downward-growing). Undoubtedly, the interactions between entities account for the emergence of significant modules, or levels, or wholes which have properties other than expected from their components. One of the difficulties is that several different hierarchies can often be found in the same set of entities. When the levels are lasting, they would seem to be formed of stable structures, but Rosen thinks that biological levels are centered on unstable equilibria.

The proceedings of the symposium will be published in book form, and will hopefully stimulate further discussion of the fascinating problems of order and disorder.

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## Immunology of Mouse Mammary Tumor Virus

Several different antigenic components have been found in the virion of the mouse mammary tumor virus (MTV). The MTV antigens fall into two classes: whole virion or coat antigens and soluble antigens. They can be demonstrated by the induction of specific antibodies in rabbits; also, mice proved to be capable of producing antibodies to some of these antigens. These new findings were brought out in a working conference on "Immunology of Mouse Mammary Tumors" held at the Institute for Medical Research, Camden, New Jersey, 11–13 November 1968.

The virion as a whole or a large subunit thereof seems to be capable of inducing precipitating serum antibodies in rabbits and agent-free mice. This reaction was reported by Phyllis Blair (University of California, Berkeley) and was confirmed by several laboratories. Virus from all mouse strains tested by Blair demonstrated a common coat antigen. Robert Nowinski (Sloan Kettering Institute, New York) reported on antibodies to a soluble antigen which presumably is located in the interior of the particle; this fact, also, was confirmed by several other groups. Louis Sibal (National Cancer Institute) reported finding two antigens after treating virion preparations with Tween-ether. With the aid of immunofluorescence, Peter Bentvelzen and J. H. Daams (Netherlands Cancer Institute, Amsterdam) found an antigen thought to be an early protein of the virus, in hemopoetic organs and in the mammary gland of infected mice. In the GR strain of mice, in which the MTV cannot be eliminated by foster nursing, this antigen was found in all organs. Using both immunological methods and bioassays, these investigators demonstrated that MTV can be released after irradiation or treatment with urethane in mouse strains which were assumed to be without MTV.

The opening session of the conference was chaired by Werner Henle (University of Pennsylvania, Philadelphia) who discussed similarities and differences of MTV and the myxoviruses. These complex viruses, rich in lipids, have much in common.

Chemically induced premalignant tissues of the mammary gland, which have been serially transplanted for more than 4 years, proved to contain new antigens, which are not MTV-derived, in spite of the presence of the virus. In this work reported by Glenn Slemmer (Institute for Cancer Research, Philadelphia), no difference in antigenicity could be detected between these premalignant tissues and the tumors which arose in them. Transplants of the premalignant tissues used to immunize the animals frequently gave rise to normal outgrowths. This was probably due to selection of a normal cell population present from the beginning in the transplant. Obviously, these normal cells do not contain the new antigens.

MTV antibody production in mice was a major topic of the conference. For many years after the discovery of the milk agent, attempts to demonstrate antibodies in mice were unsuccessful. It was believed that MTV was nonantigenic in mice. Contrary to these earlier results, MTV antibodies in mice were reported from almost all laboratories. With the possible exception of the GR strain of mice, there is no true tolerance to MTV. In most cases the antibodies were to the whole virion, or a major component of it, because treatment of the antigen with ether resulted in loss of the immunodiffusion line. Blair, who first demonstrated precipitating MTV antibodies in mice, usually used a few small inoculations of impure preparations from mammary glands without adjuvant. She was able to demonstrate antibodies to what appeared to be the whole virion. Otto Plescia and M. Menon (Rutgers University) reported on the enhancement of antigenic reactions caused by coupling the MTV virion with a strong antigen, such as bovine serum albumin. The reports from the various laboratories emphasized the dependence on methodology for detection of antibodies in mice. Some procedures seemed to induce antibodies to the whole virion coat and still others to a soluble internal antigen, as well as the coat. Weekly intramuscular inoculations of a virus in complete Freund's adjuvant in adult C57BL male mice, followed by a final booster without adjuvant, and bleeding 3 to 4 days later, gave, even after ten inoculations, no discernible antibodies by immunodiffusion test according to Dan Moore and Jesse Charney (Institute for Medical Research, New Jersey). However, when cells from an induced tumor were grown intramuscularly in isologous agent-free, C57BL/Haag mice, a good antibody response was obtained when the absorbed mouse sera were tested against purified virions. However, if the virions were pretreated with ethyl ether, no precipitation line was observed, thus indicating that the antibodies were against the whole virion or one of its major ether-sensitive components.

