Cell-Mediated Tumor Allograft Immunity: In vitro Transfer with RNA

Abstract. *Specific inhibition of migration of spleen cells from C57B1/6J mice, which had rejected A/J sarcoma-1 tumors, occurred in the presence of A/J lymph mode antigens. The migration inhibitory effect was transferable to normal C57B1/6J spleen cells by RNA extracted from lymph nodes and spleens of immunized animals.*

The rejection of the A/J mouse tumor sarcoma-1 (Sa-1) by C57B1/6J mice is considered an example of allograft immunity (*1*). Its passive transfer to normal animals with living lymphoid cells but not with serum suggests cell-mediated immunity (delayed hypersensitivity) as an underlying mechanism (*2*). Cell-mediated allograft immunity in vitro can be detected by several methods (*3*) including the well-established antigen-induced inhibition of cell migration (*4*). We now report (i) inhibition of migration of spleen cells from Sa-1 immunized C57B1/6J mice in the presence of soluble A/J antigens and (ii) transfer of this immunity to nonimmune C57B1/6J spleen cells with RNA extracts of spleens and lymph nodes from animals that had rejected Sa-1 tumors.

Sa-1 tumor cells, grown in the ascites form in A/J mice, were injected subcutaneously (2×10^6 cells per 0.1 ml) into each shoulder of adult C57B1/6J animals; second injection (10×10^6 Sa-1 cells(was given intraperitoneally 3 to 4 days later. If the animals were not used within 14 days, immunization was sustained by subcutaneous injections of 2×10^6 tumor cells every 2 weeks. Cell suspensions were prepared from the spleen of three to six mice that were gently teased in medium 199; the cells were washed five times and centrifuged at 800 rev/min each time for 5 minutes and finally resuspended in medium 199. After the last washing the cells were suspended in medium 199 containing 20 percent fetal calf serum, so that the concentration of cells was 10 percent of their packed volume.

Glass capillaries (100-μl Drummond microcaps) were filled with the cell suspension and flame-sealed at one end. The capillaries were then centrifuged at 500 rev/min for 5 minutes, cut at the cell-fluid interface, attached to glass cover slips with sterile silicone grease, and sealed with melted wax into Mackaness migration chambers. The chambers were filled either with culture medium alone or with medium containing crude A/J lymph node antigens (*5*, *6*) and were incubated at 37°C for 24 hours. The migration areas were measured with the use of a planimeter on the enlarged tracings, and the inhibition of migration was calculated from the ratio of the average area of at least four capillaries with antigen and without. Significant inhibition of the migration of spleen cells from C57B1/6J mice, which had been immunized with Sa-1 cells, was observed with the antigen obtained from normal A/J lymph nodes at concentrations of 100 μg/ml or higher (Table 1). The specificity of this reaction was demonstrated by the absence of migration inhibition when spleen cells from nonimmunized C57B1/6J animals were used. Moreover, an unrelated antigen, prepared from CBA mouse lymph nodes, did not inhibit significantly the migration of the immune C57B1/6J spleen cells (*6*).

Lymph nodes and spleens from C57B1/6J mice were frozen on Dry Ice. To 1 to 3 g of tissue was added 10 to 30 ml of freshly distilled buffer-saturated (0.01*M* sodium acetate, *p*H 5.0) phenol, containing 0.1 percent 8-hydroxyquinoline, 0.5 percent sodium dodecyl sulfate, and 4 μg of polyvinyl sulfate per milliliter. The tissues were homogenized for 15 minutes at 10°C, and an equal volume of the buffer containing bentonite (0.5 mg/ml) was added. The homogenate was heated to 55°C, cooled to 10°C, and centrifuged at 15,000 rev/min for 10 minutes. An equal volume of the buffer-saturated phenol was added to the aqueous phase, and the extraction was repeated three times. The RNA from the aqueous phase was then precipitated with three volumes of cold, 95 percent ethanol over a 12-hour period at −20°C. The material was centrifuged at 15,000 rev/min for 10 minutes, dissolved in minimum amounts of 0.3*M* sodium acetate (*p*H 5.0), and precipitated three times with three volumes of 95 percent ethanol at −20°C. The RNA concentration was estimated spectrophotometrically. Only extracts in which the ratios of optical density at 260 nm to that at 280 nm were 1.9 to 2.1 were used in transfer experiments (*7*). Mixtures of spleen cells from nonimmunized C57B1/6J mice (0.3 to 1.0×10^9 cells per 2 ml of medium 199) and RNA extracts (1 to 2 mg per 0.2 ml of medium 199) were incubated for 30 minutes at 37°C.

