ANTIGENIC MODULATION

Loss of TL Antigen from Cells Exposed to TL Antibody. Study of the Phenomenon In Vitro*

By LLOYD J. OLD, M.D., ELISABETH STOCKERT, EDWARD A. BOYSE, M.D., and JAE HO KIM, M.D.

(From the Divisions of Immunology and Biophysics, Sloan-Kettering Institute for Cancer Research, and Cornell University Graduate School of Medical Sciences, Cornell University Medical College, New York 10021)

(Received for publication 6 November 1967)

Isoantigens of the TL (thymus-leukemia) system are determined by the locus *Tla* adjacent to *H-2* in linkage groups IX (1). In normal mice, they are to be regarded as organ-specific isoantigens because they are found exclusively in one cell type, the thymocyte, and are present in some strains of mice (TL+ strains) but absent in others (TL− strains). The system is of special interest in relation to neoplasia because of the anomalous appearance of TL antigen in leukemias of TL− mice; ie, mice whose thymocytes do not contain TL antigen and which therefore are capable of forming TL antibody (2).

Table I summarizes our knowledge of the phenotypes known to be expressed in thymocytes and leukemia cells. Three antigens of the *Tla* locus have been identified, TL.1, TL.2, and TL.3. TL+ mice have one of two phenotypes, TL.2 or TL.1,2,3. The components which occur anomalously in leukemia cells are TL.1 and TL.2; TL.3 occurs only in leukemias of TL.1,2,3 mice. It follows that leukemia cells may have the phenotype (*a*) TL− in any strain, (*b*) TL.1,2 in TL− or TL.2 strains, or (*c*) TL.1,2,3 in TL.1,2,3 strains. Regarding the anomalous appearance of TL.1 and 2 in leukemia cells, we conclude that all mice have the structural genes for synthesis of these antigens, their expression in the thymocytes of normal mice being dependent on a controlling gene or genes belonging to the *Tla* locus with alleles for *expression* vs. *nonexpression*. Experiments with radiation chimeras indicate that the controlling gene exerts its effect directly within the cells which populate the thymus and not indirectly through an influence of the thymus (3). In leukemia cells, loss of normal control over TL phenotype is evident in two ways. First, TL+ cells now are disseminated in organs other than the thymus. Secondly, TL antigens 1 and 2 are found expressed in TL− mice.

The realization that TL+ leukemias occur in TL− mice, which are capable of being immunized against TL antigens and of forming high titers of cytotoxic TL antibody, initially raised the possibility of active immunization against such leukemias within the inbred strain of origin. It was later recognized that this is rendered impracticable by the occurrence of a phenomenon called "antigenic modulation" (4). Mice of TL− strains produce TL antibody

* This investigation was supported by the National Cancer Institute Grant CA 08748, Atomic Energy Commission Grant [AT (30-1)-910], and a grant from the John A. Hartford Foundation, Inc.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

when immunized with allogeneic TL+ leukemia cells; when syngeneic TL+ leukemias are now passed in such previously immunized hosts, TL antigen is suppressed and the cells are no longer sensitive to cytotoxic TL antibody and complement. This disappearance of TL antigen and its reappearance on subsequent passage in nonimmune hosts, are referred to as antigenic modulation. It can be produced passively by the transfer of TL antiserum, either by injection or by suckling on immune mothers, and it occurs in TL+ thymocytes as well as in leukemia cells (5).

TABLE I

Summary of All Known Phenotypes of Thymocytes and Leukemia Cells

Designation of mouse strain (= phenotype of normal thymocytes)	Phenotypes of leukemias in mice with phenotypes shown on the left
TL−	TL− or TL.1,2
TL.2,	TL− or TL.1,2
TL.1,2,3	TL− or TL.1,2,3

TABLE II

TL Phenotypes of Mice and Leukemias Used in This Study

Phenotype	Mouse strain	Transplanted leukemia
TL−	C57BL/6, C3H	—
TL.1,2	—	ERLD (C57BL/6 radiation-induced leukemia)
TL.2	BALB/c, 129	—
TL.1,2,3	A	RADA1 (A strain ascites leukemia: radiation-induced) ASL1 (A strain spontaneous leukemia)

In order to facilitate the investigation of antigenic modulation we have made a study of its occurrence in vitro under conditions which lend themselves to analysis of the kind that is not feasible with modulation produced in vivo.

Materials and Methods

Mice.—These were obtained from our colonies; TL phenotypes shown in Table II.

Leukemias.—Origins and TL phenotypes given in Table II (see also reference 2).

Antisera.—See Table III.

Procedure for Modulation In Vitro.—The cells were washed in medium 199 and counted. Volumes of this suspension containing the number of cells required were centrifuged at 800 rpm increasing to 1200 rpm (International Refrigerated Centrifuge Model PR-2; 269 Head) for 10 min and the cells resuspended at a concentration of 5×10^6/ml in the desired medium. Incubation was carried out in stoppered bottles at 37°C in a water bath. Exposure of the

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

cell suspensions to an atmosphere of CO_2 was found to be unnecessary in the case of leukemia cells as the acidity of the medium was maintained or more usually increased under these conditions. The bottles were shaken every 10 min to keep the cells freely suspended. The medium was 199 with the addition of either normal mouse serum (NMS) or TL antiserum. In all cases a minimum of 5% mouse serum was maintained during incubation: thus 5% NMS was added to all suspensions in which the concentration of TL antiserum used was less than

TABLE III

Antisera

TL

Immunization	Absorption	Designation
C57BL/6 *anti*-A strain leukemia ASL1	In vivo in A ♂♂ (removes *anti*-H-2^a: TL antibody is not absorbed)	*anti*-TL.1,2,3
(BALB/c × C3H)F_1 *anti*-A strain leukemia ASL1	Unnecessary: *anti*-TL is the only demonstrable antibody formed	*anti*-TL.1,3
	In vivo in (C57BL/6 × A)F_1 ♂♂ bearing advanced transplants of C57BL/6 leukemia ERLD (TL.1,2). Absorbed serum has no *anti*-TL.1 activity	*anti*-TL.3
C57BL/6 *anti*-129 thymus	Unnecessary: only TL antibody formed	*anti*-TL.2

H-2

Immunization	Test cell	Designation
C3H/An *anti*-BALB/c ascites sarcoma Meth A	A strain lymph node cells	*anti*-H-2(D)
(BALB/c × C57BL/6)F_1 *anti*-C3H ascites sarcoma BP8	A strain lymph node cells	*anti*-H-2(K)

5%. After the prescribed period of incubation the cells were immediately cooled in an ice bath to prevent or arrest modulation (see Results) and were then washed twice; these washings and all procedures thereafter being carried out in the cold, except for subsequent cytotoxic tests and tests of sensitivity to complement (C′) requiring incubation at 37°C.

