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Spontaneous Induction of Endogenous Murine Leukemia Virus-Related Antigen Expression During Short-Term *In Vitro* Incubation of Mouse Lymphocytes

(group-specific antigen/immunofluorescence/murine sarcoma cells/murine leukemia virus)

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ABSTRACT **Short-term lymphocyte cultures from mouse thymus, spleen, or lymph nodes were studied for the presence of murine leukemia virus group-specific antigens with an immunofluorescence test using rat immune sera against syngeneic cells infected with the radiation leukemia virus or its pseudotype of murine sarcoma virus and goat and rabbit antisera against purified murine leukemia virus group-specific antigen. Antigens reacting with these sera appeared in the cultured lymphocytes within 24 hr, and the proportion of immunofluorescent-positive cells increased to 25–80% by the second or third day of cultivation, concomitantly with a decrease in cell viability. The appearance of these antigens can be suppressed by inhibitors of DNA (mitomycin-C), RNA (actinomycin-D, cordycepin, and polyadenylic acid), and protein (cycloheximide) synthesis. No infectious virus could be detected by the immunofluorescence and XC-cell tests. The observed phenomenon appears to represent the spontaneous partial derepression of endogenous murine leukemia virus replication in lymphocytes during short-term *in vitro* cultivation.**

During short-term *in vitro* cultivation of mouse thymocytes and lymphocytes, we have observed quite unexpectedly that murine leukemia virus (MuLV)-related antigens, demonstrable by immunofluorescence (IF), appeared spontaneously in the cultured cells. A series of experiments relating to this observation comprises the subject of the present report.

MATERIALS AND METHODS

Animals. C57BL/Ka, C3H/DiSn, BALB/c/J, 129, AKR/J, and NIH/Swiss mice of both sexes were used at 2 months of age.

Lymphocyte Cultures. Lymphocyte suspensions prepared from thymus, spleen, and lymph nodes were cultured in 60 or 35 mm Falcon tissue culture dishes at a cell density of 2×10^7 or 7×10^6 cells per dish, respectively, in Eagle's minimal essential medium (MEM) plus 10% fetal-calf serum, supplemented with 1% 200 mM glutamine and antibiotics.

IF Test for the Detection of MuLV Antigens. Indirect immunofluorescence staining of acetone-fixed cells was carried out as described by Hilgers *et al.* (1). The percentage of fluorescent cells was counted over a range of dilutions of virus-specific immune sera. In addition to six dilutions of a given immune serum, each slide included normal serum and phosphate-buffered saline (PBS) controls. (Values presented are the means of three data points in the plateau region of the dilution curve.)

Sera. MuLV-specific immune serum 147 was obtained from Fischer inbred rats immunized against a Fischer rat transplantable lymphoma induced by the radiation leukemia virus (RadLV) (2). Sera 117 and 151 were prepared in W/Fu rats against a W/Fu fibroblast line (W/FuSL) transformed by and producing the RadLV-rescued pseudotype (3) of murine sarcoma virus [MSV(RadLV)]. Such sera are known to contain multivalent antiviral and cytotoxic antibodies (4, 5).

The two "gs-specific" immune sera prepared against the group-specific (gs) antigen fraction of MuLV (6), purified by polyacrylamide gel electrophoresis and electrofocusing (7), were kindly supplied by Dr. Raymond V. Gilden, Flow Laboratories, Inc.: one was produced in rabbits (pool-1, NIH-NCI.E, 71-2097) and contained principally intraspecies specificity; the other (F-35647) was produced in goats and contained principally interspecies specificities (8).

Fluoresceinated Antisera. Fluorescein-conjugated goat anti-rat globulin (Hyland Laboratories) was used with the rat anti-MuLV sera. Fluoresceinated goat anti-rabbit and burro anti-sheep globulins were used with the rabbit and the goat anti-MuLV-gs sera, respectively. These were a gift from Drs. M. Julius and L. A. Herzenberg, Stanford University.

