

*DEMONSTRATION OF DELAYED HYPERSENSITIVITY TO  
SOLUBLE ANTIGENS OF CHEMICALLY INDUCED  
TUMORS BY INHIBITION OF MACROPHAGE MIGRATION\**

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*Abstract.*—Sensitization to soluble antigens of chemically induced tumors is demonstrable in syngeneic strain 13 (inbred) guinea pigs by specific inhibition of macrophage migration. According to their capacity to (a) inhibit the migration of peritoneal cells from sensitized strain 13 donors or (b) release migration-inhibitory factor from their lymph node cells, the soluble antigens of three different chemically induced tumors did not cross react. This is in keeping with the absence of cross-reactivity among the same three tumors in previous tests of transplantation resistance or delayed hypersensitivity *in vivo*. Thus, inhibition of macrophage migration may provide a feasible assay *in vitro* for use in defining these soluble antigens further by physicochemical methods.

We have shown previously that soluble components of syngeneic chemically induced tumors elicit both transplantation immunity and delayed-type hypersensitivity, specific for each individual tumor, in strain 13 guinea pigs.<sup>1, 2</sup> It was hoped that the availability of an assay based on delayed-type hypersensitivity would facilitate the purification of the skin-sensitizing tumor antigens. In our initial studies with fractions prepared by various procedures, however, the interpretation of the skin reactions was complicated by nonspecific irritation at the test site. Therefore, attempts were made to supplement the skin tests with an *in vitro* assay. As we report here, the inhibition of macrophage migration, which has been shown to correlate closely with delayed-type hypersensitivity *in vivo* to a variety of antigens,<sup>3-6</sup> provides a method capable of demonstrating sensitization to the soluble antigens of chemically induced tumors.

In the macrophage-migration test, peritoneal cells from sensitized guinea pigs are packed in capillary tubes and allowed to migrate on cover slips in tissue culture chambers. When specific antigen is present in the culture medium, the migration of macrophages is inhibited. Inhibition is mediated by a soluble factor called migration inhibitory factor, which is released by lymphocytes from sensitized donors upon contact with specific antigen.<sup>7, 8</sup> Injection of purified preparations containing migration inhibitory factor into the skin of normal guinea pigs evokes induration and erythema. At early stages, the cellular reaction is primarily mononuclear. These findings have led to the suggestion that migration inhibitory factor may be a mediator of delayed-type hypersensitivity reactions *in vivo*.<sup>9</sup>

*Methods.*—Inbred guinea pigs of strain 13 were obtained from our colony. Induction of sarcomas with 3-methylcholanthrene and 9,10-dimethyl-1,2-benzanthracene and their maintenance in serial passage in strain 13 guinea pigs have been described previously.<sup>2</sup>

Antigen was prepared by homogenizing fragments of tumor tissue in saline (20% w/v) for 2 to 5 min in a Virtis 45 homogenizer. After the homogenate was clarified by centrifugation at 1000 *g* for 30 min, the supernatant was centrifuged at 100,000 *g* for 60 min. The resulting supernatant was then concentrated to one-fourth its original volume by negative pressure dialysis and then stored at  $-70^{\circ}\text{C}$ . For immunization, one volume of the soluble antigen was emulsified in one volume of Freund's adjuvant (containing 2 mg of heat-killed *M. tuberculosis* per milliliter), and a total of 0.8 ml of the mixture was injected in divided doses into the foot pads and subcutaneous tissues of guinea pigs. It should be noted that strain 13 guinea pigs immunized in this fashion are resistant to transplants of the corresponding tumor and develop delayed hypersensitivity to soluble tumor antigen.<sup>1, 2</sup>

Peritoneal exudates were induced in sensitized strain 13 guinea pigs by intraperitoneal injection of 30 ml paraffin oil (Marcol 52). The exudate was harvested 48 hr later and after washing in Hank's solution containing  $\frac{1}{2}$  unit of heparin/ml,  $5 \times 10^6$  peritoneal cells were suspended in 0.2 ml Eagle's minimal essential medium containing the antigen to be tested, and supplemented with penicillin (160 U/ml), streptomycin (160  $\mu\text{g}/\text{ml}$ ), and 10% unheated normal strain 13 guinea pig serum. One-tenth milliliter of the cell suspension was drawn into each of two capillary tubes and centrifuged at 150 *g* for 2 min. The tubes were then cut at the cell-liquid interface, placed on a cover slip in a single culture chamber, and secured with a drop of silicone grease. The chamber was filled with the medium used for suspending the cells after harvest and sealed with melted paraffin. After incubation at  $37^{\circ}\text{C}$  for 24 hr, the chambers were photographed. The extent of migration was determined by tracing projections of the negatives on paper, cutting out the area within the tracing, and weighing the paper.