Cytotoxic Tests (6–8).—Serial dilutions (0.05 ml) of antiserum were incubated with cells (0.05 ml of a suspension containing 5×10^6 cells/ml) and pooled absorbed guinea pig serum (GPS) diluted 1/3 (0.05 ml) to provide C′. After incubation for 45 min at 37°C the cells were counted in trypan blue to determine the dead (stained) cell count per cent. In every test, cells were incubated also in: (*a*) antiserum alone, and (*b*) GPS alone; unless otherwise stated these controls showed no more than 10% stained cells. The diluent was medium 199.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

The GPS was previously absorbed twice in the cold with mouse leukemia cells to remove heteroantibody (9 vol GPS: 1 vol packed cells; ×2).

Relative H-2 Absorption Capacity.—The two H-2 antisera shown in Table III were used for measurements of the H-2 content of RADA1 cells undergoing modulation (Fig. 7). 0.06 ml of H-2 antiserum (dilution determined in preliminary experiments) was absorbed with 3×10^6 RADA1 cells for 30 min in the cold with repeated shaking. The absorbed serum was titrated by the cytotoxic test against A strain lymph node cells. The result was expressed as per cent A lymph node cells dead in a cytotoxic test with the absorbed antiserum. For

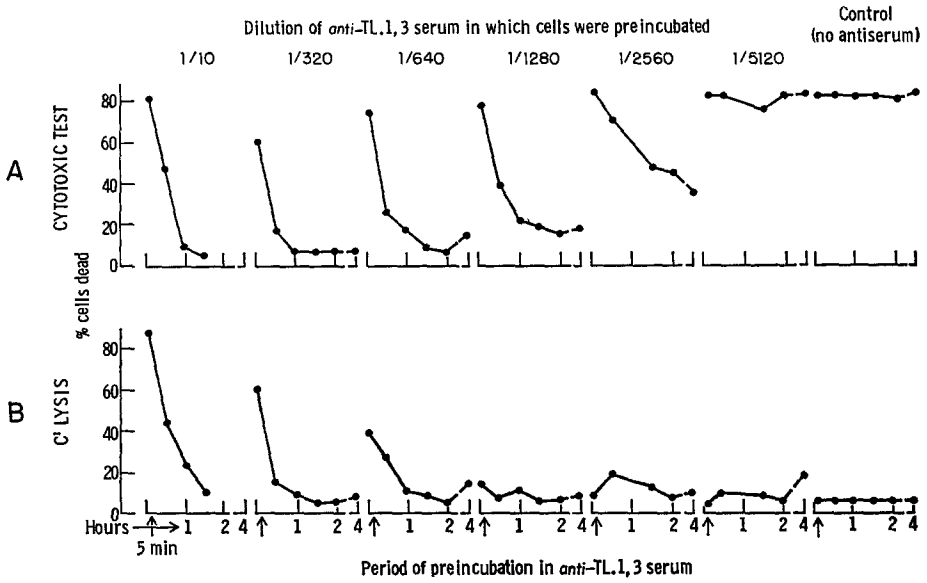

FIG. 1. Modulation of TL antigens induced in vitro by incubation of TL+ cells in TL antiserum. RADA1 (TL.1,2,3) leukemia cells were exposed to concentrations of *anti*-TL.1,3 serum ranging from 1/10 to 1/5120 for periods of 5 min to 4 hr. The cells were then washed and tested (*a*) for the presence of TL antigen, by the cytotoxic test with TL antiserum and guinea pig serum C′ (A), and (*b*) for attached antibody, by addition of C′ alone (B).

The results show (*a*) Dilutions 1/10–1/640: initial sensitization to C′ followed by loss of sensitization to C′ (B) and progressive loss of TL antigen (A). (*b*) Dilutions 1/1280–1/2560: no sensitization to C′ (B) and progressive loss of TL antigen (A). (*c*) Dilution 1/5120 and control: no sensitization to C′ (B) and no loss of TL antigen (A).

each time interval (Fig. 7) this value was determined for RADA1 cells incubated in TL antiserum (X) and for control RADA1 cells incubated in NMS (Y). An arbitrary index, the "relative H-2 absorption capacity" is given by Y/X.

Incorporation of Labeled Precursors into Nucleic Acid and Proteins.—Equal volumes of a suspension of RADA1 cells (6×10^5/ml) were incubated with thymidine-^{3}H (0.2 μc/ml, 1.9 c/mmole), uridine-^{3}H (1 μc/ml, 8.0 c/mmole), or valine-^{3}H (2.0 μc/ml, 290 mc/mmole) as a measure of DNA, RNA, and protein synthesis respectively. At specified time intervals the cells were collected and filtered through Millipore membrane filters (0.45 μ pore size). The filters were washed with cold 5% trichloroacetic acid and their radioactivity determined with a liquid scintillation spectrometer.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

RESULTS

Antigenic Modulation In Vitro.—

In initial experiments a variety of TL+ cells were tested for their ability to undergo modulation in vitro. These showed that TL+ leukemia cells readily become TL− on exposure to TL antibody, whereas TL+ thymocytes modulate very little even after several hours of exposure to TL antiserum. Of the several TL+ leukemias tested, the A strain leukemia RADA1 was selected for further experiments on the grounds that it grows in the ascitic form, carries all three TL components, and undergoes modulation rapidly and completely in response to TL antibody.