Cell Lines. BL-5 is an established C57BL/Ka mouse-embryo fibroblast line; BL-5(RadLV) is a subline of BL-5 stably infected with RadLV (9); W/7 is a C3H.SW-3T3 line; W/7-GLV is a subline of W/7 permanently infected with Gross leukemia virus (GLV); Cl.18.4 is a C3H myeloma cell line; and Tl.M.14 and El.4 Bu are both C57BL lymphoma cell lines. The three last-named cell lines, all of which express MuLV antigens, were obtained from the Cell Distribution Center, Salk Institute, La Jolla, Calif.

Virus Isolation. Moloney leukemia virus (MLV) and its murine sarcoma virus pseudotype [MSV (MLV)], harvested from the supernatant fluids of a stably infected rat fibroblast cell line, 78A1, by ultracentrifugation and sucrose gradient purification, were a gift from Owen Witte (10). The purified virus was disrupted with 0.5% Triton X-100 and used as a source of antigen in Ouchterlony double diffusion tests.

Antigen Extracts from Infected and Noninfected Cells. Extracts from cells disrupted by alternate freezing and thawing were suspended in deoxycholate-Triton X-100 KCl and purified by ultracentrifugation, as described by Parks *et al.* (11).

Separation of T- and B-lymphocytes. Thymus-dependent lymphocytes (T-cells) from mouse lymph nodes were isolated by removing the B-lymphocytes by nylon wool column filtration (12). Bursa-equivalent lymphocytes (B-cells) were isolated from the same source by cytotoxic destruction of the T-cells with anti-θ serum plus complement, followed by the isolation of dead and live cells on albumin gradients (13).

Abbreviations: MuLV, murine leukemia virus; RadLV, radiation leukemia virus; MSV, murine sarcoma virus; MSV (RadLV), RadLV-rescued pseudotype of MSV; MLV, Moloney leukemia virus; MSV(MLV), the murine sarcoma virus pseudotype of MLV; gs, group-specific antigen; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; IF, immunofluorescence; T-cells, thymus-dependent lymphocytes; B-cells, bursa-equivalent lymphocytes.

TABLE 1. *Appearance of neoantigens in C57BL/Ka lymphocytes after in vitro incubation*

Tissue	Fresh Cells		Incubated cells*	
	% MuLV+	% Viability	% MuLV+	% Viability
Thymus	0	96	43	47
Spleen	1	85	45	46
Lymph node (axillary, inguinal, popliteal cervical)	0	90	74	16
Lymph node (mesenteric)	2	92	73	26

* 48 hr at 37°.

The number of dead cells was determined by trypan blue exclusion.

Metabolic Inhibitors. Mitomycin-C, cycloheximide, cytosine arabinoside, cordycepin (Sigma Chemical Co., St. Louis), actinomycin-D (Cosmegen, Merck), and polyadenylic acid (Schwarz-Mann) were used. Their effects were monitored by the incorporation of the relevant radioactive precursors: [^{3}H]thymidine (6.7 Ci/mM), [^{3}H]uridine (50.2 Ci/mM), and [^{3}H]leucine (40.4 Ci/mM), all from New England Nuclear Corp. For the leucine labeling studies, leucineless MEM with 1% bovine-serum albumin replaced fetal-calf serum. The labeled cells were collected on glass filter pads; the pads were washed with PBS, trichloroacetic acid (TCA), and ethanol, and dried; and their radioactivity was measured in a POPOP-PPO-toluene scintillation system.

RESULTS

Appearance of MuLV-Related Antigens in Mouse Lymphocyte Cultures. Lymphocytes from the thymus, spleen, or lymph nodes of C57BL/Ka mice were incubated at 37° for 48 hr and processed for immunofluorescence. Lymphocytes containing cytoplasmic MuLV-related antigens detectable by IF were invariably observed after incubation, and comprised between 25 and 80% of the total lymphocyte population (Table 1). A small percentage (0.5–3%) of IF-positive cells could be demonstrated in lymphocyte suspensions from mesenteric nodes and spleen prior to incubation. We believe that these are antibody-producing cells detected through cross-reaction of their surface immunoglobulins with the fluoresceinated goat anti-rat serum. In contrast, no MuLV-positive cells could be detected in fresh C57BL/Ka thymocyte suspensions in approximately 60 replicate experiments.

