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In Vitro Transformation of Lymphoid Cells by Abelson Murine Leukemia Virus

(murine fetal liver/B-lymphocytes/surface immunoglobulin/mercaptoethanol/ murine lymphosarcoma)

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ABSTRACT **Cell cultures prepared from fetal murine liver were infected by Abelson murine leukemia virus. After about 2 weeks, proliferating cells of lymphoid morphology appeared in some of the cultures. Addition of 2-mercaptoethanol to the initial culture medium greatly enhanced the appearance of the lymphoid cells. Immunoglobulin determinants were evident on the cells in some cultures. Continuous passage of the cells in certain cultures was possible and the passaged cells could form tumors after animal inoculation. Because Abelson murine leukemia virus is able to induce *in vitro* malignant transformation of lymphoid cells, it probably causes leukemia by directly affecting cellular growth control.**

The type-C RNA tumor viruses cause leukemia in a large number of species, yet the mechanism of leukemogenesis has remained unclear partly for lack of *in vitro* transformation systems for hematopoietic cells. Several isolates of murine leukemia virus, typified by the Moloney leukemia virus (M-MuLV) and Gross virus, cause tumors of lymphoid tissues originating in the thymus after a latent period of 3–6 months (1). These viruses replicate *in vitro* but do not transform the infected cells or cause alteration in cellular growth control.

Abelson and Rabstein (2) isolated an RNA tumor virus, Abelson leukemia virus (A-MuLV), which, in association with M-MuLV, causes a rapidly progressive lymphosarcoma or leukemia that does not involve the thymus. The tumor cells all lack θ antigen (3), an antigen found on M-MuLV-induced tumors (4). Some of the A-MuLV-induced tumors are probably of bone marrow-derived (B-cell) origin because they have immunoglobulin (Ig) on their surface (3). The majority of the cells, however, have neither Ig nor the C3 complement receptor of B-cells (3) and may represent a population of malignant null cells.

Scher and Siegler (5) have shown that A-MuLV directly transforms NIH/3T3 cells *in vitro*. A-MuLV was shown to be defective for replication and to require a helper virus, such as M-MuLV, in order to replicate (5).

Since A-MuLV has a transforming activity for 3T3 cells and causes the rapid appearance of lymphoid tumors *in vivo*, this agent was used to study the effect of a leukemia virus on hematopoietic cells. Murine fetal liver contains a population of cells able to restore complete hematopoietic function to lethally-irradiated allogeneic recipients (6) and, therefore, represents a rich source of hematopoietic stem cells. We now show that A-MuLV infection of fetal liver cell cultures induces rapid proliferation of lymphoid cells some of which have Ig determinants on their surface. These cells can be subcultured *in vitro* and induce tumors in animals.

Abbreviations: M-MuLV, Moloney leukemia virus; A-MuLV, Abelson leukemia virus; B-cell, bone marrow-derived lymphocyte Ig, immunoglobulin.

MATERIALS AND METHODS

Cells and Culture Methods. Cell cultures were prepared from the livers of embryos from 12- to 16-day pregnant Swiss or Balb/c mice. Livers from the embryos of one mother were pooled for each experiment. The livers were shredded using a stainless steel mesh and the cells were suspended in medium containing RPMI-1640 and 20% fetal calf serum (Irvine Scientific Co.) that had been heat inactivated at 56° for 30 min. Antibiotics were not used. In some experiments, 2-mercaptoethanol (Aldrich Chemical Co.) was added to a final concentration of 50 μM. About 7.5×10^5 nucleated cells were plated in 30 mm plastic petri plates (Nunc) in 1.5 ml of medium.

The cultures were maintained at 37° in a humidified 5% CO_2 atmosphere. They were fed weekly by the addition of 1 ml of medium. When medium completely filled the dish, spent medium was centrifuged to retain any cells that might be in the medium and the cells were returned to the cultures.