Fig. 1 shows an experiment in which RADA1 cells were incubated in various concentrations of *anti*-TL.1,3 serum for up to 4 hr. In the range 1/10–1/640 modulation was complete within 90 min; ie, at this time the cells were no longer sensitive in the cytotoxic test with TL antiserum and C′ (Fig. 1, A). In these concentrations of TL antibody the cells were initially sensitized to lysis by C′ but progressively lost their sensitivity on further incubation (Fig. 1, B). In the range 1/1280–1/2560 modulation was incomplete and there was virtually no sensitization to C′. At a concentration of 1/5120 there was neither modulation nor sensitization to C′. The cytotoxic titer of this antiserum (50% end point) for RADA1 cells was 1/256 under the standard conditions of the cytotoxic test, which are comparable with those of the modulation test.

Inhibition of Antigenic Modulation.—

These experiments were directed to the question whether modulation is a passive process consequent on the attachment of TL antibody, or whether it requires active metabolic participation on the part of the cell.

Influence of temperature: In Fig. 2 it is seen that reduction of the incubation temperature to 0°C completely inhibited modulation. This is not explained by failure of attachment of TL antibody at 0°C because the cells were fully sensitized to C′ under these conditions (Fig. 2). At 22°C modulation was incomplete.

Effect of metabolic inhibitors and X-radiation: RADA1 cells were incubated for 1–2 hr at 37°C with various metabolic inhibitors and tested for their ability to undergo modulation when subsequently exposed to TL antibody. Two of these, actinomycin D and iodoacetamide, completely inhibited modulation at concentrations that had no effect on viability during the period of the test (5 μg/ml in both cases). The capacity of RADA1 cells to modulate was abolished by exposure to 5 μg/ml actinomycin D for 1 hr; lesser concentrations, or shorter periods of exposure, produced proportionately less impairment (Fig. 3). The other compounds tested did not inhibit modulation, even in some instances at levels producing decreased viability in controls: puromycin (10 μg/ml and 20 μg/ml); chloramphenicol (100 μg/ml); *p*-fluorophenylalanine

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

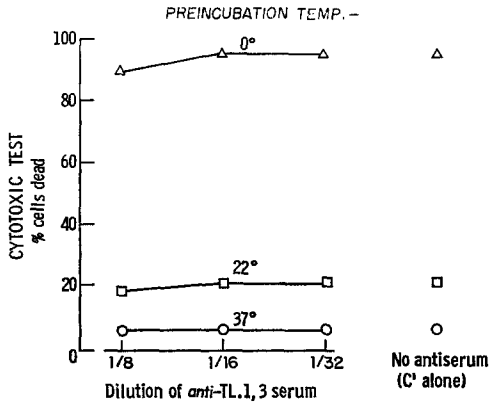

FIG. 2. Temperature-dependence of antigenic modulation. Cytotoxic tests with RADA1 (TL.1,2,3) leukemia cells previously incubated with *anti*-TL.1,3 serum 1/10 for 2 hr at 0°C (ice bath), 22°C (room temperature), or 37°C, and washed twice in the cold. Control: the per cent viability of RADA1 cells after preincubation at each temperature remained >95.

Modulation was complete at 37°C, incomplete at 22°C, and did not occur at 0°C.

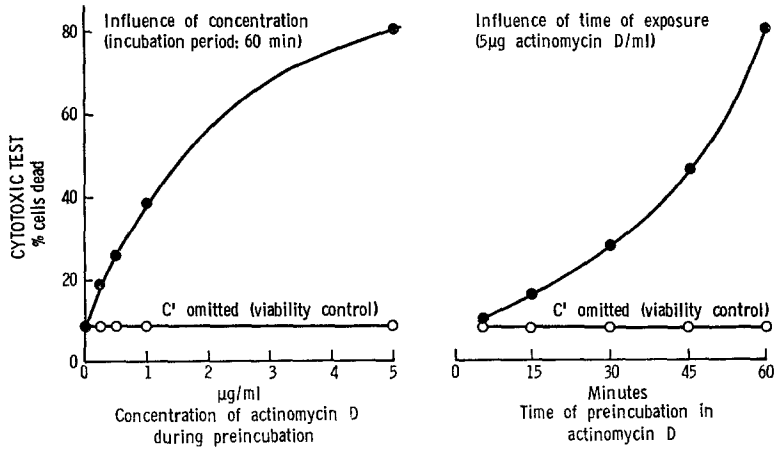

FIG. 3. Inhibition of antigenic modulation by actinomycin D. Step 1. Incubation of RADA1 (TL.1,2,3) leukemia cells in actinomycin D—cells then washed twice in the cold.

Step 2. Incubation in *anti*-TL.1,3 serum 1/320 for 1½ hr—cells then washed twice in the cold.

Step 3. This figure. Cytotoxic tests with *anti*-TL.1,3 serum.

Curves for sensitivity to C′ alone (not shown) were similar to those in this figure. Controls in which normal mouse serum was substituted for TL antiserum in step 2 were included for every point; these showed normal sensitivity to TL antibody and C′ and no sensitivity to C′ alone.

Modulation is completely inhibited by exposure of cells to 5 μg actinomycin D/ml for 1 hr and is partially inhibited by lower concentrations or shorter periods of incubation.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

(0.5 mg/ml and 1 mg/ml); cycloheximide (10 μg/ml and 20 μg/ml); deoxyadenosine (0.5 mg/ml); 5-fluorodeoxyuridine (FUDR 1 μg/ml); 1-β-D-arabinofuranosylcytosine (ara-C; 10 μg/ml and 20 μg/ml); hydrocortisone 21-phosphate, disodium salt (0.5 mg/ml and 1 mg/ml). Four of these inhibitors were tested for their influence on DNA, RNA, and protein synthesis in RADA1 cells (under the same conditions as those used for testing their influence on modulation) as indicated by rates of incorporation of tritium-labeled precursors. As anticipated, actinomycin D produced major and immediate suppression of RNA synthesis, followed by suppression of DNA and protein synthesis becoming virtually complete at 2 hr. The most immediate and pronounced effect of iodoacetamide (a potent inhibitor of SH-containing enzymes) in the 1st hr, was on DNA synthesis, with moderate depression of protein synthesis, and little effect on RNA synthesis. FUDR curtailed DNA synthesis immediately, with virtually no effects on RNA and protein synthesis during a period of 4 hr. At concentrations near the toxic level for RADA1 cells puromycin gave only moderate suppression of protein and RNA synthesis, therefore little significance can be attached to the results obtained with this agent. The results with FUDR and ara-C permit the conclusion that modulation is not dependent on DNA synthesis; from the results with actinomycin D there is a strong indication that RNA synthesis *is* necessary; adequate evaluation of the necessity for protein synthesis awaits evidence as to whether the relevant compounds used in this study in fact selectively inhibit protein synthesis in RADA1 cells.