In another immunizing procedure, male and female mice from several strains were given a single, intraperitoneal injection of purified virus in complete Freund's adjuvant. A small booster dose of virions was given 90 days later. (This procedure has been shown by Sibal *et al.* [*Proc. Soc. Exp. Biol. Med.* **127**, 726 (1968)] to be effective in producing good titer antiserum to Rauscher virus.) Seven days after the booster inoculation, all the sera contained antibodies. Females in all strains gave stronger immunodiffusion lines than their littermate males. Strains BALB/c and RIIIf gave stronger lines than did types Af and C57BL.

The effect of thymectomy was reported by Edmund Yunis (University of Minnesota). Thymectomizing neonatal mice of high cancer strain C3H caused a decrease in incidence and a delayed development of mammary tumors. The question of tolerance and tumor development was discussed at some length. It was hypothesized that MTV-associated antigens cause a breakdown in tolerance followed by (i) a virus-host cell interaction; (ii) damage associated with immune response; and (iii) development of malignancy. The way in which thymectomy delays and decreases tumor incidence may be in

decreasing the rate of tolerance breakdown. The introduction into neonatally thymectomized mice of lymphoid cells from MTV-negative mice facilitated the development of malignancy, whereas MTV-positive cells were much less effective. Yunis believes that the development of malignancy depends on the existence in the host, first of a state of tolerance for viral-induced antigens, followed by a breakdown of tolerance. It was concluded that the thymus plays an important role in MTV infection, tolerance, and tumor development. However, ultrastructural observations by Karl Hollmann (College de France, Paris) of thymuses from high and low cancer strains indicated that the replication of B particles was found only in one strain—New Zealand black, a strain noted for autoimmune disease.

Substantial progress in decreasing the time required for assaying MTV was reported. Ouchterlony-type immunodiffusion plates are now being used in all laboratories to test for the various viral antigens, and Nowinski has shown that the milk from individual mice of high cancer strains contains a soluble MTV antigen, designated as S1, when tested against antiserum of rabbits immunized with purified virus. Milk from mice of low cancer strains known to be free of infectious virus were free of S1 antigen. A combination of biological and immunodiffusion (I.D.) assays, which overcomes the insensitivity of the latter, was reported by Jesse Charney (Institute for Medical Research, New Jersey). The MTV infectivity of any milk or other material can be titered by inoculating young, agent-free mice with serial dilutions of the infective preparations and testing the milk by I.D. after the first, second, and third litters. Fully-infected C57BL/Haag mice show positive I.D. lines in 50 percent of the milks at the first lactation, in 70 percent at the second lactation, and 87 percent at the third lactation. Approximately 85 percent of all mice that show positive I.D.'s at the second or third lactation eventually develop tumors. Mice negative at the third lactation remained free of tumors. Thus, an infectivity test for MTV can be made in 12 weeks (from inoculation to first lactation) or 18 weeks (to third lactation). In an experiment where groups of mice were inoculated at 2, 4, 8, and 12 weeks of age, it was found that when inoculated at 8 weeks, few were positive at the first lactation and still fewer were positive when inoculated at 12 weeks of age. However, by the third lactation all groups were 80 to 90 percent positive irrespective of when they were inoculated. The time required to reach the point where 50 percent had positive milks was 16, 14, 11, and 9 weeks for the respective groups inoculated at 2, 4, 8, and 12 weeks of age. When newborns were given the same inoculum, only 31 percent were positive even at the third lactation.