The following controls were included: (1) peritoneal cells from sensitized donors in medium without antigen, (2) peritoneal cells from sensitized donors in medium containing antigen prepared from a tumor of different antigenic type, and (3) peritoneal cells from nonsensitized donors in medium containing tumor antigen. Sometimes the cells from sensitized donors migrated better in the presence of an unrelated tumor antigen than in medium without antigen. Therefore, the results were evaluated by comparing the migration of cells from sensitized donors in the presence of the homologous antigen with that occurring in a medium containing antigen prepared from an antigenically unrelated tumor.

In attempts to induce the production of migration inhibitory factor by incubating lymph node cells from immunized donors with the relevant antigen, the procedure was as follows: the cervical, axillary, and inguinal lymph nodes were teased out in Hank's balanced salt solution supplemented with penicillin (160 U/ml), streptomycin (160  $\mu\text{g}/\text{ml}$ ), and 3% unheated normal guinea pig serum. The released cells were passed through a 100-mesh stainless steel sieve, washed, and suspended in Eagle's minimum essential medium containing glutamine, penicillin, streptomycin, and 10% strain 13 normal guinea pig serum. They were then transferred to Leighton tubes, each tube containing  $15 \times 10^6$  cells in 1.5 ml medium. Soluble tumor antigen was added at concentrations ranging from 5 to 20 vol %. As controls, lymph node cells were incubated in the absence of tumor antigen, with an unrelated tumor antigen, or with tuberculin PPD. After 24 hr at  $37^{\circ}\text{C}$ , the cells were sedimented by centrifugation and the supernatant assayed for migration inhibitory factor. The results were evaluated by comparing the migration of peritoneal cells from nonsensitized donors in supernatant from lymph node cultures incubated with antigen to that occurring in supernatant from lymph-node cultures incubated without antigen; in the latter case, antigen was added to the supernatant after removal of the lymph-node cells.

**Results.**—The results of experiments with soluble antigen from three tumor lines (MC-D, MC-E, and DMBA-B) are presented in Table 1, and may be summarized as follows:

- (1) The migration of peritoneal cells from strain 13 guinea pigs immunized

with soluble antigens of chemically induced tumors was specifically inhibited when the corresponding soluble antigen was present in the incubation medium. If we accept as significant an inhibition of migration greater than 20 per cent (the experimental variation was less than that), significant inhibition was found in 23 of 26 attempts. Within a range of 5 to 20 volumes per cent of antigen the degree of inhibition appeared to be dose dependent.

TABLE 1. *Macrophage migration tests: Inhibition of migration of peritoneal cells from specifically immunized strain 13 guinea pigs by soluble antigens derived from syngeneic chemically induced tumors.*

Antigen	Concentration (volume per cent)	Percent migration*	Mean	No. positive/ no. trials
DMBA-B	5	57, 70	63	2/2
	10	27, 43, 50, 51, 55, 80	58	5/6
	20	21, 30, 38, 46, 50, 57	40	6/6
MC-E	10	25, 27, 45, 60, 68	57	5/5
	20	18, 24, 37	26	3/3
MC-D	10	44		1/1
	20	72		1/1
PPD	25 $\mu$ g/ml	10, 10, 19, 21, 21	16	5/5

In two additional experiments, soluble antigen preparations from DMBA-B and MC-E precipitated upon thawing and were clarified before use by centrifugation, the sediment being discarded. The supernatant did not cause inhibition of migration.

\* (a) for tumor antigens: (migration area with homologous tumor antigen)/(migration area with unrelated tumor antigen)  $\times$  100. (b) for PPD: (migration area with PPD)/(migration area without PPD)  $\times$  100.

(2) No cross-reactivity was found between the soluble antigens of MC-D, MC-E, and DMBA-B. As mentioned above, antigen prepared from a tumor of unrelated antigenic type, if it had any effect, appeared to enhance rather than to inhibit macrophage migration. The antigenic specificity of the three tumors as demonstrated by the macrophage-migration technique corroborates our previous finding that the same tumors fail to cross-react in tests of transplantation resistance or delayed hypersensitivity *in vivo*.<sup>2</sup>

(3) Tuberculin PPD used as a control antigen was found to cause a greater degree of migration inhibition than the soluble tumor antigens. This is to be expected because PPD at the concentration used is an extremely potent antigen in the macrophage migration system.<sup>3-6</sup>

After establishing the direct inhibitory effect of the soluble tumor antigen on peritoneal cells from specifically sensitized donors, attempts were made to induce the production of migration inhibitory factor by incubating lymph-node cells from immunized donors with the relevant antigen and subsequently testing the supernatant on peritoneal cells from normal donors. An example of a single test is shown in Figure 1, and the results are summarized in Table 2. Migration inhibitory factor was detected in 8 of 13 trials. Induction was specific because unrelated tumor antigen did not cause migration inhibitory factor release. However, the indirect migration test gave less predictable results than the direct test of antigen on peritoneal cells from sensitized donors. It is possible that the relatively crude antigen preparation contained enzymes which degrade migration inhibitory factor or destroy receptors on the surface of the sensitized lymphocytes.