None of the metabolic inhibitors affected the sensitivity of the cells to TL antibody and C′ in the cytotoxic test, as might be the case if the turnover of TL antigen were rapid and maintenance of the TL phenotype therefore highly dependent on continuous synthesis of protein or nucleic acid. Exposure of modulated RADA1 cells to actinomycin D did not cause reversion of the phenotype to TL+. Thus the TL− phenotype, once induced, is stable, at least over a period of 4 hr.

5000 R X-radiation had no effect on the capacity of RADA1 cells to modulate.

Synthesis of DNA, RNA, and Protein in Cells Undergoing Modulation.—

Antigenic modulation was not accompanied by demonstrable changes in net synthesis of DNA, RNA, or protein, as tested by uptake of radioactive precursors over periods of 15 min to 4 hr, although this does not exclude discrete changes in the synthesis of particular products which may be obscured by the exceedingly high metabolic activity of these leukemia cells.

Modulation of TL Antigens by TL Antisera of Various Specificities.—

The four TL antisera available are *anti*-TL.1,2,3; *anti*-TL.1,3; *anti*-TL.2; and *anti*-TL.3 (Table III). So far it has not been possible to prepare mono-

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

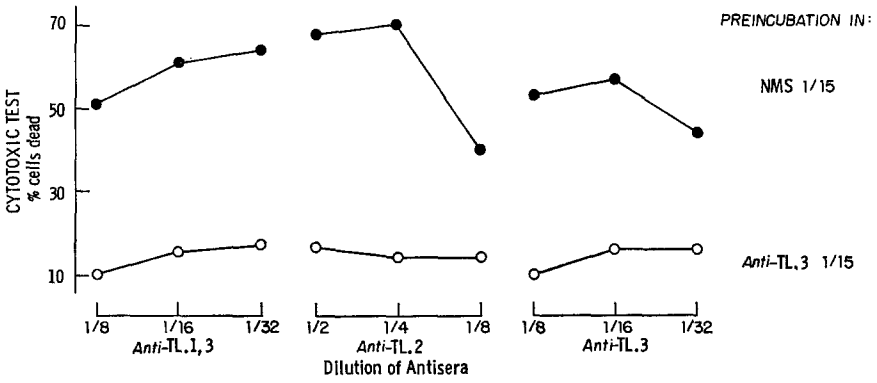

FIG. 4. Cytotoxic tests with RADA1 (TL.1,2,3) leukemia cells previously incubated for 1½ hr with *anti*-TL.3 serum. (NMS, normal mouse serum.)

As a consequence of exposure to anti-TL.3 serum the cells are no longer sensitive to anti-TL.1, anti-TL.2, or anti-TL.3.

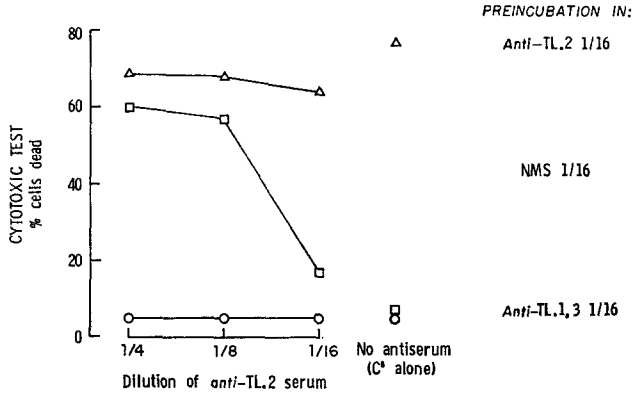

FIG. 5. Cytotoxic tests with RADA1 (TL.1,2,3) leukemia cells previously incubated for 1½ hr in normal mouse serum (NMS), in *anti*-TL.1,3 serum, or *anti*-TL.2 serum.

TL.2 is not modulated by anti-TL.2.

specific *anti*-TL.1. In previous experiments in vivo (5), TL.1,2,3 cells were completely modulated by *anti*-TL.1,3 serum, indicating that modulation of TL.2 takes place as a consequence of modulation of TL.1 and 3 and does not require the presence of *anti*-TL.2. In the following experiments with RADA1 cells the various TL antisera were tested for their capacity to modulate TL antigens of both homologous and nonhomologous specificity.

Modulation of all TL components by anti-TL.3: The preparation of *anti*-TL.3 serum is given in Table III. No residual TL.1 antibody can be detected in this serum in cytotoxic tests with the standard TL.1 test cell ERLD. Never-

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

theless in order to ensure absence of *anti*-TL.1 the serum used for modulation in the experiment shown in Fig. 4 was absorbed a second time in C57BL/6 mice bearing leukemia ERLD (see Table III). This doubly absorbed *anti*-TL.3 serum produced modulation of all three components, TL.1, TL.2 and TL.3.

Inability of anti-TL.2 to induce modulation: Preincubation of RADA1 cells with *anti*-TL.2 serum did not produce modulation of TL.2 (Fig. 5).

Although it may be presumed that TL.1 and TL.3 antigens also are not modulated by *anti*-TL.2, this is difficult to demonstrate directly. In the first place, cells sensitized by *anti*-TL.2 do not modulate and consequently remain susceptible to C′ lysis; they therefore cannot be used in direct cytotoxic tests with *anti*-TL.1,3 serum. Furthermore, *anti*-TL.2 partially blocks the uptake of these antibodies, which complicates the demonstration of TL.1 and TL.3 antigens by the method of absorption (the cells having already taken up *anti*-TL.2). However the possibility that TL.1 and TL.3 might be modulated by *anti*-TL.2 is rendered remote by the observation that addition of *anti*-TL.2 actually impedes modulation by *anti*-TL.1,3 serum (see below).