Table 1. Assay procedures for MTV bioactivity and MTV antigens.

| Test | Time |
|---|---|
| *Bioactivity* | |
| Development of tumors | 10 to 20 months |
| Development of nodules | 16 to 20 weeks |
| Development on antigen in milk | 9 to 16 weeks |
| *Antigens* | |
| Immunodiffusion | 1 to 4 days |
| Hemagglutination inhibition | 1 day |

A hemagglutination-inhibition test for MTV antigen was reported by Louis Sibal and William Feller (National Cancer Institute). After adsorption of Ficoll-$D_2O$ gradient, purified viral antigen onto tanned sheep RBC, the cells are agglutinated by rabbit antibody to MTV. Inhibition of this reaction by preparations containing MTV forms the basis of a sensitive, quantitative assay for MTV in mouse milk. This rapid test for MTV antigen is much more sensitive than any of the Ouchterlony-type tests, being sensitive for milks diluted to more than $10^{-3}$. Just which antigen or antigens are involved is not yet clear but pretreatment with ether increases the sensitivity.

Improved methods for preserving and isolating MTV were reported by William Hall (National Cancer Institute). Large batches of mouse milk can be separated on a sucrose gradient in a zonal rotor (Anderson B XIV) without precipitation of the virions into a pellet. Pelleting and resuspension are known to disrupt many of the particles. In Hall's procedure the concentrated virion band from the zonal rotor is layed over tubes containing a Ficoll-heavy water gradient. This second centrifugation results in a narrow band which appears to be highly purified, biologically active virus particles.

Treated in this fashion the particles band with a buoyant density of 1.156 g/cm$^3$ and have a calculated sedimentation coefficient of 1400*S*. If, however, the virions are isolated by use of Ficoll gradient alone, without use of sucrose and heavy water, according to Moore, they have a buoyant density of about 1.10 g/cm$^3$ and a sedimentation coefficient of about 900*S*. The particles are not homogeneous in density nor in sedimentation coefficient. Their apparent densities and sedimentation rates are sensitive to diffusible molecules and osmotic changes since they are membranous sacs containing a high percentage of water. As they move down in a heavy-water gradient, their apparent density is increased by exchange of heavy for light water. Sucrose, by osmotic action, causes a decrease in particle volume and thus an increase in apparent density. Sucrose molecules may also enter the aqueous compartment or adhere to the membranes.

Chester Southam (Sloan-Kettering Institute, New York) described techniques for demonstrating cell-based immunity to tumor cells in vitro. By time-lapse photography he demonstrated how the sensitized lymphocytes attacked tumor cells growing in monolayer cultures.

Vittorio Defendi (University of Pennsylvania, Philadelphia) presented data on the antigens of the SV40-polyoma system and provided new techniques for studying MTV.

A lively session on MTV assay procedures was chaired by W. Ray Bryan (National Cancer Institute). A review and a statistical analysis of data on an assay based on the early development of hyperplastic alveolar nodules in inoculated mice was presented by William Hall and John Gart (National Cancer Institute) and John Verna (Melpar, Inc., Falls Church, Virginia). Plans were made to compare the new assay methods based on testing milks at early lactations by Ouchterlony and hemagglutination inhibition procedures with the nodule test so that a standard test can be recommended (Table 1).

The bioactivity test for the development of antigen in milk depends on one of the antigen tests. The sensitivity of the three bioactivity tests are about the same. They can detect activity in C3H or RIII milk at dilutions of $10^{-5}$ to $10^{-7}$. The immunodiffusion test is sensitive at milk dilutions of no greater than $10^{-1}$ and the hemagglutination test at dilutions of more than $10^{-3}$.

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