ANTIGENIC MODULATION IN VITRO

I. Fate of Thymus-Leukemia (TL) Antigen-Antibody Complexes Following Modulation of TL Antigenicity from the Surfaces of Mouse Leukemia Cells and Thymocytes*

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Cell surface antigens of the thymus-leukemia (TL)[1] system in the mouse are detectable only on thymocytes and cells of certain leukemias (1–3). Although TL antigens are not expressed on thymocytes of all mouse strains, leukemias developing in TL− strains may be phenotypically TL+, suggesting that TL structural genes may be present in all mouse strains, and possibly in all cells (2). The appearance of TL antigens on thymocytes and leukemia cells presumably results from activation or de-repression of these structural genes by regulatory genes (4).

Initial attempts to immunize TL− mice against their own TL+ leukemias by making them hyperimmune to TL antigens were unsuccessful, and when leukemia cells were recovered from these mice they had become phenotypically TL− (1). The cells remained TL− as long as they were passaged continually in hyperimmune mice, but regained the TL+ phenotype when returned to nonimmunized mice. This reversible loss of TL antigenicity has been termed antigenic modulation, and occurs rapidly when cells are exposed to TL antiserum either in vivo (1, 5) or in vitro at 37°C (6), cell surface antigenicity being measured by the cytotoxic activity of a subsequent exposure of cells to fresh TL antiserum in the presence of lytic complement (C′).

Analysis of antigenic modulation in vitro suggested to Old et al. (6) that modulated TL antigen-antibody complexes were lost from the cell surface by a process requiring cellular metabolic activity. Loss of these complexes could presumably occur either by exfoliation from the cell surface or internalization by pinocytosis. Yu and Cohen (7) have suggested that pinocytosis may account for the apparent loss of modulated TL antigens.

Although antigenic modulation has not been demonstrated in the same

manner for other surface antigens on mouse lymphoid cells, *H-2* antigens on
leukemia cells have been modulated with a combination of *H-2* antibody and
antimouse immunoglobulin (Ig) antibody (8, 9), while surface Ig on mouse
myeloma cells has been modulated directly with anti-Ig antibody (8). In both
cases, pinocytosis of cell-bound antibody complexes was considered the most
likely explanation for modulation.

We have examined modulation of TL antigenicity from mouse leukemia cells
and thymocytes by cytotoxicity, immunofluorescence, and immunoelectronmi-
croscopy, and have found that most modulated TL antigen-antibody complexes
remain on the cell surface, displaced topographically into aggregates or "caps"
(10), while a smaller proportion is internalized by pinocytosis.

Materials and Methods

Mice.   These were obtained from our colonies and included strains A (phenotype TL.1,2,3; *H-2ᵃ*)
and C57BL/6 (TL−; *H-2ᵇ*), and the congenic stocks, A/TL− (TL−; *H-2ᵃ*), C57BL/6/TL+ (TL.1,2,3;
H-2ᵇ), and C57BL/6/H-2ᵏ (TL−; *H-2ᵏ*).

Mouse Alloantisera.   Two mouse alloantisera specific for TL.1,2,3 antigens, (A/TL− ×
C57BL/6)F₁ anti-A strain spontaneous leukemia ASL1 (anti-ASL1) and A/TL− anti-A strain thymus
(antithymus) were used. Antithymus antiserum was used in experiments in which absolute
specificity to TL.1,2,3 antigens was critical, since anti-ASL1 antiserum might occasionally contain
leukemia-specific antibodies (11) or antibodies to C-type viruses present in ASL1 cells (T. Aoki,
personal communication) which might cross-react with viruses at the surfaces of leukemia cells used
as test cells in these experiments. Both antisera were absorbed twice with (A/TL− × C57BL/6)F₁
thymus cells to remove autoantibodies (12) and absorbed twice with C57BL/6/TL+ or (A × C57BL/6/
TL+)F₁ spleen cells to eliminate possible *H-2*-related histocompatibility antibodies not detectable
by cytotoxicity (13). After absorption, both antisera were tested on a variety of cell types by the cyto-
toxicity test and by indirect immunofluorescence, and were found to be highly specific for TL. 1, 2, 3
antigens. An alloantiserum specific for *H-2ᵇ* antigens was prepared by immunizing (C57BL/6/H-2ᵏ
× A)F₁ mice with the C57BL ascites leukemia EL4, while an anti-*H-2ᵈ* alloantiserum was prepared
by immunizing C57BL/6 mice with the BALB/c ascites tumor Meth A.