Infection of Cell Cultures. A-MuLV was prepared from a cloned NIH/3T3 nonproducer cell line (ANN-1) (5) infected with clonal M-MuLV (7). A-MuLV virus preparations had titers of 5×10^3 to 5×10^4 focus-forming units/ml by the NIH/3T3 transformation assay (5) and a titer of 1 to 2×10^6 plaque-forming units by the XC plaque assay with NIH/3T3 cells (8). These preparations induce Abelson leukemia in animals (unpublished data). For treating mock-infected control cultures, conditioned medium was harvested from rapidly growing cultures of an A-MuLV transformed-nonproducer cell line (ANN-1) (5). Clonal M-MuLV, grown on NIH/3T3 cells, had a titer of 1 to 5×10^6 plaque-forming units/ml after dilution in ANN-1 medium. These virus preparations induced thymic lymphoma in mice (unpublished data). Both virus preparations and the conditioned medium were filtered through 0.45 μm Millipore filters prior to use. Cells cultures were infected 1–4 hr after plating with 0.5 ml of A-MuLV stock, M-MuLV, or treated with 0.5 ml of conditioned medium.

Detection of Surface Immunoglobulin. The presence of Ig on the surface of transformed cells was detected by direct

TABLE 1. *Transformation of hematopoietic cells by A-MuLV*

		Virus replication			
		Transformed cultures		Nontransformed cultures	
	Cellular transformation	A-MuLV (FFU/ml)	M-MuLV (PFU/ml)	A-MuLV (FFU/ml)	M-MuLV (PFU/ml)
A-MuLV	Yes	$1–5 \times 10^3$	$5–10 \times 10^5$	$<10^1$	$5–10 \times 10^3$
M-MuLV	No	—	—	0	$5–10 \times 10^3$
Conditioned medium	No	—	—	0	0

Supernatants of all cultures were removed 3 weeks post infection and tested for the presence of focus-forming virus (A-MuLV) and plaque-forming virus (M-MuLV) using the NIH/3T3 transformation assay and the XC plaque assay.

FFU, focus-forming units; PFU, plaque-forming units.

immunofluorescent staining. Cells proliferating in the medium of transformed cultures were centrifuged and 5×10^6 cells were suspended in 0.2 ml of Dulbecco's modified Eagle's medium supplemented with 2% heat inactivated fetal calf serum. Fluorescein-conjugated anti-mouse Ig (0.1 ml) was added and the cells were incubated on ice for 20 min. The cells were centrifuged, washed in a 100-fold excess of phosphate buffered saline and mounted for microscopic examination. For all experiments, control spleen and thymus cell suspensions from freshly sacrificed mice were included. In all cases, thymus cell suspensions contained no cells which reacted with the fluorescein-conjugated antibody. Spleen suspensions contained 35–50% Ig positive cells.

Fluorescein-conjugated anti-mouse Ig was obtained from three sources. An equal mixture of anti-IgA, -IgM, and -IgG (Meloy Laboratories) was used in most experiments. Two preparations of anti-mouse Ig prepared from ammonium sulphate precipitation of whole mouse serum (Progressive Laboratories and Dr. E. Unanue, Harvard Medical School) were used in several experiments. All preparations gave comparable results.

Tumor Induction. Adult Balb/c mice were injected intraperitoneally with 0.5 ml of Balb/c lymphoid cells that had been transformed by A-MuLV and passaged with mercaptoethanol. Some mice received a priming injection of either 0.5 or 1 ml of pristane (2,6,10,14-tetramethylpentadecane) (Aldrich Chemical Co.) intraperitoneally 1–3 weeks prior to the injection of the cells. Moribund animals were sacrificed and autopsied. Smears of ascitic fluid and touch preps of tumors were made and stained with Wright-Giemsa. In addition, tumors were fixed and prepared for histology (9).

RESULTS

Transformation of Fetal Liver Cells. Murine fetal liver cells from Balb/c or Swiss mice were infected with A-MuLV which was rescued from the nonproducer cell line ANN-1 by M-MuLV. Mock-infected cultures were treated with medium conditioned by growth of the non-producer cell line. Parallel cultures were infected by M-MuLV alone. M-MuLV was diluted 1:10 in ANN-1 conditioned medium because cultures plated in the absence of conditioned medium showed poor survival.

Cultures infected with A-MuLV were sacrificed at intervals post-infection and the morphology of the cells in the monolayer and medium was examined after Wright-Giemsa staining. Twenty-four to 48 hr after plating, monolayers composed of epithelioid and fibroblastic cells had been formed. A significant number of cells with the morphologic characteristics of macrophages was also seen. Twenty-four to 72 hr after plating, cells of the erythroid series were the predominant nonadherent cell type. After 4–6 days, neutrophilic granulocytes became the predominant nonadherent cell type. These granulocytes were present until 10–14 days post-infection.