IF-positive cells first became detectable near the end of the first 24 hr and increased rapidly in number during the second 24-hr period (Fig. 1). There was an inverse relationship between the percentage of MuLV-positive cells and the percentage of viable cells. After separation of dead and live cells on albumin gradients, the dead-cell fraction always contained a higher percentage of MuLV-positive cells. Thus, in one typical run, only 26 (35%) of 75 "light fraction" (living) cells were IF-positive, *versus* 81 (89%) of 91 "heavy fraction" (dead) cells. Moreover, pre-irradiation of the cells (1,000 R *in vitro*) immediately before incubation accelerated both the appearance of MuLV-related antigens and the onset of cell death. However, killing of thymic cells by heating to 56° for 30 min or by exposure to pH 10 for 60 min failed to yield MuLV-related antigen expression.

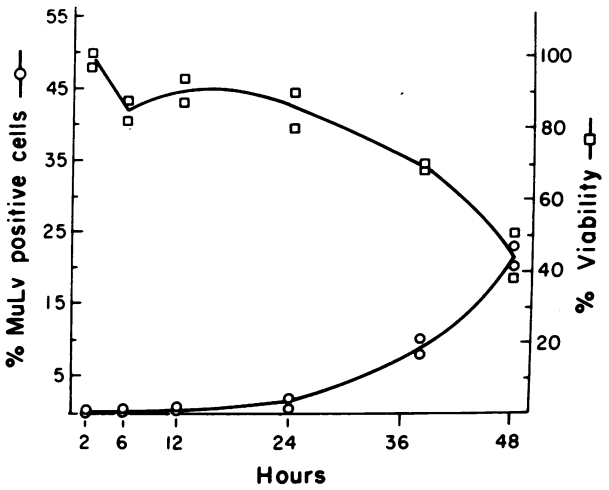

FIG. 1. The kinetics of the appearance of MuLV-related antigens during *in vitro* incubation of C57BL mouse lymphocytes. O——O = % IF-positive cells, using serum 117; □——□ = % viable cells.

T-cells were separated from B-cells in a lymph-node cell suspension (as described in *Materials and Methods*), yielding preparations with 92% and 3% θ-positive cells, respectively. When the two purified fractions were incubated for 48 hr, both T- and B-lymphocytes expressed the MuLV-related antigens to about the same extent (46 and 56% IF-positive cells, respectively).

In 24- to 72-hr cultures of C57BL/Ka bone-marrow, a small proportion (1–10%) of cells became IF-positive, corresponding roughly to the percentage of lymphocytes in normal mouse bone marrow. Long-term embryo fibroblast cultures from low-leukemia strains such as C57BL and C3H have remained consistently IF-negative for MuLV antigens, unless infected with exogenous MuLV (14).

Specificity of the Antigen(s) Appearing in Cultured Lymphocytes. Frozen-thawed cell suspensions were prepared from fresh and cultured C57BL/Ka thymocytes, BL-5(RadLV) cells, and from a RadLV-induced C57BL/Ka thymic lymphoma. An aliquot (6 to 7 × 10^7 cells) of each suspension was mixed with 0.5 ml of 1:100 diluted rat anti-MuLV serum (no. 117) and incubated at 4° for 60 min. The incubated sera were then serially diluted and reincubated with BL-5(RadLV) cells, which are regularly 100% IF-positive (9, 14). Whereas, adsorption on fresh thymocytes had little or no effect on serum titer, adsorption on cultured thymocytes significantly decreased the anti-MuLV titer of the serum, though less effectively at low dilutions than the BL-5(RadLV) cells or the thymoma cells (Fig. 2).

The neoantigen appearing in incubated mouse lymphocytes could also be detected with the "gs-specific" immune sera (Table 2). A somewhat lower percentage of IF-positive cells were detected with the rabbit than with the goat anti-MuLV-gs serum. Good agreement was obtained with all five sera against all nine target cells; normal rat, goat, and rabbit sera were consistently negative (Table 2).

