells (Fig. 1). A significant reduction in area was consistently found when concentrations of 2.5% sensitized lymph node cells were added to unsensitized peritoneal exudate cells. In 11 experiments, the average migration at 24 hr measured 53 ± 7% compared to controls. Concentrations in excess of 2.5% lymph node cells yielded only a slight further reduction in migration. Assays employing 1.25% sensitized cells were ineffective in diminishing the

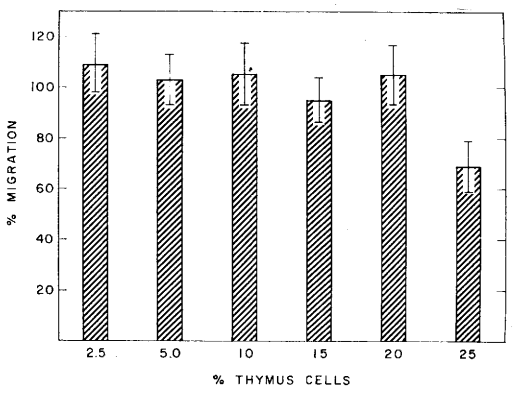

FIG. 2. Inhibition of migration of unsensitized peritoneal exudate cells with added thymic cells. Only in those assays employing 25% thymic cells was a positive cell-mediated response observed.

migration of unsensitized peritoneal exudate cells.

In contrast to the efficacy of lymph node cells in mediating this response, thymus and marrow cells proved considerably less effective in this assay. Using graded concentrations of thymus cells, ranging from 5 to 20%, no measurable inhibition of peritoneal exudate cells migration was observed (Fig. 2). The area of migration, employing 20% thymus cells from sensitized animals, averaged 105 ± 12%. A reduction in migration was observed in assays using 25% thymus cells, suggesting the presence of a few immunologically competent cells in thymus cell suspensions.

Similar results were obtained with suspensions of bone marrow cells. It should be noted that the guinea pig marrow contains between 30 and 35% cells morphologically classified as small lymphocytes. However, concentrations of marrow cells ranging from 7.5 to 20% proved to be ineffective in inhibiting macrophage migration (Fig. 3); using 20% concentrations, the average value was 86 ± 12%. In contrast, concentrations of 25% marrow cells yielded measurable inhibition; the mean value in these determinations was 67 ± 13%.

To further evaluate the activity of different lymphoid cells in cellular immunity, graded numbers of cells from tuberculin sensitized animals were used in passive transfer experiments. These results are summarized in Table I. Whereas 25 × 10^6 lymph node cells consistently led to conversion of skin reactivity, suspensions of thymic and marrow cells proved considerably less potent in their ability to transfer delayed hypersensitivity.

Discussion. The development of a population of immunologically competent "T" type lymphocytes depends on the integrity of both the thymus and the marrow (1–4). However, as illustrated by the present investigations, the lymphoid cells populating these "central" tissues are considerably less potent in tests of cellular immunity than a comparable number of lymph node cells. Employing a sensitive and highly specific *in vitro* assay of cellular immunity, the inhibition of macrophage migration (7, 8), both thymus and marrow cells proved to be weak effectors; these results were further substantiated by *in vivo* cell transfer experiments.

The inhibition of macrophage migration *in vitro* can be effected by relatively few sensitized lymphocytes. Previous studies by David *et al.* (11) showed that the addition of 2.5% PEC from sensitized donors to exudates from normal animals were capable of effecting a positive response. Further, Bloom and Bennett (8), using column separated cells, observed inhibitory activity with as few as 0.6% lymphocytes. Based on these data, it has been suggested that one sensitized lymphocyte is potentially capable of inhibiting the

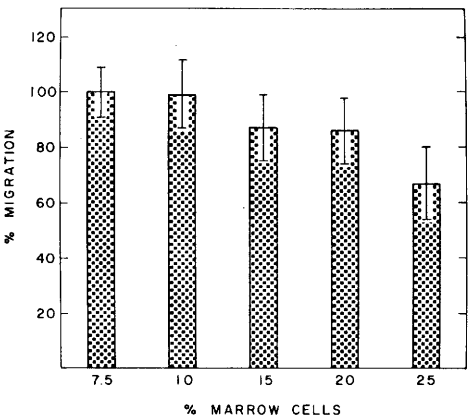

FIG. 3. The activity of graded concentrations of bone marrow cells in inhibiting the migration of normal PEC. Except at concentrations of 25%, intramedullary cells were ineffective in producing positive responses.

TABLE I. Cell Transfer Experiments.

Source of cells	No. of cells transferred $\times 10^6$	No. of recipients	No. with positive skin tests[a]
Lymph nodes	10	7	3
	25	4	3
	50	11	11
	100	2	2
Thymus	100	5	0
	200	6	1
	250	4	0
Bone marrow	100	7	0
	150	3	0
	200	5	1
	250	13	10
	500	5	2

[a] Skin tested 48 hr after ip transfer of sensitized cells. Reaction evaluated 24 hr later; response considered positive if there was in excess of 5 mm induration.

migration of several thousand macrophages (6).

In the present investigations, the addition of as few as 2.5% lymph node cells from sensitized donors produced a significant inhibition in the migration of normal PEC. As noted, the average migration in these assays was similar to that calculated with sensitized PEC alone. Concentrations in excess of 2.5% resulted in only a slight further reduction in the area of migration; in contrast, no inhibition was observed in tests employing 1.25% lymph node cells. Thus, by these criteria, it is apparent that lymph node suspensions contain an appreciable number of sensitized "T" type lymphocytes which are capable of mediating this *in vitro* response.