Rabbit Antisera.   Rabbits were immunized with mouse IgG or Fc fragments of mouse IgG purified
according to Hämmerling and Rajewsky (14).

Fractionation of Antisera.   (*a*) The IgG fraction of rabbit antimouse IgG antiserum was obtained
by precipitation with 40% ammonium sulfate and chromatography on a diethylaminoethyl
(DEAE)-Sephadex A-50 column, and concentrated by diaflo filtration (Amicon Corp., Lexington,
Mass.). F(ab')₂ fragments were prepared by pepsin digestion of this IgG fraction, and were purified
specifically with immunoadsorbents (15). (*b*) Fab' fragments of anti-ASL1 antiserum were prepared
according to Lamm et al. (16), except that initial fractionation of the antiserum was achieved by
precipitation with 40% ammonium sulfate. These preparations were shown to be essentially free of
intact bivalent antibody by immunodiffusion (15).

Fluoresceination of Antisera.   Purified IgG fractions of rabbit antisera were conjugated to
fluorescein isothiocyanate (FITC) by the method of Cebra and Goldstein (17). The molar
fluorescein:protein ratio of rabbit antimouse IgG/FITC antibody was 1.8, while the ratio of rabbit
antimouse Fc fragments/FITC antibody was 2.6. A crude IgG fraction of anti-ASL1 antiserum,
obtained by precipitation with 40% ammonium sulfate, was conjugated to fluoresceinyl thioureido
caproic acid (FTC) by reaction with a water-soluble carbodiimide derivative (18). The molar
fluorescein:protein ratio of this conjugate was 0.6.

Procedure for Modulation In Vitro.   The radiation-induced A strain leukemia RADA1 (TL. 1, 2, 3;
H-2ᵃ), in ascites form, and thymocytes from the congenic mouse stock C57BL/6/TL+ (TL. 1,2,3;
H-2ᵇ) were used as test cells. Cell suspensions in medium 199 containing 2% gamma globulin-free
fetal bovine serum (FBS; Grand Island Biological Co., Grand Island, N. Y.) were washed twice and
viability was determined by trypan blue dye exclusion. Thymus cells were obtained in suspension by

mincing thymus tissue with scissors in a few drops of medium and collecting the supernate. Only cell suspensions with greater than 90% viability were used in these experiments. For modulation, 5×10^6 cells were suspended in 1 ml of medium containing 2% FBS and antiserum in the appropriate concentration, in a glass centrifuge tube. It was found unnecessary to incubate cells under tissue culture conditions, since the incubation medium remained close to its initial value of pH 7.4 when cells were maintained in a water bath-shaker at 37 °C for more than 4 h. The tubes were also manually shaken every 5 min to insure that all cells remained freely suspended. For controls, antiserum was omitted or replaced with pooled normal mouse serum (NMS). After the appropriate length of incubation, cells were immediately cooled in an ice bath and washed twice in the cold.

Cytotoxicity Test (19, 20). Cells (0.05 ml of a suspension containing 5×10^6 cells/ml) were incubated with 0.05 ml of serial dilutions (1/100 to 1/100,000) of antiserum and 0.05 ml of guinea pig or rabbit serum as a source of lytic C' for 45 min at 37°C. After incubation, the percentage of dead cells was determined by uptake of trypan blue dye. For each test, controls included incubating cells with antiserum alone (serum control) or C' alone (C' control). Incubations were in medium 199 without serum supplements.

Complement (C') for the cytotoxicity test. Thymocytes were tested with either: (a) pooled rabbit serum from individual animals with low natural cytotoxicity for mouse lymphoid cells and high C' activity (21) (rabbit C'); or (b) pooled guinea pig serum with low toxicity for mouse lymphoid cells and high C' activity (nontoxic guinea pig C'). RADA1 cells were tested with either: (a) pooled guinea pig serum toxic for normal mouse lymphoid cells but nontoxic for leukemia cells (toxic guinea pig C'); (b) rabbit C' absorbed with mouse lymphoid cells to decrease its natural toxicity for these cells and permit its use in higher concentrations on leukemia cells, absorption being done in the presence of EDTA to bind divalent cations and prevent loss of C' during absorption (21) (absorbed rabbit C'); or (c) nontoxic guinea pig C'.