Dense areas of small round cells were noted in a few of the A-MuLV-infected cultures 10–15 days post-infection. In such cultures, nonadherent cells proliferated rapidly, becoming the predominant cell type in the culture and reaching a density of 3 to 5×10^6 cells per ml in 5–7 days. For the purposes of discussion we will refer to the process of generating these cells as "transformation" and to the cells as "transformed" cells.

In cultures injected with M-MuLV alone or mock-infected, development of cell types followed the same pattern as in the A-MuLV-infected cultures for the first 10 days. Thereafter, no evidence of transformed cells was ever found. The granulocytic cells disappeared by day 14 leaving adherent fibroblastic and epithelioid cells which could be maintained for 3 months or more.

Media from all cultures were assayed for the presence of infectious virus. A-MuLV-infected cultures replicated both A-MuLV and M-MuLV (Table 1). Cultures containing the transformed non-adherent cells produced much higher titers of both A-MuLV and M-MuLV than A-MuLV-infected cultures that were not transformed. M-MuLV-infected cultures replicated M-MuLV with an efficiency comparable to the M-MuLV component in A-MuLV-infected cultures which did not transform.

Cell cultures from Balb/c and Swiss mice transformed with equal efficiency.

Morphology of Transformed Cells. Cells transformed by A-MuLV had the appearance of lymphoblasts when examined by light microscopy (Fig. 1). The majority of these cells had a relatively large nucleus with a poorly defined nucleolus. The small rim of cytoplasm contained no granules but vacuoles were often noted. The leukocyte peroxidase reaction, used to identify cells of the myeloid series, was consistently negative. These *in vitro* transformed cells resembled closely the ascites form of tumors induced *in vivo* by A-MuLV.

To confirm the lymphoid nature of these cells, a representative transformed culture was fixed and processed for electron microscopy (10) at 2.5 weeks after infection. Approximately 95% of the cells had lymphoid characteristics (Fig. 2) whereas 5% had the appearance of macrophages. The lymphoid cells had a relatively large nucleus with scanty cytoplasm. The