In contrast to the efficacy of lymph node cells as initiators of this *in vitro* response, thymic lymphocytes proved relatively ineffective. The area of migration in those assays employing concentrations as high as 20% thymic cells from tuberculin positive animals did not significantly differ from the controls. It may be inferred that a minimum number of immunologically reactive cells are present in suspensions of thymocytes as 25% concentrations did promote some degree of inhibition. These data suggest that thymic lymphoid cells are approximately 1/10 as active as a comparable number of lymph node cells in mediating cellular immunity.

Results obtained in assays for MIF are in agreement with the comparatively poor response of thymocytes when challenged with the nonspecific mitogen, phytohemagglutinin (PHA) (9). This test also assesses the status of cellular immunity (12). Studies using guinea pig thymic cells indicate that the response to PHA constitutes only about 5% that observed with an equal number of lymph node cells. In another assay of cellular immunity, the release of lymphotoxin by PHA-stimulated lymphocytes, thymocytes also proved ineffective (13). Thus, by three *in vitro* measures of delayed hypersensitivity, the lymphoid cells of the thymus are, at best, only minimally active as initiators of this type of immunity.

The failure of thymic lymphocytes to produce MIF in response to PPD cannot be attributed to an inability of antigens to gain access to the interior of this organ. Clark has shown that many antigens are capable of entering the thymus parenchyma (14); thus, it appears that the inability to respond can be attributed to intrinsic differences in the immunologic activity of thymocytes as compared to lymph node cells.

The weak immunologic activity of thymocytes as initiators of *in vitro* cell-mediated response appears to be relevant to an assess-

ment of their physiologic role. It is generally established that the thymus is required to develop a population of peripheral lymphocytes that are competent in initiating this class of immune responses (4). However, it would appear that the majority of lymphoid cells in the thymus are not active in mediating this response; either these cells must undergo further maturation outside of the thymus, thereby developing into immunologically competent cells, or else subserve other functions. Perhaps one such function is to condition marrow derived cells, either by direct interaction or by a humoral influence to develop in immunologically competent units. Previous observations have suggested that marrow–thymus interaction, *in vitro*, can lead to augmentation of PHA response, thus tending to support the concept of *in vivo* cellular interaction (9).

It is noteworthy that marrow cells are also ineffective in inhibiting macrophage migration. Studies by both Davies *et al.* (15) and McGregor (16) indicate that marrow derived cells ultimately differentiate into immunologically competent lymphocytes. However, as indicated in these experiments, concentrations of 20% marrow cells from immunized animals are incapable of effecting this *in vitro* response. This failure cannot be attributed to a deficit of lymphoid cells; approximately one-third of the marrow cells are morphologically classified as small lymphocytes. In assays employing 25% marrow cells, measurable inhibition in migration was observed; this suggests the presence of a minor population of immunologically competent cells in suspensions of bone marrows. Whether these cells represent circulating lymphocytes admixed with the marrow suspensions or constitute immunologically competent cells intrinsic to the marrow itself cannot be determined by these data. However, they do provide evidence for the weak immunologic activity of intramedullary lymphoid cells.

These observations are also concordant with the markedly impaired response of marrow lymphoid cells to PHA. Studies using either whole marrow suspensions (17) or lymphoid rich fractions (18) have shown that the proliferative response of intramedullary lymphoid cells to PHA is considerably less than that observed with lymph node cells.

In vivo confirmation of the weak cell-mediated immunologic activity of thymic and marrow lymphoid cells was obtained in transfer studies. Delayed hypersensitivity responses to tuberculin protein could be consistently transferred with as few as 25×10^6 lymph node cells; in contrast, thymic and marrow cells proved relatively ineffective in conferring immunity on unsensitized recipients.

Thus, by both *in vitro* and *in vivo* criteria, these data suggest that the majority of thymic and marrow lymphoid cells do not possess the capacity to initiate cell-mediated immune responses. However, based on these observations, there appears to be a second but numerically small population of cells in these central lymphoid organs which are immunologically active. Whether this latter group arises locally as the result of maturation of progenitors or represents circulating "T" type lymphocytes which have migrated into these sites cannot be determined from these data. Further, the ultimate fate of the major component remains to be elucidated; perhaps under appropriate circumstances some of these cells ultimately mature into immunologically active units. However, it is apparent that the majority of lymphoid cells populating either the thymus or the marrow seem unable to mount cell-mediated immune responses, thereby further confirming the heterogeneity of lymphocytes with respect to their immunological activity (1).

Summary. Different populations of guinea pig lymphoid cells demonstrate marked heterogeneity in their ability to elaborate an *in vitro* chemical mediator of cellular immunity, migration inhibition factor. A concentration of 2.5% lymph node cells obtained from animals demonstrating cutaneous reactivity to tuberculin protein effectively inhibited the migration of peritoneal exudate cells from normal donors. Thymic and bone marrow cells were relatively ineffective in this assay; no significant reduction in the area of migration was observed with cell concentrations as high as 20%. The presence of a sec-

ond but numerically small population of immunologically active cells in both these central lymphoid organs was suggested by the inhibition of migration in assays using 25% cells from sensitized donors. Results of these *in vitro* assays were further confirmed by *in vivo* cell transfer experiments.

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