The cells were centrifuged, resuspended in culture medium, and subjected to migration tests with or without the A/J lymph node antigen as described above. Absence of migration inhibition was observed with cells treated with RNA from nonimmunized animals. However, significant inhibition of migration of cells treated with RNA from immune animals (*8*) was obtained

Table 1. Inhibition of migration of C57Bl/6J spleen cells with A/J lymph node antigens. The percent inhibition of migration is equal to $[1-(M+/M-)] \times 100$, where $M+$ is the average migration area with antigen and $M-$ is the average migration area without antigen.

Antigen (μg/ml)	Percent inhibition of migration			
	Normal cells	Mean ± S.D.	Immune cells	Mean ± S.D.
500	1.8; 7.5; 18; 23.5	12.7 ± 4.9	42.3; 51.5; 52	46.6 ± 3.8
300	−4.5; 9; 9.5; 19; 21.5	10.9 ± 4.5	29; 39.5; 40; 43; 47.5	39.8 ± 3.0
100	−4.5; 1; 4; 6.5; 7; 9	3.8 ± 2.0	26; 35; 35; 36.5; 38.5	34.2 ± 2.1

Table 2. Results of double-blind transfer experiments with RNA extracts.

Unknown RNA* (fraction No.)	Percent inhibition of migration†		Source of RNA	
	A/J antigen (100 μg/ml)	A/J antigen (200 μg/ml)	Suggested by experiment	Actual RNA provided
1	28.7	37.7	Immune	Immune
2	30.3	42.4	Immune	Immune
3	28.6	37.1	Immune	Immune
4	10.0	10.5	Normal	Normal
5	11.1	4.5	Normal	Normal
6	35.5	42.0	Immune	Immune
7	30.7	55.3	Immune	Immune
8	56.5	58.7	Immune	Immune

* Four batches of RNA, one from normal and three from immunized mice, were provided to the coders (*10*), who in turn divided them into eight "unknown" fractions. † Calculated with formula given in Table 1.

with the A/J antigens [on several occasions, such experiments were unsuccessful (*6, 9*)]. To establish objectively the validity of these findings, we performed double-blind experiments (*10*); the results of these experiments demonstrated unambiguously that (i) on the basis of the activity transferred it was possible to correctly determine the identity of unknown RNA extracts and (ii) immune RNA extracts were capable of transferring migration inhibitory capacity to nonimmune cells (Table 2). The precise chemical nature of the "transfer" factor or factors (*11*) present in immune RNA extracts remains to be elucidated.

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References and Notes

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5. Lymph nodes from A/J or CBA mice were homogenized at room temperature in distilled water with a Tri R glass homogenizer, (50 ml of water for the lymph nodes of ten mice). The homogenate was centrifuged at 15,000 rev/min for 30 minutes, and the supernatant was dialyzed against distilled water (two changes of 6 liters) at 4°C for 24 hours. The nondialyzable portion was clarified by centrifugation, lyophilized, and stored at −20°C.
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8. Normal or immune RNA refers to RNA extract prepared from lymph nodes and spleens of nonimmunized C57B1/6J mice or from mice immunized with Sa-1.
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10. RNA fractions for double-blind experiments were coded by Drs. L. Gyenes and P. Ganguli, Department of Microbiology and Immunology, University of Montreal, Montreal, and tested for transfer activity by V. L.
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Innervation of the Hamster Harderian Gland