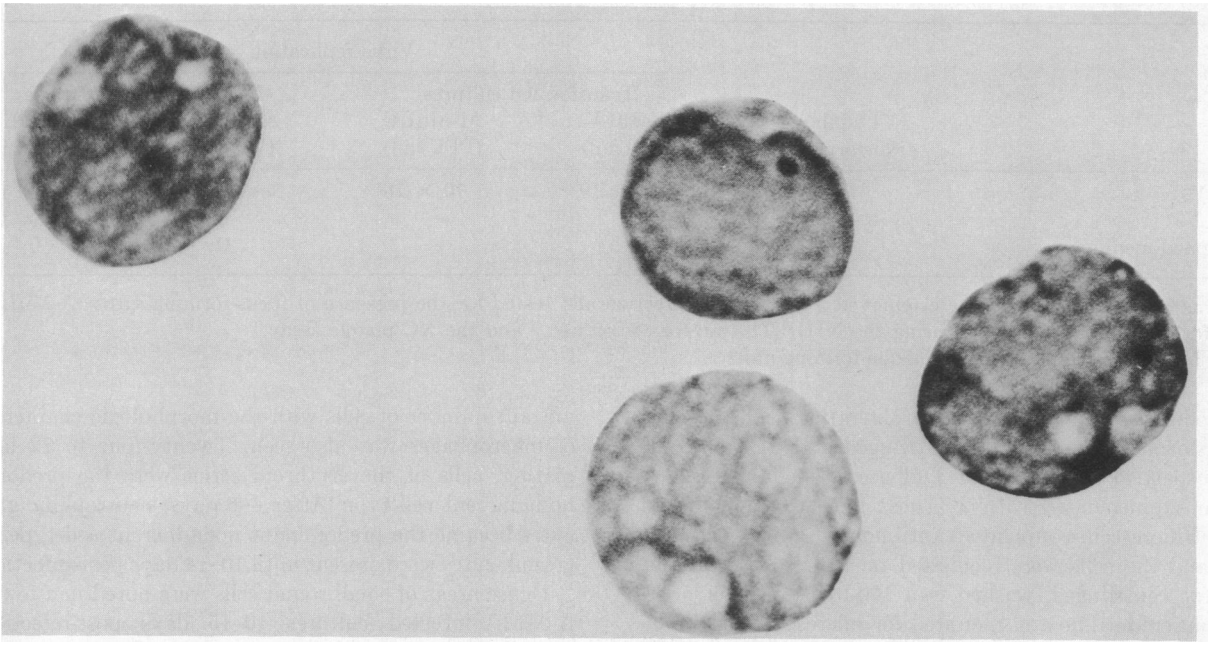

FIG. 1. Transformed cells stained with Wright-Giemsa. These cells have the characteristic appearance of lymphoblasts (×1000).

chromatin was of a fine pattern and an ill-defined nucleolus was often evident. The cytoplasm contained many free ribosomes and little rough endoplasmic reticulum. No phagocytic vacuoles, lysosomes, or glycogen granules were noted.

Enhancement of Transformation. Under the culture conditions used above, A-MuLV was able to transform lymphoid cells in only 3–4% of the infected cultures. To attempt to increase the frequency of transformation, we added 2-mercaptoethanol to the culture medium because this reducing agent has been shown to enhance the viability of normal lymphocytes *in vitro* (11) and is a requirement for the culture of some lymphoid tumor cells (12, 13). From 70–100% of A-MuLV infected cultures grown in medium containing 50 μM mercaptoethanol become transformed (Table 2). Transformed cells were not detected in the M-MuLV-infected or mock-infected mercaptoethanol-treated cultures in any experiments.

Although the frequency of cellular transformation was enhanced by the addition of mercaptoethanol, mercaptoethanol did not affect the latent period for the transformation event. The initial rate of lymphoid cell proliferation in mercaptoethanol-treated and untreated cultures was also approximately equal with the cells reaching densities of 3 to 5 × 10^6 cells per ml 5–7 days after transformation was first noted.

TABLE 2. *Effect of mercaptoethanol on transformation of lymphoid cells*

	Frequency of transformation*	
	With mercaptoethanol	Without mercaptoethanol
A-MuLV	21/30	1/30
M-MuLV	0/15	0/15
Conditioned medium	0/15	0/15

* Number of cultures with transformed lymphoid cells/total cultures infected.

The ability of the transformed lymphoid cells to proliferate after subculture, however, depended upon the presence of mercaptoethanol. Cells from mercaptoethanol-treated cultures were harvested after achieving a density of 3 to 5 × 10^6 cells per ml and were routinely passaged every 3–4 days by transferring 5 to 10 × 10^4 cells per ml into 10 cm plates. An approximately 10-fold growth of cells occurred between passages. Cells from cultures grown without mercaptoethanol could be passaged only after maintenance at high density for long periods of time (up to 2 months). While every mercaptoethanol-treated culture tested could be readily subcultured, only 1 of 9 untreated cultures produced cells which could be passaged. Passaged cultures from either source are relatively homogeneous, with lymphoblasts proliferating in the culture medium and no adherent cells.

In a significant proportion (up to 50%) of the A-MuLV-infected untreated fetal liver cells, a second, nonlymphoid cell was noted 3–4 weeks after infection. These cells appeared in mercaptoethanol-treated cultures infrequently but never appeared in M-MuLV-infected or mock-infected cultures. They were larger than lymphoid cells and had many fine basophilic cytoplasmic granules. Their nucleus was occasionally acentric with a sharply defined nucleolus. These cells, which resemble mast cells, may represent a second class of A-MuLV transformants.

TABLE 3. *Presence of immunoglobulin on A-MuLV-transformed lymphoid cells*

Source of lymphoid cells	Frequency of immunoglobulin positive cultures*	% Cells positive in positive cultures
Transformed culture − mercaptoethanol	4/6	60–90
Transformed culture + mercaptoethanol	2/11	60–70

* Number of cultures with positive cells/total cultures tested.

