Migratory Inhibition of Sensitized Peritoneal Macrophages by Tumor-Specific Transplantation Antigen in Morris Hepatoma 5123 (35197)

ANDREAS P. KYRIAZIS, ROBERT W. WISSLER, AND KATTI DZOGA

Department of Pathology, The University of Chicago, and the Argonne Cancer Research Hospital,[1] Chicago, Illinois 60637

The inhibition of migration of macrophages as an indicator of the presence of delayed hypersensitivity to an homologous antigen has been extensively studied (1–3). Both David (4) and Bloom and Bennett (5, 6) have advanced the hypothesis that sensitive peritoneal exudates or lymph node lymphocytes, upon interacting with antigen *in vitro*, elaborate a low molecular weight protein, the migration inhibitory factor (MIF), which reacts with the macrophages to suppress their migration. This phenomenon has been shown to be highly specific in detecting the presence of the sensitizing antigen. Due to this specificity the study of the presence of a specific antigenic component in a mixture of various macromolecular substances is feasible. With this in mind, we have applied the method of inhibition of migration of sensitized peritoneal macrophages in studying the presence of a specific tumor transplantation antigen in Morris hepatoma 5123, and its absence in normal liver tissue from which the tumor originated.

Materials and Methods. Animals. Adult male rats of both Buffalo and Sprague-Dawley strains, their weight ranging from 300 to 350 g, were used throughout.

Glassware. All glassware was siliconized (Silicad No J-600, Clay Adams) to prevent cells from adhering to it.

Antiserum production. Antiserum to the tumor was produced in a special hybrid strain of rabbits as described by Wissler *et al.* (7).

Absorption of antiserum. Absorption of antiserum was performed as follows. Normal serum and homogenates of livers, spleens, lungs, and kidneys from 10 Buffalo rats were pooled separately. To 2 ml of antiserum, 0.5 ml of each pool was added at room temperature. The mixture was kept overnight at 4°. Then it was centrifuged at 5000 rpm for 1 hr and the supernatant was removed. The collected supernatant was tested in gel immunodiffusion plates against normal tissue and tumor extracts. The plates were kept at room temperature for 72 to 96 hr under continuous inspection. Antisera which reacted with normal tissue extracts were absorbed for a second or even a third time. In most of the cases, however, the second absorption was adequate to eliminate any nonspecific reaction with normal tissues.

Antigen preparation. Morris hepatoma tumors of the line 5123 (45th transplant generation) growing in Buffalo rats were removed, minced with scissors, and passed through a stainless steel hand-operated press homogenizer to remove most of the connective tissue. The homogenate was suspended in ice-cold saline–0.3 *M* sucrose, and cell disruption was carried out by freezing the homogenate in a bath of Dry Ice–acetone. Cell membranes were isolated according to the method described by Mann *et al.* (8). The isolated cell membranes were incubated with papain (crude papain type II, Sigma) for 1 hr at 37°. Digestion was interrupted by adding iodoacetamide to the incubation mixture to a final concentration of 0.05 *M*. The digested membranes were centrifuged in a Spinco preparatory ultracentrifuge at 52,038*g* for 1 hr. The supernatant was dialyzed overnight in a phosphate buffer (0.01 *M*, pH 6.2) and fractionated in a CM-Sephadex C-25 column (0.9 × 30 cm). Stepwise elution gave the chromatogram shown in Fig. 1. Antigenic activities of the eluted peaks were studied in Ouchterlony microdiffusion slides and with

[1] Operated by the University of Chicago for the United States Atomic Energy Commission.