FIG. 1.—Production of migration inhibitory factor by lymph-node cells from strain 13 guinea pigs immunized with soluble antigen from syngeneic tumor MC-E in complete Freund's adjuvant. The lymph-node cells were incubated in medium (a) without tumor antigen (LY.), (b) with soluble antigen from tumor MC-E (LY. + MC-E), (c) with soluble antigen from tumor DMBA-B (LY. + DMBA-B), or (d) with tuberculin PPD (LY. + PPD). The supernatants of the lymph-node cultures were then assayed for migration inhibitory factor on peritoneal cells from nonsensitized Hartley guinea pigs.

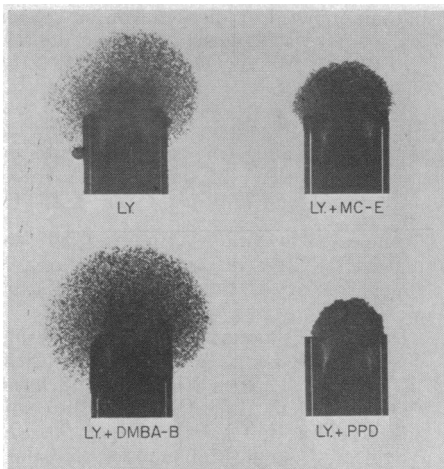


TABLE 2. Indirect macrophage migration tests: Inhibition of migration of peritoneal cells from nonimmunized Hartley guinea pigs by the supernatant of lymph node cultures from specifically immunized strain 13 guinea pigs after incubation with soluble antigens derived from syngeneic chemically induced tumors.

Antigen	Concentration (vol. %)	Percent migration*	No. positive/no. trials
DMBA-B	10	85	0/1
	20	26, 69, 80, 94, 97	2/5
MC-E	5	47	1/1
	10	66, 70, 76	3/3
	20	66, 103	1/2
MC-D	20	44	1/1

In two additional experiments, soluble antigen preparations from DMBA-B and MC-E precipitated upon thawing and were clarified before use by centrifugation, the sediment being discarded. The supernatant did not induce the release of MIF.

\* (Migration area with supernatant from lymph-node culture incubated with homologous tumor antigen)/(migration area with supernatant from lymph-node culture incubated in medium alone; tumor antigen added after removal of lymph-node cells)  $\times$  100.

*Discussion.*—Most studies using the technique of macrophage migration have dealt with measurements of hypersensitivity to soluble protein antigens.<sup>3-9</sup> It has also been applied to the detection of transplantation immunity in the mouse using living allogeneic cells as the source of antigen.<sup>10</sup> The technique has now been shown to be useful for the study of tumor antigens in guinea pigs, yielding results that parallel those obtained by transplantation techniques and by skin tests for delayed hypersensitivity. Immunological analysis of chemically induced tumors has been severely hampered by the lack of serological methods capable of detecting the transplantation antigens of these tumors. This deficiency has inhibited attempts to isolate and characterize the soluble antigens of guinea pig tumors. The results of the present studies indicate that the macrophage migration technique may provide a suitable *in vitro* assay for this purpose.

*Note added in proof:* After this manuscript had been submitted, two reports appeared which are related to the work presented here. Kronman *et al.* showed that cells of a guinea pig

hepatoma induced by diethylnitrosamine, when mixed with peritoneal exudate cells from specifically sensitized syngeneic donors, inhibited the migration of these cells. (Kronman, B. S., H. T. Wepsic, W. H. Churchill, Jr., B. Zbar, T. Borsos, and H. J. Rapp, *Science*, **165**, 296 (1969).)

Malmgren *et al.* reported that the migration of peritoneal exudate cells from guinea pigs sensitized with allogeneic tumor cells was inhibited when these tumor cells were mixed with the peritoneal cells. (Malmgren, R. A., E. C. Holmes, D. L. Morton, C. L. Yee, J. Marrone, and M. W. Myers, *Transplantation*, **8**, 485 (1969).)

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