Immunofluorescence. For indirect membrane fluorescence staining following modulation, cells were fixed with 2% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS), for 15 min at 0°C, washed with PBS, and 10^6 cells were incubated in 0.05 ml vols with rabbit antimouse IgG/FITC antibody (100 µg/ml) or rabbit antimouse Fc fragments/FITC antibody (100 µg/ml) in PBS for 30 min at 0°C. Cells were washed, resuspended in 50% glycerol in PBS, and examined at room temperature with a Leitz Orthoplan microscope (E. Leitz, Wetzlar, Germany) equipped with an Osram HBO 200W mercury lamp, and BG38 excitor, KP490 interference, and K530 barrier filters. For direct membrane fluorescence, cells were modulated with anti-ASL1/FTC antibody, washed, fixed with paraformaldehyde, and examined in the same manner as indirect fluorescence.

Immunoelectronmicroscopy. The rabbit F(ab')$_2$ hybrid antibody, antimouse IgG/anti-southern bean mosaic virus (SBMV), and SBMV were prepared according to Hämmerling et al. (22). Following modulation, cells were fixed with paraformaldehyde as described above, then 5×10^6 cells were incubated in 0.1 ml of medium 199 containing 100 µg/ml of hybrid antibody for 30 min at 0°C. After washing twice, the cells were incubated similarly with SBMV (100 µg/ml), washed, and then fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4. Subsequent preparation for electron microscopy was as described previously (15).

Results

Characteristics of Antigenic Modulation In Vitro. Incubating RADA1 cells with anti-ASL1 (anti-TL.1,2,3) antiserum at 37°C resulted in a rapid loss of TL.1,2,3 antigenicity as measured by the cytotoxicity test using toxic guinea pig serum as a source of lytic C' (Fig. 1). Loss of antigenicity was directly proportional to the concentration of antiserum in the modulating incubation, and was nearly complete within 1 h when dilutions of 1/100 or less were used. The initial cytotoxic titer (the dilution at which 50% of the test cells are killed) of this antiserum on RADA1 cells using this C' was 1/5,000. During the modulation or preincubation, and in all subsequent experiments, cell viability remained greater than 90%, and there was less than a 10% loss of antigenicity when NMS was

substituted for TL antiserum. Since modulation of TL antigenicity was nearly as rapid and complete at an antiserum dilution of 1/100 as at 1/10, in subsequent modulation experiments with RADA1 cells this antiserum was used at a dilution of 1/100. By contrast, after incubation of RADA1 cells (H-2^a = H-2^{dk}) with anti-H-2^d antiserum for 4 h under similar conditions, about 85% of the initial amount of H-2^d antigenicity remained demonstrable by cytotoxicity.

Modulation of TL antigenicity from TL+ thymocytes and RADA1 cells had similar characteristics, except that thymocytes modulated considerably more slowly (Fig. 2). For comparison of modulation on these two cells, cytotoxicity was
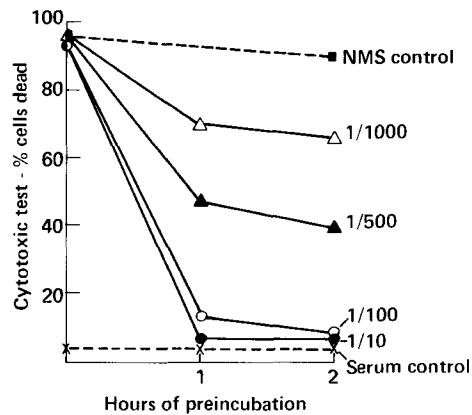

Fig. 1.   Effect of TL antiserum dilution on modulation of TL antigenicity from RADA1 leukemia cells. Cells were preincubated with dilutions of anti-ASL1 antiserum from 1/10 to 1/1,000, or with a 1/10 dilution of normal mouse serum (NMS control), at 37°C, then residual TL antigenicity on the cell surface was measured by cytotoxicity, using fresh anti-ASL1 antiserum (diluted 1/100) and toxic guinea pig C' (diluted 1/3). Cells not preincubated were tested by cytotoxicity directly. Cell viability was assessed by preincubating cells with antiserum (1/10), then testing the cytotoxicity of fresh antiserum (1/100) in the absence of C' (serum control).
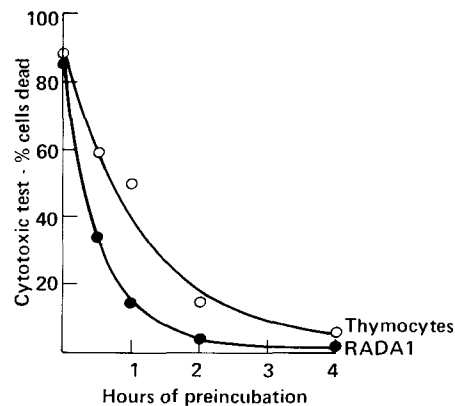