Cultured C57BL thymocytes fixed on slides were treated with rat anti-MuLV serum (no. 151) and subsequently with the goat gs-specific antiserum (F35647), or first with the goat anti-gs serum and subsequently with the rat anti-MuLV serum. Binding of the second antibody to the cells was detected by use of the appropriate fluoresceinated antiserum and compared with that of cells treated with the second im-

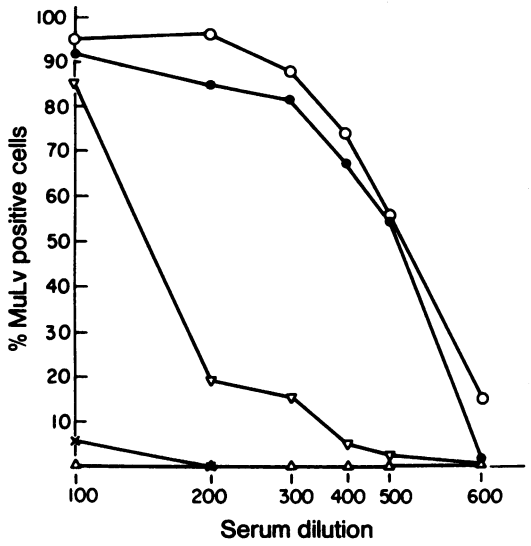

FIG. 2. Residual titer of rat anti-MuLV serum, measured in BL-5(RadLV) cells, after adsorption by: O = untreated serum; ● = normal thymocytes; ▽ = cultured thymocytes; × = thymoma cells; △ = BL-5(RadLV) cells.

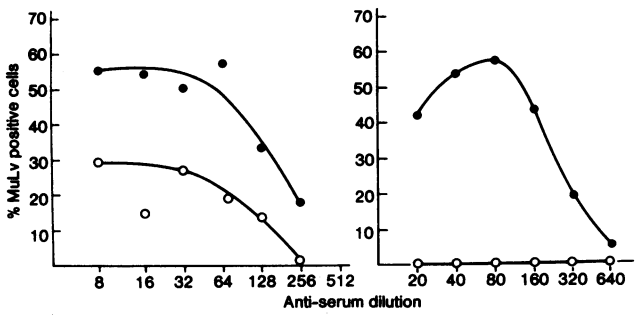

FIG. 3. Partial blocking of the binding of rat anti-MuLV serum to incubated thymocytes by anti-purified gs-antigen serum and complete blocking of anti-gs antigen serum binding by rat anti-MuLV serum. *Left:* ● = rat anti-MuLV serum alone; O = anti-gs-antigen serum, followed by rat anti-MuLV serum. *Right:* ● = anti-gs-antigen serum alone; O = rat anti-MuLV serum followed by anti-gs-antigen serum.

mune serum alone. The rat anti-MuLV serum completely blocked the reaction of the cells with the goat anti-gs serum (Fig. 3), whereas the goat anti-gs serum had only a partial blocking effect on the reaction with the rat anti-MuLV serum, demonstrating that some of the incubated thymocytes express not only the main protein (gs) antigen of MuLV, detected by both sera, but also sufficient levels of other antigens to be detected by the multivalent rat anti-MuLV sera alone.

Crude extracts prepared from normal and incubated C57-BL/Ka thymocytes, from BL-5(RadLV) cells, and from BL-5 cells, and purified MLV disrupted with Triton X-100 were used as antigens against rat anti-MuLV serum (no. 151) in an Ouchterlony double diffusion test. The incubated thymocytes gave lines of identity both with BL-5(RadLV) and with the disrupted MLV. No precipitation line was detected with normal thymocytes or with the noninfected BL-5 cells (Fig. 4).