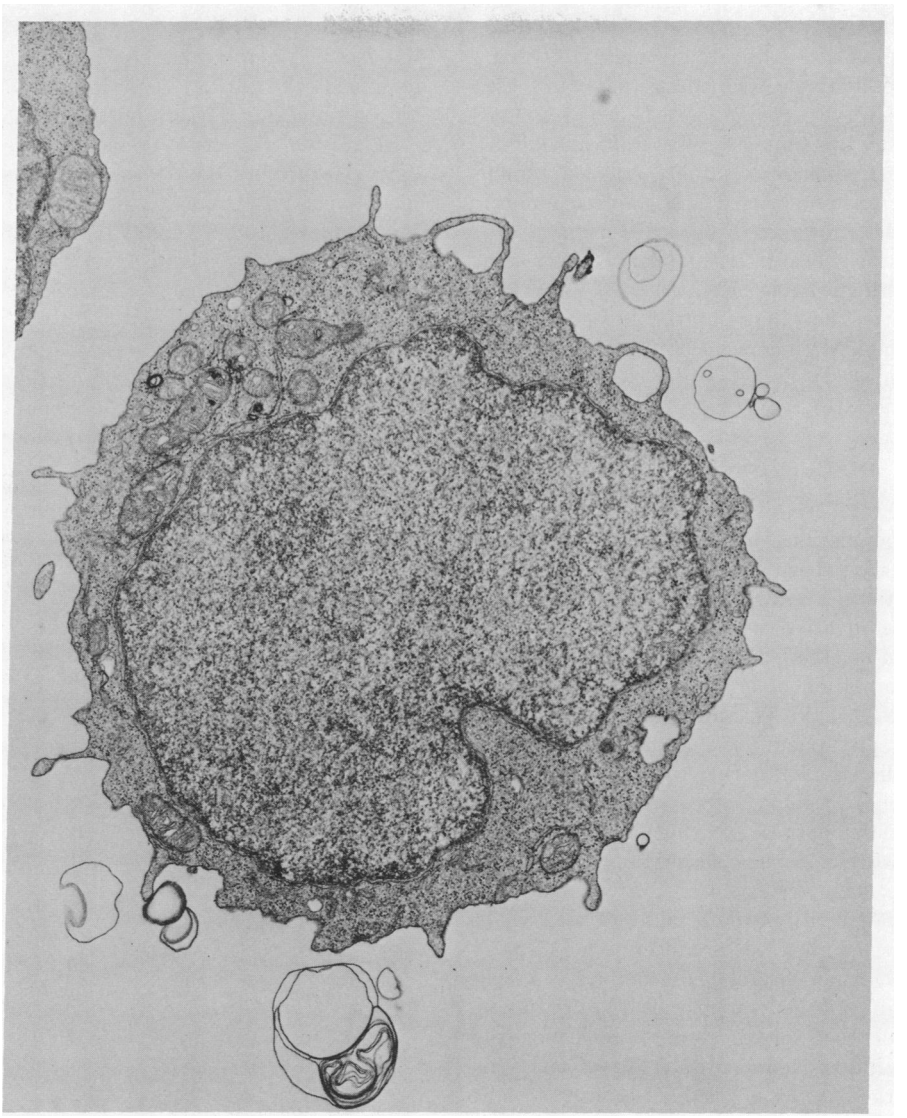

FIG. 2. Electron micrograph of a transformed cell. Cells were fixed in 2.5% glutaraldehyde and post-fixed in 1.3% osmium tetroxide (10) (×22,000).

Immunoglobulin on the Surface of Transformed Cells. The A-MuLV transformed lymphoid cells were screened for the presence of surface Ig, a characteristic of B-cells. A high percentage of the transformed fetal liver cultures grown in the absence of mercaptoethanol had cells present in the medium with Ig determinants on their surface (Table 3). In such positive cultures, 60–90% of the cells had Ig. Only 20% of the cultures grown in the presence of mercaptoethanol had surface Ig. As in cultures not treated with mercaptoethanol, 60–70% of the cells in the positive cultures had Ig determinants. Thus, B-cells were initially present in these transformed cultures. Two pools of passaged cells lacked detectable surface Ig at one month after initial subculture. Cytotoxicity assay (14) of one of these pools, derived from a transformed Balb/c fetal liver culture demonstrated that these cells also lacked the θ antigen characteristic of thymus-derived lymphocytes (T-cells) (4). These cells had the H-2^d histocompatibility antigen found in Balb/c mice. This mass culture of A-MuLV transformants may be a population of null cells.