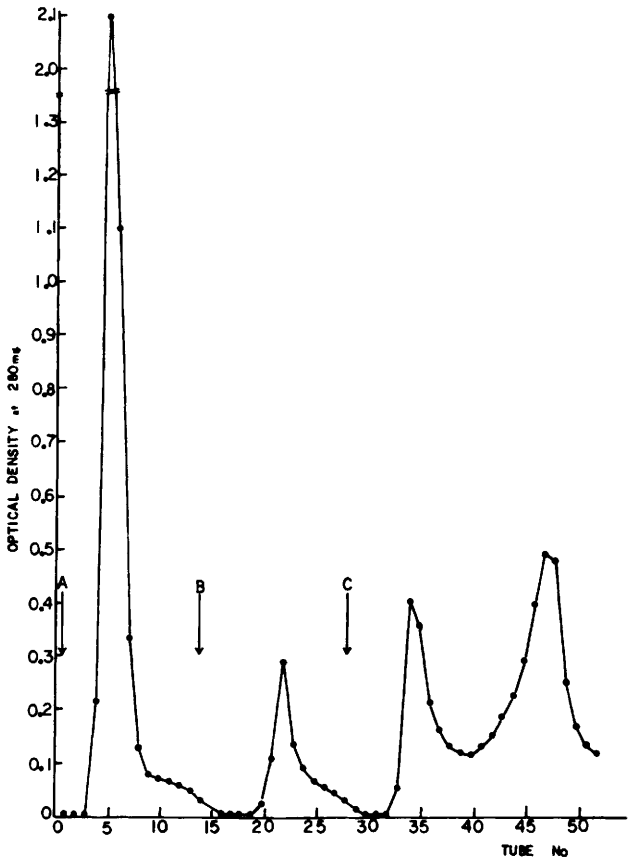

FIG. 1. Fractionation of papain digested tumor cell membranes on CM-Sephadex C-25 column. Stepwise elution: phosphate buffer, pH 6.2; (A) 0.01 *M;* (B) 0.05 *M;* (C) 0.1 *M;* column bed, 0.9 × 30 cm.

immunoelectrophoresis. Specific antigenic activity, as demonstrated by using repeatedly absorbed antisera to the tumor, was present in the first peak. This was the partially purified tumor antigen (PPTA) used in the present study. Normal liver tissue from the same strain of rats in which the tumor was growing was also processed as described above. The final product was the normal liver antigen (NLA).

Sensitization. Two-tenths ml of PPTA (protein concentration in PPTA, 8 mg/ml) was mixed with an equal volume of Freund's incomplete adjuvant (Difco Lab.) and half that volume of *Bordetella pertussis* vaccine[2] (Lilly, strain 37790, Lot T-59 187, Eli Lilly and Co., Indianapolis, Indiana). Each Sprague-Dawley rat was sensitized with 0.5 ml of the antigen emulsion injected intradermally in 6–9 spots.

Peritoneal macrophage preparation and migration. Ten days after sensitization, peritoneal macrophage production was stimulated by injecting 15 ml of a light mineral oil (Marcol 52, Enco) into the peritoneal cavity of each rat. Control rats received the same amount of oil. Both sensitized and control animals were bled to death 72 hr after the injection of oil, and the peritoneal content was washed twice with about 100 ml of Eagle's minimal essential medium[3] (MEM) containing 100 units of crystalline penicillin/ml. The washings were filtered through

[2] Petussis vaccine was obtained from Dr. D. A. Rowley's laboratry of the Department.

[3] Medium 199 was compared with MEM in thes step. There was no appreciable difference between results obtained with MEM and 199.

TABLE I. Statistical Analysis of the Mean Migration Areas of Peritoneal Macrophages from Normal and Sensitized Animals Before and After the Addition into the Culture Medium of NLA[a] or PPTA.[b]

Groups compared		Difference between means	Change (%)	$p_{(t)}$
Normal nonsensitized	Normal nonsensitized + NLA	1.30	4.20	>0.05
Normal nonsensitized	Normal nonsensitized + PPTA	1.07	3.50	>0.10
Normal nonsensitized	Sensitized	3.52	11.54	≤ 0.001
Sensitized	Sensitized + NLA	11.08	41.06	<0.001
Sensitized	Sensitized + PPTA	24.45	90.62	<0.001

[a] Normal liver antigen.

[b] Partially purified tumor antigen.

multilayered cheese cloth, and the aqueous phase was separated from the oil in a separatory funnel. The cell-containing aqueous phase was centrifuged in an international centrifuge at 1200 rpm for 10 min to sediment the cells. The packed cells were washed twice with MacCoy's 5a medium by centrifuging them at 900 rpm for 5 min each time, and finally incubated for 30 min at 37° in a 1:10 dilution (cells to medium). At this stage a differential cell count showed 80–85% macrophages with about 95% viable cells as indicated by exclusion of trypan blue (9). Small microcapillary tubes were filled with the cell suspension, sealed at one end with flame and centrifuged for 1 min in an International microcapillary centrifuge model MB to pack the cells. The filled tubes were cut at the cell fluid interface, and the cell-containing portion was mounted in MacKanness-type chambers of 1-ml capacity (10). Two capillary tubes were fixed in position in each chamber with silicon grease. The chambers were sealed with silicon grease and filled with MacCoy's 5a medium containing 30% fetal calf serum. All chambers were incubated for 48 hr. The areas of macrophage migration were read 24 hr after the incubation had started using an inverted unitron phase contract microscope.