Lack of Infectious Virus in the Lymphocyte Cultures. Supernatants of the thymocyte cultures, as well as the incubated thymocytes themselves, were seeded on C3H-3T3 [Fv-1^n; (15)], BL-5 (Fv-1^b), UCI-B (16), and NRK (17) cells, and the cultures were serially passaged. The presence of infectious virus was tested by IF (1, 14), focus formation (18), and the XC-cell assay (19, 20) *in vitro* and by IF assay *in vivo* (Declève *et al.*, in preparation). All of these tests have been negative, indicating that complete infectious virus is not produced during the short-term incubation of uninfected mouse lymphocytes.

Experiments with Enzymes and Metabolic Inhibitors. Fresh thymocytes from C57BL/Ka mice were treated with trypsin or neuraminidase (21–23). No IF-positive staining could be detected in either fixed or unfixed cells after such enzyme treatment. However, very low levels of such antigens were detected *in vivo* in some low-leukemia strains by Parks *et al.* (11), using the more sensitive radioimmunoassay technique.

Suppression of RNA and DNA synthesis by increasing concentrations of actinomycin-D and mitomycin-C, respectively, was accompanied by concomitant inhibition of the appearance of the viral antigens. However, cytosine arabinoside, which also blocks DNA synthesis, had no effect (Table 3). Cordycepin and polyadenylic acid, which block MuLV replication (24, 25), were both strongly inhibitory (Table 3, Fig. 5). Small concentrations of cycloheximide inhibited protein synthesis and MuLV antigen expression, both effects being fully

TABLE 2. *Percentage of IF-positive cells with different anti-MuLV sera and different indicator cells*

Sera	Thymocytes		Indicator cells						
	Incubated*	Fresh	BL-5 (RadLV)	BL-5	W/7(GLV)	W/7	Cl.18.4	Tl.M.14	El.4.Bu
Rat α-MSV(RadLV)-induced sarcoma cells (151)	59.0	0.0	100.0	0.0	92.0	0.0	100.0	100.0	100.0
Rat α-MSV(RadLV)-induced sarcoma cells (117)	54.5	0.0	100.0	0.0	N.D.†	N.D.	N.D.	N.D.	N.D.
Rat α-RadLV-induced lymphoma cells (147)	52.0	0.0	100.0	0.0	N.D.	N.D.	N.D.	N.D.	N.D.
Normal rat	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Goat α-MuLV *gs*	54.0	0.6	100.0	0.0	88.0	N.D.	100.0	94.0	N.D.
Normal goat	0.0	0.5	0.0	0.0	0.0	N.D.	0.0	1.0	N.D.
Rabbit α-MuLV *gs*	35.0	0.6	84.0	0.0	N.D.	N.D.	N.D.	N.D.	N.D.
Normal rabbit	0.2	0.8	0.0	0.0	N.D.	N.D.	N.D.	N.D.	N.D.

For the sources of the other cell lines, see *Materials and Methods.*

* C57BL/Ka thymocytes incubated for 48 hr.

† N.D. = not done.

TABLE 3. *The effect of metabolic inhibitors on the appearance of MuLV-related antigens in cultured lymphocytes*

	cpm [^{3}H]-uridine	cpm [^{3}H] thymidine	% MuLV + cells
A. *Actinomycin-D, 0–48 hr*			
0.1 μg/ml	56	—	22.0
1.0 μg/ml	32	—	23.0
10.0 μg/ml	30	—	12.8
25.0 μg/ml	23	—	6.7
50.0 μg/ml	13	—	4.6
Control	547	—	45.6
B. *Mitomycin-C, 0–48 hr*			
0.1 μg/ml	—	198	44.0
1.0 μg/ml	—	105	24.3
10.0 μg/ml	—	40	11.0
25.0 μg/ml	—	36	5.1
50.0 μg/ml	—	42	9.7
Control	—	218	30.0
C. *Cytosine arabinoside, cordycepin, 0–24 hr*			
Cytosine arabinoside, 2×10^{-5} M	23,382	162	18.2
Cordycepin, 200 μg/ml	31,602	10,287	8.7
Control	78,509	4,389	18.3

reversible (Table 4). Poly(A) was added to the cultures for different 12-hr periods during the course of a 48-hr incubation after which the cultures were processed for IF. The results (Fig. 5) suggest that the essential translational events occur between 12 and 24 hr, in good agreement with the kinetics of appearance of the antigen (Fig. 1). Thus, these experiments indicate that the MuLV-related antigens in the cultured lymphocytes are expressed as the consequence of an active biosynthetic process.