Inhibition of Antigenic Modulation by Anti-TL.2 Serum.—

It was noted that *anti*-TL.1,3 serum was consistently more effective than *anti*-TL.1,2,3 serum in inducing modulation in vitro. For this reason most experiments were performed with *anti*-TL.1,3 serum. The subsequent finding that *anti*-TL.2 is incapable of inducing modulation suggested that the relatively poor modulating capacity of *anti*-TL.1,2,3 serum might be attributable to its *anti*-TL.2 content. Fig. 6 shows that this interpretation is correct. Removal of *anti*-TL.2 from an *anti*-TL.1,2,3 serum by absorption with TL.2 thymocytes rendered it as effective as the standard *anti*-TL.1,3 serum. Control absorption with TL− thymocytes had no effect. In other experiments it has been shown that preincubation with *anti*-TL.2 serum renders RADA1 cells less sensitive to modulation by *anti*-TL.1,3 serum.

Changes in H-2 Antigen Accompanying Antigenic Modulation.—

Antigenic modulation of TL+ leukemia cells in vivo results in an increase in demonstrable H-2 antigen (5). *H-2* is a compound locus determining a series of antigens (9). "D" and "K" are the original designations of two strong H-2 antigens determined by opposite poles of the locus, the two ends of the locus therefore being referred to as the D end and the K end. With respect to *Tla* the order is *Tla*:*H-2*(D):*H-2*(K) (1, 10).

Cells undergoing modulation in vitro also showed the increase in H-2 antigen (Fig. 7) and this was confined to H-2(D) antigen with no change in H-2(K) antigen. Modulation of TL antigen was apparently complete in approximately 1 hr, but the H-2(D) content of the cells continued to rise for 4½ hr.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

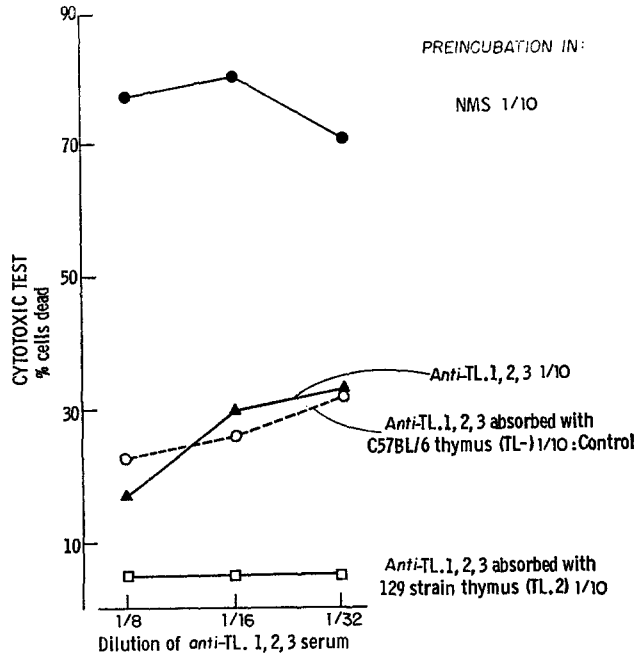

FIG. 6. TL.2 antibody retards modulation by *anti*-TL.1,3 antibody. Cytotoxic tests with RADA1 (TL.1,2,3) leukemia cells preincubated for 1½ hr in TL antiserum containing *anti*-TL.2 or lacking *anti*-TL.2. (NMS, normal mouse serum.)

The modulating capacity of an anti-TL.1,2,3 serum is increased by first absorbing out anti-TL.2.

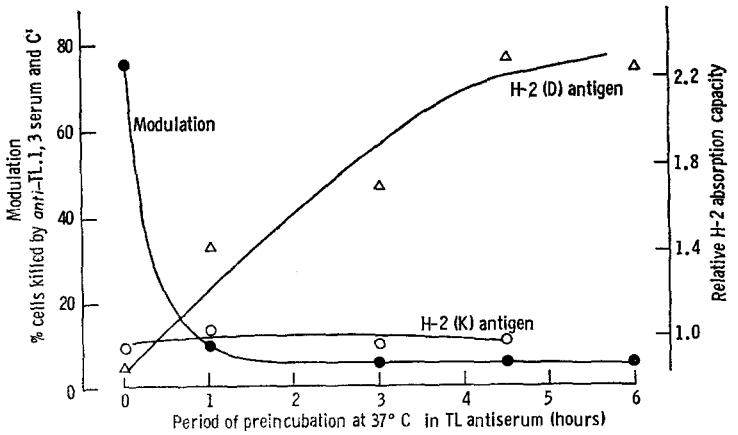

FIG. 7. When antigens TL.1,2,3 of RADA1 leukemia cells are suppressed by TL antibody (antigenic modulation) H-2 isoantigens of the D region are increased in amount, but H-2 isoantigens of the K region are not affected. (For calculation of relative H-2 absorption capacity see Materials and Methods).