Fig. 2.   Comparison of modulation of TL antigenicity from RADA1 cells and C57BL/6/TL + thymocytes. Both cell types were preincubated with anti-ASL1 antiserum (1/100) at 37°C, then residual TL antigenicity was measured by cytotoxicity using fresh antiserum (1/100) and nontoxic guinea pig C' (1/4).

measured using nontoxic guinea pig C'. The cytotoxic titer of anti-ASL1 antiserum was about 1/3,000 on RADA1 cells and 1/5,000 on thymocytes using this C' source, indicating a greater amount of TL antigenicity on thymocytes. While modulation of RADA1 cells was about 50% complete after 20 min, a comparable degree of modulation of thymocytes was reached only after 55 min. Modulation of TL antigenicity from thymocytes, like RADA1 cells, occurred nearly as rapidly and completely at an antiserum dilution of 1/100 as at a dilution of 1/10.

Immunofluorescence of Modulation. The fate of cell surface-bound TL antibody during modulation was visualized on RADA1 cells and thymocytes by immunofluorescence, using anti-ASL1/FTC antibody in the modulating preincubation. Since conjugation to FTC resulted in a decrease in the cytotoxic titer of anti-ASL1 antiserum on RADA1 cells to 1/40, it was used at a dilution of 1/10. Following incubation for a particular length of time, one aliquot of cells was examined by fluorescence microscopy, while the residual TL antigenicity of another aliquot of cells from the same sample was measured by cytotoxicity.

Correlated immunofluorescence and cytotoxicity results are presented in Figs. 3-6 and Fig. 9. After 15 min of preincubation, fluorescent label was distributed uniformly over the entire surface of all cells, but was also concentrated immediately beneath the cell surface at several locations, presumably in pinocytic vesicles (Fig. 3). Approximately 80% of the initial TL antigenicity remained on cells from this sample (Fig. 9). After 30 min, the cell surface was labeled uniformly except for occasional large aggregates, while considerable label had been internalized in cytoplasmic vesicles (Fig. 4). TL antigenicity was reduced to about 60% of its initial level by this time (Fig. 9). Label on the cell surface was completely aggregated into large "patches" after 1 h of preincubation (Fig. 5), with patches on 27% of the cells concentrated on one side of the cell into "caps" (10) (Fig. 9). Internalized label appeared to be concentrating within one region of the cell in many cases. Only 20% of the initial level of TL antigenicity remained on cells from this sample. By 2 h, with antigenicity reduced to 6% of its initial level, 70% of the cells displayed caps of label, with patches more generally distributed over the surfaces of the remainder of the cells (Figs. 6 and 9). Label inside the cells was usually concentrated in a region approximately opposite from the location of the capped surface label. The distribution of label on the surface and inside cells after 4 h of preincubation was similar to that at 2 h, caps having formed on nearly 90% of the cells (Fig. 9).

Similar results were obtained when RADA1 cells were modulated with antithymus antiserum (Fig. 9), although internalized antibody could not be visualized since this antibody was detected by indirect fluorescence staining with rabbit antimouse IgG/FITC antibody. Antithymus antiserum had a titer of 1/160 on RADA1 cells using toxic guinea pig C', and was used at a dilution of 1/10 in the modulating incubation. Labeling patterns were also similar inside and on the surfaces of thymocytes modulated with anti-ASL1/FTC antibody, although patch and cap formation, like modulation, occurred more slowly than on RADA1 cells. After 4 h, the labeling pattern, extent of capping, and degree of modulation on thymocytes was comparable to RADA1 cells after 2 h.