The Appearance of MuLV-Related Antigens in Lymphocytes from Other Mouse Strains and Other Species. The six mouse strains studied were: C57BL/Ka (Fv-1^b, G_{IX}-, low leukemia); C3H/DiSn (Fv-1^n, low leukemia); BALB/c (Fv-1^b, G_{IX}-, low leukemia), which apparently harbors three different endogenous C-type viruses (26); NIH Swiss (random-bred, Fv-1^n, low leukemia); 129 (Fv-1^n, G_{IX}^{+++}); and AKR/J (Fv-1^n, G_{IX}^{++}, high leukemia), which releases virus without induction (27, 28). The thymocytes of all of these strains expressed MuLV-related antigens after short-term incubation *in vitro* (Fig. 6). Unlike the other strains, thymocyte suspensions of

TABLE 4. *Reversibility of the inhibition by cycloheximide of MuLV-related antigen expression in cultured mouse thymocytes*

	cpm [^{3}H]TdR	cpm [^{3}H]UR	cpm [^{3}H]Leu	% MuLV + cells
Experiment 1, 0–24 hr				
Cycloheximide*	15,584	59,097	3,541	0.2
Control	4,389	78,509	17,733	18.3
Experiment 2, 0–36 hr				
Cycloheximide*	10,502	17,919	15,957	15.3
Control	557	15,748	17,135	29.0

* 1.4×10^{-5} M; in Experiment 2 cycloheximide was washed out of the cultures after 12 hr of incubation, followed by an additional 24-hr incubation to permit comparison with the 24-hr control data of Experiment 1.

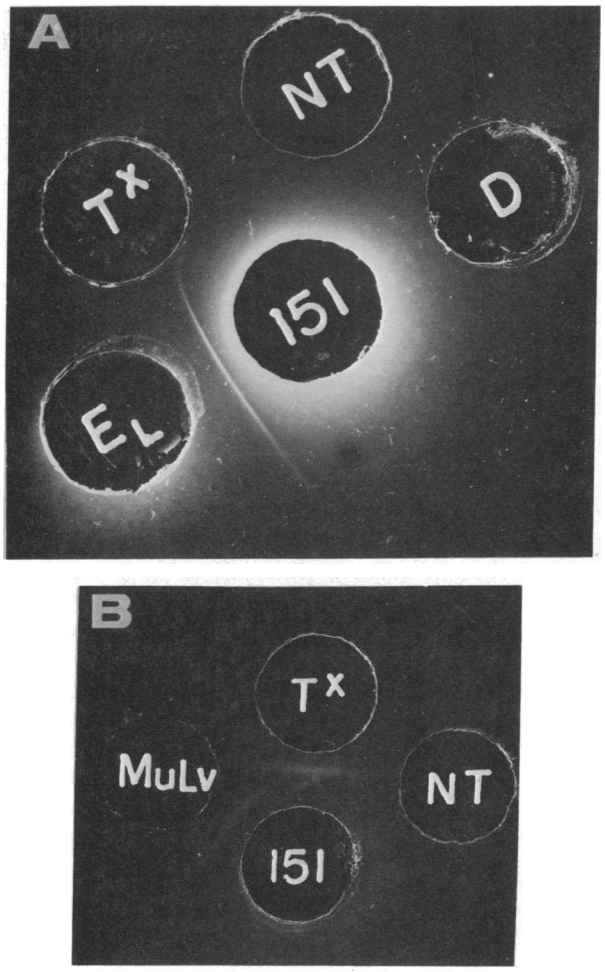

FIG. 4. Double diffusion experiments. The contents of the wells are labeled as follows: *151*, rat anti-MuLV serum 151; *MuLV*, MSV(MLV); *Tx*, incubated thymocytes; *NT*, normal thymus cells; *EL*, BL-5(RadLV) cells; and *D*, BL-5 cells.