Tumor Induction by the Transformed Lymphoid Cells. Transformed lymphoid cells isolated from Balb/c fetal liver and subsequently passaged *in vitro* were injected intraperitoneally into adult Balb/c mice to assay for tumorigenicity. Because pristane has been shown to enhance passage of primary Abelson tumors in Balb/c mice (3), some mice received a priming injection of 0.5 or 1 ml of pristane 1–3 weeks prior to the injection of the A-MuLV-transformed cells. Solid tumors and ascites developed in all of the mice within 24 days of cell inoculation (Table 4).

TABLE 4. *Tumorigenicity of A-MuLV transformed lymphoid cells*

Inoculum (cells/mouse)	Mean latent period (days)		Frequency of tumors*	
	Pristane-primed	Unprimed	Pristane-primed	Unprimed
10×10^6	10	11	9/9	7/7
1×10^6	12	18	4/4	14/14

* Number of animals with tumor/number of animals examined.

Histologic examination of the tumors showed that they were composed of lymphoblasts infiltrating connective tissue, muscle, fat, and pancreatic tissue. The mitotic index in these rapidly growing tumors was approximately 1.6%. The ascites fluid also contained many lymphoblasts. No significant difference was noted in the frequency or latent period of tumor development between pristane-primed and unprimed mice. The solid tumor masses, however, were larger in pristane-primed animals. Both the solid tumors and ascites cells were morphologically indistinguishable from A-MuLV-induced tumors passaged *in vivo*. The tumors appear to be of donor origin because virus-induced tumors do not arise before the sixth week in adult mice (2), and A-MuLV does not cause ascites in unprimed mice (15).

DISCUSSION

A-MuLV infection of fetal liver is able to induce the continuous growth of populations of lymphoid cells. These lymphoblastoid cells have been maintained in culture for more than 30 generations, and are malignant because they cause rapidly growing invasive tumors. We consider these properties to define an *in vitro* transformation event induced by A-MuLV. Transformation of lymphoid cells *in vitro* by A-MuLV demonstrates that this virus can act directly to alter the growth properties of lymphoid cells and therefore probably causes leukemia by directly interfering with normal growth and differentiation.

The A-MuLV stock used in these experiments was a mixture of A-MuLV and M-MuLV. No transformation was demonstrable by M-MuLV alone nor was transformation demonstrated in cultures treated with medium that had been conditioned by A-MuLV transformed nonproducer cells (ANN-1). Thus, the A-MuLV component rescued from ANN-1 cells appears to be responsible for the lymphoid cell transformation.

Mercaptoethanol increases the transformation frequency of A-MuLV-infected cultures. This reducing agent may act either by increasing the number of viable cells susceptible to transformation or may allow more transformed cells to replicate and become evident. We do not know if transformed cells appearing in mercaptoethanol-treated cultures are functionally different from those in untreated cultures.

The presence of Ig on the surface of some transformed cells indicates that these cells are of B-cell origin and further supports the finding that the A-MuLV can cause B-cell tumors (3). Most A-MuLV tumors lack Ig, C3 receptor, and θ antigen indicating that they are malignant null cells. Some of our A-MuLV-transformed cells are also similar to null cells because they lack Ig determinants and θ antigen.

In vitro stimulation of hematopoietic cell growth has been achieved in other systems although complete transformation to malignant cells has not been previously reported. Avian myeloblastosis virus has been shown to transform myeloid cells from chick embryo (16, 17). As with the A-MuLV complex, this virus is probably a mixture of defective transforming virus and helper (18). Although avian myeloblastosis virus alters the growth of myeloid cells *in vitro*, these cells do not form tumors in animals (19). The Epstein-Barr virus allows the establishment of permanent lines of human or primate lymphoid cells (20, 21). The tumorigenicity of these cells has not been conclusively established (22).

Sklar *et al.* (23) have previously infected short-term (4–6 days) cultures of mouse splenocytes with A-MuLV. After inoculation into animals, the cultured cells developed into lymphoblastic tumor cells. Their system differs from ours in that cells from pristane-primed adult animals were infected and animal inoculation was required for transformation to be recognized.

The work presented here demonstrates that A-MuLV causes lymphoid cells to undergo a malignant transformation *in vitro*. The early events in lymphoid cell transformation by leukemia virus can now be studied under controlled conditions.

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