Peritoneal cells from each animal of the control and sensitized group were placed in incubation chambers, respectively. The following cell-culture groups were established: nonsensitized control without antigen; nonsensitized control with NLA; nonsensitized control with PPTA; sensitized (control) without antigen; sensitized with homologous (PPTA) antigen; sensitized with heterologous (NLA) antigen.

Tissue cultures (subgroups) from five control and five sensitized animals were tested simultaneously at any time under identical experimental conditions.

After the completion of the experiment the subgroups from each one group were statistically compared. Since there was no significant difference among subgroups of each one group all subgroups were combined for further statistical evaluation.

Whenever PPTA or NLA was used its concentration was adjusted to 100 μg of protein/chamber.

Results. The results of the migration test are given in Fig. 2, and Table I. Each point on the diagram (Fig. 2) represents the migration of cells from a single capillary tube, the figures in backets being one standard deviation from the mean. Table I shows the difference between means, the percentage change, and the level of significance for each set of compared groups.

The migration of nonsensitized-control cells is practically unaffected by the addition into the incubation medium of either NLA or PPTA (Table I). The observed decrease of migration by 1.3 and 1.1 mm^2, percentage changes of 4.2 and 3.5, respectively, is not significant at the 95% level of significance.

Comparing, however, the migration patterns of nonsensitized and sensitized-control groups we find that the sensitized cells respond differently. There is a definite decrease in migration of sensitized cells even in the absence of sensitizing antigen from the incubation medium. The 11.5% change is found

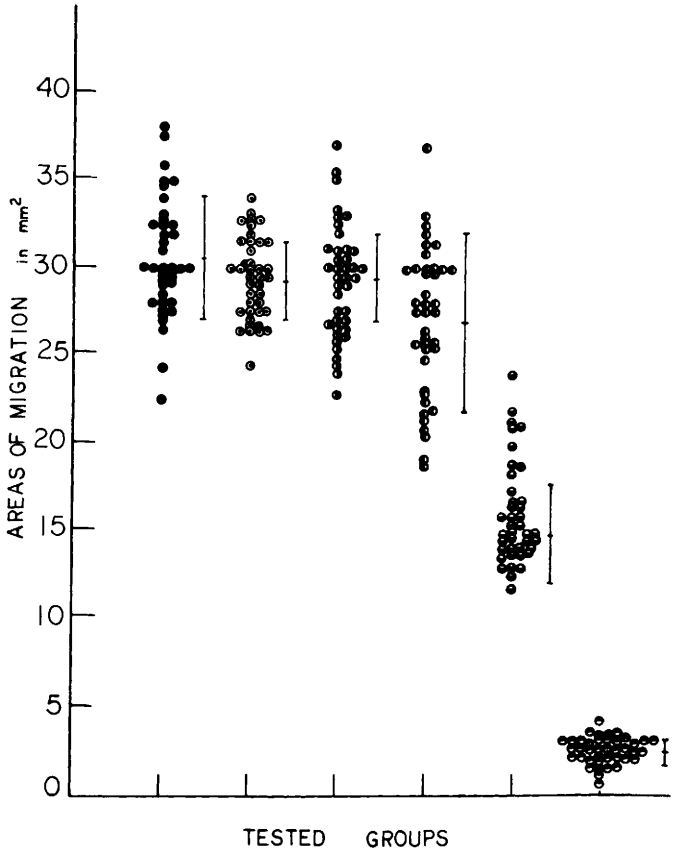

FIG. 2. Migration patterns of peritoneal macrophages from normal and sensitized animals before and after the addition into the culture medium of liver (NLA), or tumor (PPTA) antigen. Each point represents the migration of cells from a single capillary tube. Brackets encompass one standard deviation around the mean. (●), normal nonsensitized cells; (⊙), normal nonsensitized cells + PPTA; (◑), normal nonsensitized cells + NLA; (◐), sensitized cells without antigen; (◒), sensitized cell + NLA; (◓), sensitized cells + PPTA.

to be significant.

The migration of the same sensitized cells is further decreased when challenged with NLA. The addition of heterologous antigen into the incubation medium reduces migration by 11.1 mm^2, a percentage change of 41.1 This same cell preparation responds maximally to the presence of homologous antigen (PPTA). The mean migration value is only 2.5 mm^2 as compared to the 27.0 mm^2 of the sensitized-control cells, and 16.0 mm^2 of the same group challenged with the heterologous antigen (NLA). This value represents a 90.6% decrease in migration. The effects of various concentrations of challenging antigens upon the migration of sensitized cells were then evaluated. It was found that the response of sensitized peritoneal macrophages decreased with decreasing amounts of NLA. At a final concentration of 40 μg of NLA no inhibitory effect could be detected.