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

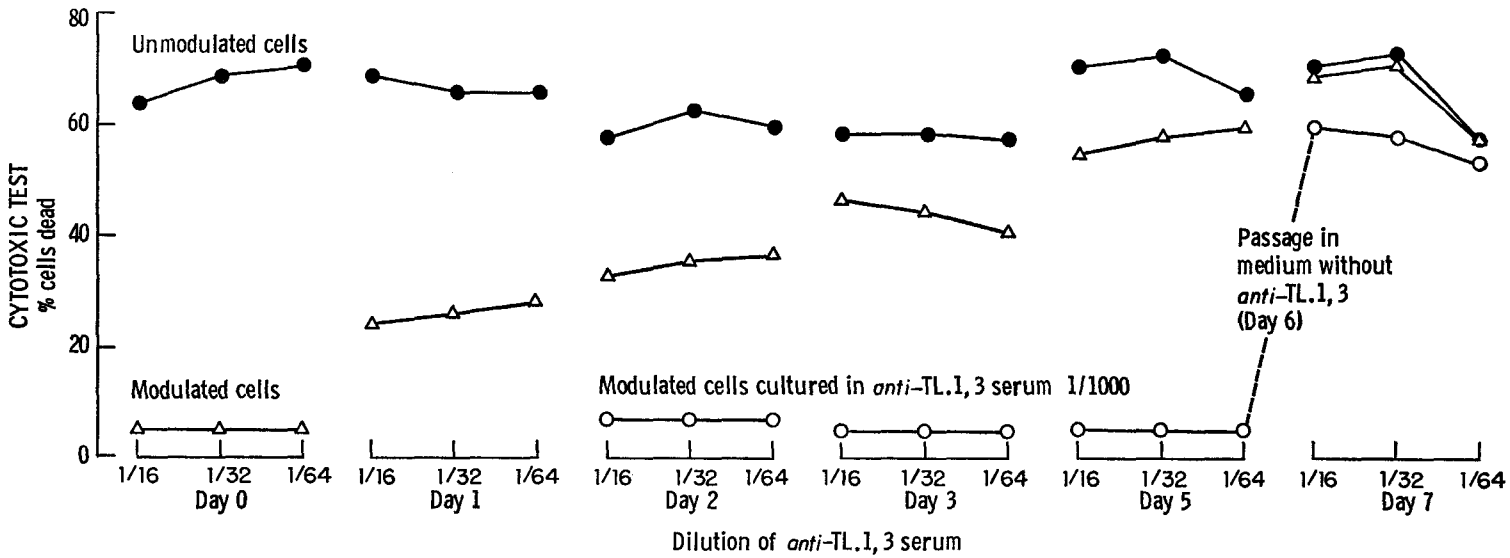

FIG. 8. Return of TL phenotype in cells modulated in vivo and then cultured in vitro without TL antibody. Cytotoxic tests with RADA1 (TL.1,2,3) leukemia cells maintained in culture for 1–7 days after being obtained from mice treated the previous day with either 3 ml of normal mouse serum (control unmodulated cells), or *anti*-TL.1,3 serum (modulated cells). The doubling time of the cultured cells was approximately 24 hr. The initial cell concentration was 2×10^5/ml and passage to fresh medium was performed when the cell concentration exceeded 1×10^6 ml. Medium: MEM (minimum essential medium) + "nonessential amino acids" + 15% heat-inactivated fetal bovine serum (with or without *anti*-TL.1,3 serum—see figure).

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

Restoration of TL Phenotype after Antigenic Modulation.—

It was known from previous work that modulated cells from immunized hosts become TL+ again when passed in nonimmunized hosts (4) but the rate of reversion was unknown except for the observation that it could be completed during a single passage in a nonimmune mouse. With the object of determining how rapidly the TL+ phenotype returns, RADA1 cells modulated in vivo were washed four times to remove TL antibody present in the ascitic fluid and then were cultured in vitro (Fig. 8). Some restoration of sensitivity to TL antibody was apparent after 24 hr in culture and by 7 days the cells were indistinguishable from unmodulated cells by the direct cytotoxic test with TL antibody. The average doubling time of RADA1 cells in culture, whether modulated or not, is 18–24 hr. Thus the cells underwent six or more divisions before TL antigenicity was fully restored. A line of the original modulated cells was maintained in medium to which 1/1000 *anti*-TL.1,3 serum had been added. These remained completely TL−. After 5 days in culture, antibody was omitted and the cells became TL+, in this case more rapidly (Fig. 8). Unmodulated RADA1 cells showed no change in sensitivity to TL antibody in the cytotoxic test as a result of culture in vitro (Fig. 8).

DISCUSSION

Antigenic modulation was first observed in TL+ leukemias inoculated into actively immunized TL− hosts of the strain of origin (4). Passive immunization with TL antiserum was then shown to be effective in inducing modulation and this revealed that both TL+ thymocytes and TL+ leukemias of TL+ strain mice (which cannot be *actively* immunized) are susceptible to antigenic modulation (5). The process can now be studied in vitro, eliminating the difficulties of analysis in vivo. Several factors are seen to influence modulation of TL antigen in vitro.

Cell Type on which the Antigen is Situated.—Of the two cell types bearing TL antigen, TL+ thymocytes and TL+ leukemia cells, modulation in vitro is more readily demonstrable in the latter. Under similar conditions thymocytes also undergo modulation, but only partially and after longer periods of incubation, although complete antigenic modulation of thymus cells can be produced in vivo by transfer of TL antiserum (5). There are also differences among various TL+ leukemias in the rate at which they undergo modulation. These pronounced differences in modulation rate, according to cell type, suggest that modulation is an active cellular process occurring most rapidly in cells with high metabolic activity.

Metabolic State of the Cells.—The conclusion that modulation is an active cellular process is borne out by its abolition at reduced temperature, by actinomycin D and by iodoacetamide. (Inhibition of DNA synthesis did not abolish modulation; the interpretation of the results with inhibitors of protein synthesis is not clear; more data are required on the influence of these compounds on protein and nucleic acid synthesis in RADA1 cells.)

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

Concentration of TL Antibody.—The rapidity of modulation is independent of antibody concentration over a wide range. At limiting dilutions of antiserum it may occur in the absence of concomitant sensitization to C′. The quantity of antibody required for modulation therefore is presumably less than that needed to sensitize the cell to lysis by C′ under the specified conditions of the cytotoxic test. The implication is that modulation can take place although not every TL site on the cell is occupied by antibody, but this has not yet been critically demonstrated.