Abstract. *Harderian glands of male and female hamsters have nerve endings associated with the secretory cells, myoepithelial cells, and the blood vessels. The nerve endings adjacent to the myoepithelial cells also have myoneural junctions.*

The Harderian gland is a compound tubuloalveolar gland, located on the posterior aspect of the eyeball of animals possessing a third eyelid. The ducts of the gland open on the deep surface of the nictitating membrane (*1*). The function of the Harderian gland is not too well understood. At present the Harderian gland is thought to function in some unknown way in controlling pineal activity in immature animals (*2*). Previous investigations of the mammalian Harderian glands showed two major cell types, the secretory cells and the myoepithelial cells. The structure of these cell types is well documented (*3–7*). However, to our knowledge there are no reports on the innervation of the Harderian gland at the ultrastructural level. Our observations of the innervation of the Harderian gland are of twofold significance: (i) to our knowledge, it has never been reported before in any of the ultrastructural investigations of the Harderian gland and (ii) it may help to explain how the Harderian gland is regulated.

Four-week-old hamsters (*Mesocricetus auratus* Waterhouse) of both sexes were killed by cervical dislocation. The Harderian glands were immediately removed, cut into small pieces, and fixed in 5 percent phosphate-buffered glutaraldehyde (*p*H 7.3) for 4 hours, followed by fixation in 1 percent phosphate-buffered osmium tetroxide (*p*H 7.3) and 1 percent aqueous uranyl acetate for 1 hour each. The fixations were carried out at 4°C. The samples were then dehydrated in a graded series of ethanol, followed by propylene oxide, and embedded in Epon 812. Thin sections in the range of 600 to 800 Å were cut with a diamond knife on a Reichert ultramicrotome. The sections were stained with uranyl acetate and lead citrate. They were then examined in a Hitachi HU-11A electron microscope operating at 50 kv.

Unmyelinated nerve fibers follow the blood vessels in the interlobular area of the Harderian gland. Nerve endings with characteristic dense-cored vesicles are observed in the connective tissue adjacent to blood vessels (Fig. 1). Nerve endings are also present in ap-

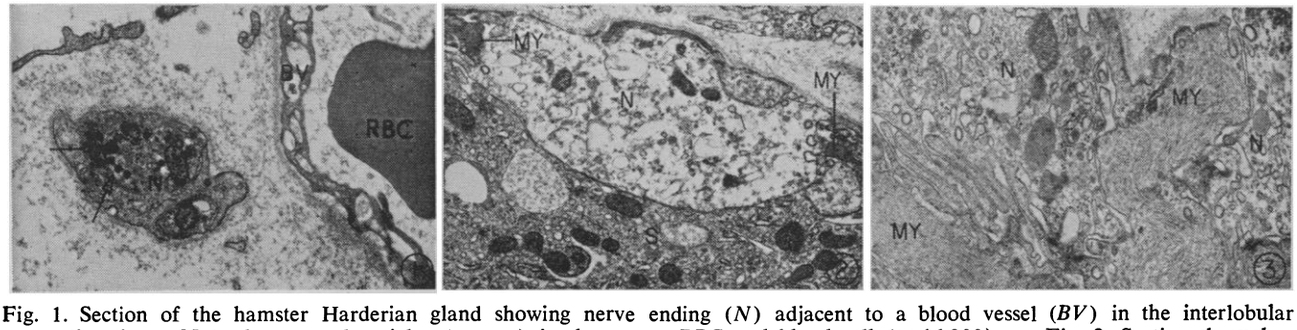

Fig. 1. Section of the hamster Harderian gland showing nerve ending (*N*) adjacent to a blood vessel (*BV*) in the interlobular connective tissue. Note dense-cored vesicles (arrows) in the nerve; *RBC*, red blood cell (× 16,300). Fig. 2. Section through a male hamster Harderian gland showing nerve ending (*N*) in close association with a secretory cell (*S*) and cytoplasmic extensions of the myoepithelial cells (*MY*) (× 9,400). Fig. 3. Section of a female hamster Harderian gland showing close association between nerve endings (*N*) and myoepithelial cells (*MY*) (× 11,400).