Variations in concentration of PPTA were found to be less critical in inhibiting migration of these cells. Sensitized cells responded well even at a concentration of 30 μg of PPTA. Below this level, however, PPTA was less effective, and concentrations as low as 20 μg were inadequate in inhibiting the migration of sensitized cells.

Discussion. Although the mean migration values of control nonsensitized cells show a slight variation in the presence of either NLA or PPTA, they remain within the 95% confidence limits and the observed differences can-

not be considered as significant.

On the other hand, the difference between the mean values of control nonsensitized and control sensitized cells is much more pronounced, the migratory ability of the sensitized cells being less than that of the nonsensitized cells. It is evident that sensitization has a suppressing effect upon migration. This observation has also been reported by Taubler and Mudd who observed that staphylococcal-sensitive cells from splenic explants failed to migrate as well as nonsensitive cells (11).

The decreased migration of sensitized cells when challenged with NLA may be the expression of the presence of antigenic cross-reactivity between hepatoma and normal liver tissue. However, by further diluting both NLA and PPTA the migration values of the sensitized cells challenged with NLA were restored to that of unchallenged cells while the same dilution did not increase the migration of sensitized cells to which the PPTA was added. A 1:3 dilution of the challenging antigen seemed adequate in greatly reducing the nonspecific suppression of migration. By this method it was possible to clearly dissociate the tumor-specific reaction and to reach a point where the test reflected only the tumor-specific activity. These effects of dilution may suggest that there are quantitative rather than qualitative differences between normal liver tissue and hepatoma. Although this hypothesis cannot be rejected completely, the results of carefully controlled immunoelectrophoretic studies and a further step in the purification of PPTA do not support it. Furthermore, the results of the present study confirm previous observations which indicate by more indirect means the presence of a specific tumor transplantation antigen in the Morris hepatoma 5123 (12).

While this work was in progress, Kronman *et al.* used the inhibition of migration of sensitized macrophages to demonstrate the presence of tumor-specific antigens in a guinea pig hepatoma (13).

All of these data clearly demonstrate that the inhibition of migration of macrophages by sensitized lymphocytes may be helpful in studying the presence of tumor-specific transplantation antigens. Its usefulness, however, cannot be critically appraised until more information is available from application in different systems.

Summary. The inhibition of the migration of sensitized peritoneal macrophages has been used in studying a specific tumor transplantation antigen in Morris hepatoma 5123 and its absence in normal liver tissue from which the tumor originated.

Migration of peritoneal macrophages sensitive to the tumor was greatly inhibited by the addition into the incubation medium of a fraction of the solubilized tumor cell membranes. Use of solubilized membranes from normal liver cells were much less effective in inhibiting the migration of sensitized peritoneal macrophages.

The specificity of the inhibition was demonstrated by using decreasing concentrations of the tested antigens. By this method it was possible to reach a point where the test was reflecting only the tumor-specific activity.

1. David, J. R., Al-Askari, S., Lawrence, H. S., and Thomas, L., J. Immunol. **93**, 264 (1964).

2. David, J. R., Lawrence, H. S., and Thomas, L., J. Immunol. **93**, 274 (1964).

3. David, J. R., J. Exp. Med. **122**, 1125 (1965).

4. David, J. R., Proc. Nat. Acad. Sci. U.S.A. **56**, 72 (1966).

5. Bloom, B. R., and Bennett, B., Science **153**, 80 (1966).

6. Bloom, B. R., and Bennett, B., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **27**, 13 (1968).

7. Wissler, R. W., Baxter, R. A., Flax, M. M., La Via, M. F., and Talmage, D. W., Cancer Res. **16**, 761 (1956).

8. Mann, D. L., Rogentine, N. G., Jr., and Faley, J. L., Nature (London) **217**, 1180 (1968).

9. Gorer, P. A., and O'Gorman, P., Transplant. Bull. **3**, 142 (1956).

10. Mackaness, G. B., J. Pathol. Bacteriol. **64**, 429 (1952).

11. Taubler, J. H., and Mudd, S., J. Immunol. **101**, 550 (1968).

12. Wissler, R. W., Craft, K., Kesden, O., Polisky, B., and Dzoga, K., *in* "Advance in Transplantation" (J. Dausset, J. Humbeurger, and G., Mathe, eds.), p. 539. Williams and Wilkins, Baltimore (1968).

13. Kronman, B. S., Wepsic, H. T., Churchill, W. H., Jr., Zbar, B., Borsos, T., and Rapp, H. J., Science **165**, 296 (1969).

Received July 22, 1970. P.S.E.B.M., 1971, Vol. 136.