Specificity of TL Antibody.—Cells of the phenotype TL.1,2,3 become TL− when exposed to *anti*-TL.3, showing that modulation of components TL.1 and TL.2 can occur despite absence of corresponding antibody from the serum used for modulation. All three TL specificities may well be on one molecule (11) in which case modulation may be envisaged as loss of this entire molecule following the attachment of antibody. On the other hand, the attachment of TL antibody to this site does not inevitably entail modulation, because *anti*-TL.2 does not induce modulation, in fact the attachment of *anti*-TL.2 hinders modulation. Monospecific *anti*-TL.1 serum is not available and so its capacity to modulate TL.2 and TL.3 is unknown. However, as TL.1,2 leukemias of TL− strains are modulated by *anti*-TL.1,3 it is clear that TL.1 and 2 are modulated by *anti*-TL.1. It seems self-evident that *anti*-TL.3 cannot modulate a TL.1,2 cell, there being no site for attachment of antibody. Thus *anti*-TL.3 can modulate TL.1 if it can be attached to the cell (Fig. 4) but in the case of TL.1,2 cells this is not so. Modulation of TL.1 in TL.1,2 cells can therefore safely be ascribed to *anti*-TL.1.

Many features of modulation exclude passive mechanisms such as blocking of TL antigenic sites by antibody that lacks complement-fixing properties and consequently is not lytic (12). This and other passive mechanisms, including antibody-induced resistance of red cells to immune lysis (13), are ruled out by several observations. (*a*) Over a wide range of TL antibody concentrations the cells are fully sensitized before they undergo modulation. Thus the fixation of lytic antibody is demonstrably *not* blocked. The only known ways of producing modulation without an initial transient phase during which the cells are sensitive to lysis by C′ are to use low concentrations of TL antibody that suffice for modulation but sensitize to C′ only weakly or not at all, or to use antibody fragments that do not bind C′.[1] (*b*) TL antigens 1 and 2 of TL.1,2,3 cells are modulated by antiserum lacking specificities 1 and 2. It is improbable that *anti*-TL.3 could block the attachment of lytic *anti*-TL.1, in fact we have found that attachment of *anti*-TL.3 does not impede absorption of *anti*-TL.1 (unpublished data). (*c*) As described above, modulation is strongly depressed by thermal or chemical inhibition of metabolic activity, and its rate depends upon the type of cell which is carrying the TL antigens, all of which is not in keeping with blocking by antibody. (*d*) Application of fluorescein-labeled

[1] Lamm, M., E. A. Boyse, L. J. Old, B. Lisowska-Bernstein, and E. Stockert. 1968. Modulation of TL (thymus-leukemia) antigen by Fab-fragments of TL antibody. Manuscript submitted for publication.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

antiglobulin serum has shown that cells modulated in vivo, in actively or passively immunized hosts, are not coated with globulin (unpublished data). (*e*) Modulated cells show a selective increase in H-2(D) antigen which is not explicable in terms of blocking antibody.

Antigenic modulation can therefore be viewed confidently as an actual loss of TL components from the cell surface.

Another mechanism now excluded by the rapidity of modulation in vitro is the selection of a TL− fraction of the population. Modulation in vitro cannot be other than an adaptive change affecting the cell population as a whole.

An apparently close parallel to antigenic modulation is the serotype transformation which takes place in *Paramecia* exposed to various changes in their environment, in particular to type-specific antiserum (14). As with modulation, the serotype change is adaptive rather than selective and depends on active metabolism. In both phenomena the lost antigen is the product of a known gene and in neither case is loss of the antigen brought about by genetic mutation. Nevertheless there are clear differences between the two systems. Serotype change in *Paramecia* can be induced by factors other than antibody (temperature, nutritional deficit, X-radiation, chemical agents), it proceeds relatively slowly over a period of several cell divisions, and it involves the appearance of a formerly unexpressed alternative antigen. Antigenic modulation in contrast can be induced only by antibody (as far as is known), it occurs rapidly and independently of cell division, and it is not accompanied by the appearance of a *new* antigen (the change in H-2 antigen accompanying modulation being only quantitative, and even this may be only apparent, for it may possibly result from removal of steric interference imposed upon the H-2(D) site by TL components of the membrane).

Modulation has not been observed in other systems of isoantigens and so appears to be peculiar to TL. A second unique feature of the TL system is the anomalous appearance of antigen in strains of mice that do not possess it in normal tissue (TL− strains). Whereas modulation is characteristic of TL antigens *as a class*, all three components being capable of modulation, anomalous appearance in leukemia cells of TL− mice is restricted to antigens TL.1 and TL.2. Thus TL.3 antigen can undergo modulation but it never appears in leukemias of mice that are TL.3 negative. In fact each of the three components has distinctive properties, which are summarized in Table IV.

The appearance of TL.1 and TL.2 antigens in the leukemias of probably all mouse strains implies that structural genes for these antigens are present in all mice, the phenotype being governed by the locus identified in linkage group IX, *Tla*, which has alleles for expression vs. nonexpression of structural TL genes. As there can be no alleles of the TL.1 and TL.2 structural genes it follows that normal TL− mice not only lack antigens TL.1 and TL.2 but have no genetically homologous products of this part of the *Tla* locus. This inter-

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

pretation is supported by the finding that the content of H-2(D) antigen in TL− thymocytes is higher than in TL+ thymocytes of the same H-2 type (5).

Expression of TL in thymocytes depends not only on their genotype but also upon residence in the thymus, since TL+ cells are not found elsewhere. Experiments with chimeras show that the thymic environment of TL− mice induces expression of TL in thymocytes of appropriate genotype and so the thymic influence presumably is common to all mice (1, 3). It is not known whether expression of TL antigen is a secondary consequence of the differentiation of immigrant cells within the thymus or whether the *Tla* locus is directly activated by a thymic factor. Whatever the explanation the persistence of the TL phenotype in disseminated leukemia cells indicates that they no longer depend upon the thymus for expression of TL. This is further emphasized by

TABLE IV

Distinctive Properties of the Three TL Antigens

Antigen	Modulation by the homologous antibody	Modulation by non-homologous antibody	Anomalous appearance in leukemia cells
TL.1	+	+	+
TL.2	−	+	+
TL.3	+	?	−

the retention of TL antigen by leukemia cells in continuous culture, an observation indicating also that TL antigens are not acquired secondarily in the manner of such blood group antigens as Lewis in man and J in cattle (see reference 15).