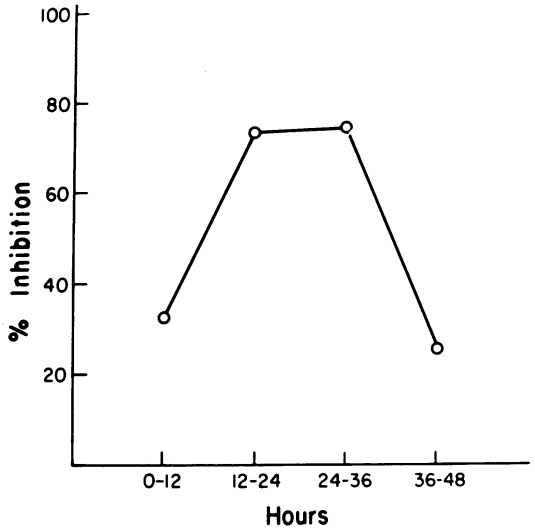

FIG. 5. The inhibitory effect of polyadenylic acid (1.0 mM expressed on a monomer basis), added at different times during thymocyte incubation, on the expression of MuLV-related antigens.

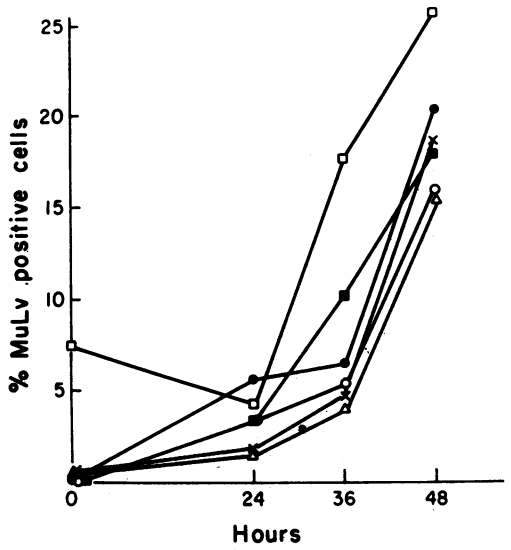

FIG. 6. The appearance of MuLV-related antigens in thymocytes of different mouse strains. □ = AKR/J; ● = C57BL/Ka; ■ = NIH/Swiss; O = C3H/DiSn; × = BALB/c; Δ = 129.

strain AKR/J contained a small percentage of MuLV-positive cells, even before *in vitro* incubation. Thus, the partial expression of MuLV-related antigens during short-term incubation of lymphocytes appears to be a rather general phenomenon in the mouse. We have also demonstrated the appearance of MuLV-related antigens during the short-term incubation *in vitro* of thymocytes from BN, Fischer, and Lewis rats, and from two human thymuses. The rat thymocyte activity is presumably unrelated to the C-type virus particles released by established cell lines derived from newborn rat thymus, since the latter reportedly failed to react with rat anti-MuLV sera (29, 30).

DISCUSSION

Neoantigens which react with rat immune sera prepared against MuLV-infected syngeneic cells and with antisera against the "purified gs fraction" of MuLV are apparently synthesized *de novo* as the result of some spontaneous induction process which occurs when mouse lymphoid cells are transferred to the *in vitro* environment. It is not yet established whether this process is identical with the spontaneous onset of sustained C-type virus replication in established cultures of AKR (27) or BALB/c (31) embryo fibroblasts or of NZB lymphoid cells (32), or the induction of MuLV replication *in vivo* or *in vitro* by X-irradiation (2, 27, 33), carcinogenic chemicals (34, 35), halogenated pyrimidine analogs (36, 37), and graft-versus-host reactions (38, 39).

No infectious virus could be detected during the course of these short-term incubations. However, in the absence of added mitogens, freshly explanted lymphocyte cultures are essentially stationary, and most of the cells have a relatively short life span. The possibility remains open that complete virus synthesis might occur in the incubated mouse lymphocytes if replication could be sustained during more protracted periods of cultivation.

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