It is evident that both intracellular and environmental factors regulate expression of TL. One of the two known external factors, the thymic influence, appears as a physiological regulator of TL expression in normal cells. The significance of the other, TL antibody, is unknown in the context of normal physiology, although reasons have been given for the conclusion that it is *not* the physiological suppressor of TL antigen in the thymus of TL− strains (1). With regard to internal control we must recognize both the maintenance of the TL− phenotype in *all* cells outside the thymus and also the retention of the TL− phenotype by thymocytes themselves in mice of those strains that are TL−. Both manifestations of control are lost in TL+ leukemias, the TL+ phenotype now being expressed in cells outside the thymus and in mice whose normal thymocytes are TL−.

Little can be said at the moment about the mechanism by which TL antibody brings about the phenotypic change TL+ → TL−. The initiating event is the attachment of antibody to antigen at the cell surface, of that there can

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

scarcely be any doubt, but how this leads to loss of the antigen is unknown. A critical question is whether modulation at a particular site requires the attachment of antibody to that site or whether modulation of *all* sites on a cell can be initiated by attachment of antibody to only *some* of them. The first of these possibilities implies a surface mechanism in which antibody fixation leads to release of antigen, which is either exfoliated or drawn into the cell. The second implies the setting in train of cellular events which lead to reversal of the phenotype. The dependence of modulation on metabolic processes, manifested by its abolition at low temperature and by actinomycin D, does not especially favor either mechanism as both might require continuation of adequate cellular metabolism.

SUMMARY

Antigenic modulation (the loss of TL antigens from TL+ cells exposed to TL antibody in the absence of lytic complement) has been demonstrated in vitro. An ascites leukemia, phenotype TL.1,2,3, which modulates rapidly and completely when incubated with TL antiserum in vitro, was selected for further study of the phenomenon. Over a wide range of TL antibody concentrations modulation at 37°C was detectable within 10 min and was complete within approximately 1 hr. The cells were initially sensitized to C′ by their contact with antibody, thereafter losing this sensitivity to C′ lysis together with their sensitivity to TL antibody and C′ in the cytotoxic test. The capacity of the cells to undergo modulation was abolished by actinomycin D and by iodoacetamide, and by reducing the temperature of incubation to 0°C. Thus modulation apparently is an active cellular process. Antigens TL.1,2, and 3 are all modulated by *anti*-TL.1,3 serum and by *anti*-TL.3 serum. This modulation affects all three TL components together, even when antibody to one or two of them is lacking. *Anti*-TL.2 serum does not induce modulation and in fact impairs modulation by the other TL antibodies. The influence of the TL phenotype of cells upon the demonstrable content of H-2 (D region) isoantigen, first shown in cells modulated in vivo, has been observed with cells modulated in vitro. Cells undergoing modulation show a progressive increase in H-2 (D region) antigen over a period of 4 hr, with no change in H-2 antigens of the K region. Restoration of the TL+ phenotype of modulated cells after removal of antibody is less rapid than TL+ → TL− modulation and may require several cell divisions.

BIBLIOGRAPHY

1. Boyse, E. A., L. J. Old, and E. Stockert. 1965. The TL (thymus leukemia) antigen. A review. *In* Immunopathology, IVth International Symposium. P. Grabar and P. A. Miescher, editors. Schwabe & Co., Basel, 23.
2. Old, L. J., E. A. Boyse, and E. Stockert. 1963. Antigenic properties of experimental leukemias. I. Serological studies *in vitro* with spontaneous and radiation-induced leukemias. *J. Natl. Cancer Inst.* **31**:977.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024

3. Schlesinger, M., E. A. Boyse, and L. J. Old. 1965. Thymus cells of radiation chimeras: *TL* phenotype, sensitivity to guinea pig serum, and origin from donor cells. *Nature.* **206**:1119.
4. Boyse, E. A., L. J. Old, and S. Luell. 1963. Antigenic properties of experimental leukemias. II. Immunological studies *in vivo* with C57BL/6 radiation-induced leukemias. *J. Natl. Cancer Inst.* **31**:987.
5. Boyse, E. A., E. Stockert, and L. J. Old. 1967. Modification of the antigenic structure of the cell membrane by thymus-leukemia antibody. *Proc. Natl. Acad. Sci. U.S.* **58**:954.
6. Gorer, P. A., and P. O'Gorman. 1956. The cytotoxic activity of isoantibodies in mice. *Transplant. Bull.* **3**:142.
7. Boyse, E. A., L. J. Old, and E. Stockert. 1962. Some further data on cytotoxic isoantibodies in the mouse. *Ann. N. Y. Acad. Sci.* **99**:574.
8. Boyse, E. A., L. J. Old, and I. Chouroulinkov. 1964. Cytotoxic test for demonstration of mouse antibody. *In* Methods in Medical Research. Year Book Medical Publishers, Chicago. **10**:39.
9. Gorer, P. A., and Z. B. Mikulska. 1959. Some further data on the H-2 system of antigens. *Proc. Roy. Soc. London, Ser. B.* **151**:57.
10. Boyse, E. A., L. J. Old, and S. Luell. 1964. Genetic determination of the TL (thymus leukemia) antigen in the mouse. *Nature.* **201**:779.
11. Davies, D. A. L., E. A. Boyse, L. J. Old, and E. Stockert. 1967. Mouse isoantigens: Separation of soluble TL (thymus-leukemia) antigen from soluble H-2 histocompatibility antigen by column chromatography. *J. Exptl. Med.* **125**:549.
12. Bloch, K. J. 1965. Heterogeneity in biologic functions of antibodies: implications for immunologic tumor enhancement. *Federation Proc.* **24**:1030.
13. Möller, G. 1964. Isoantibody-induced cellular resistance to immune haemolysis *in vivo* and *in vitro*. *Nature.* **202**:357.
14. Beale, G. H. 1957. The antigen system of *Paramecium aurelia*. *In* International Review of Cytology VI. G. H. Bourne and J. F. Danielli, editors. Academic Press, Inc. New York.
15. Race, R. R., and R. Sanger. 1962. Blood Groups in Man. F. A. Davis, Philadelphia. 4th edition. Chapter 10.

Downloaded from http://rupress.org/jem/article-pdf/127/3/523/1652445/523.pdf by guest on 24 April 2024