These results clearly demonstrate that modulation of TL antigenicity from the
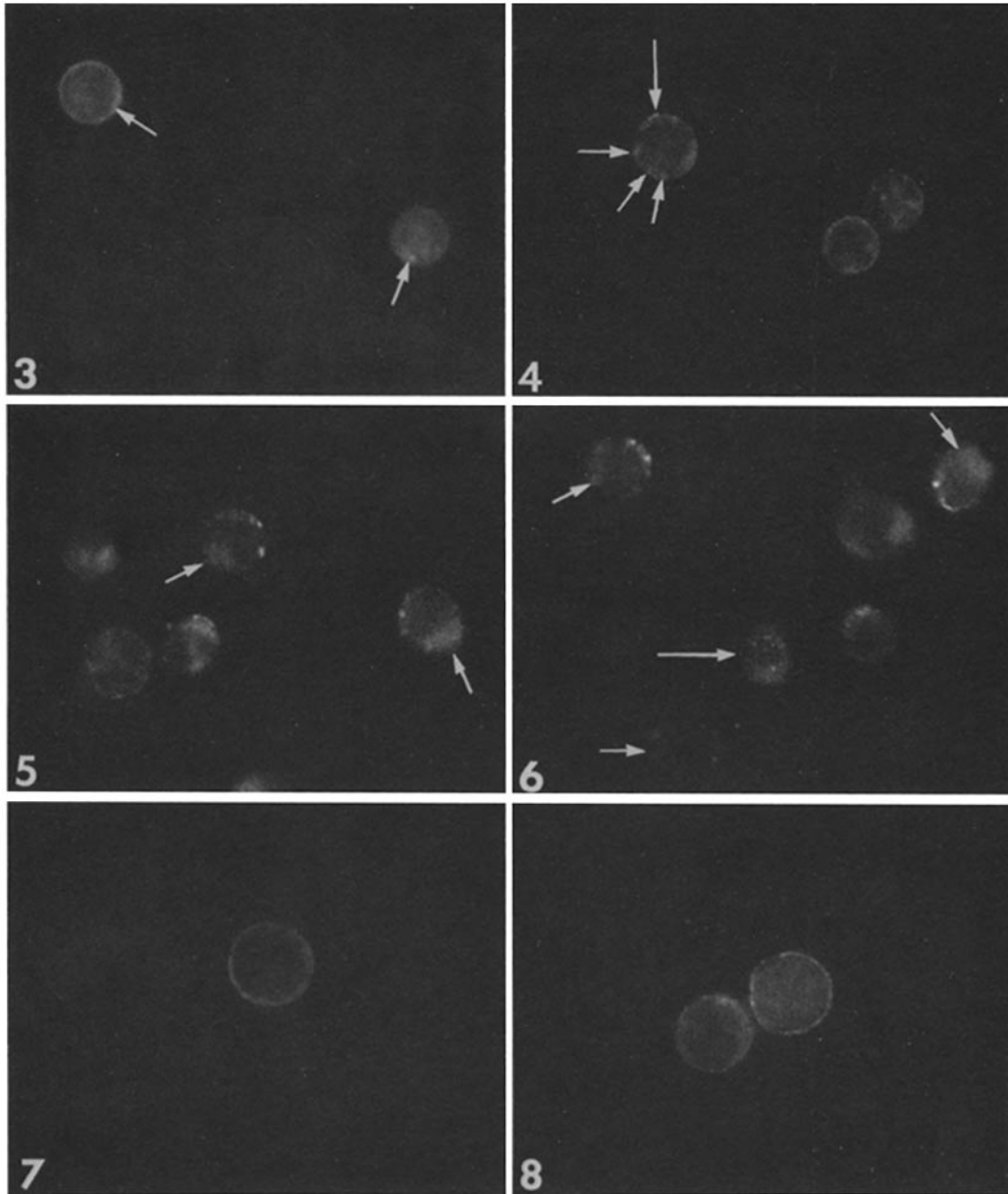
FIGS. 3-6. Direct immunofluorescence of modulation of TL antigenicity from RADA1 cells with anti-ASL1/FTC antibody at 37°C. × 900. Immunofluorescence and cytotoxicity results from the same samples are presented in Fig. 9.

FIG. 3. Preincubated for 15 min. The cell surface is labeled uniformly, with label also concentrated at several locations immediately beneath the cell surface (arrows).

FIG. 4. Preincubated for 30 min. Label is distributed uniformly over the cell surface except for occasional large aggregates (long arrow); label has also been internalized at several locations (short arrows).

surfaces of RADA1 cells and thymocytes with bivalent antibody is accompanied by progressive displacement of labeled antibody complexes from an initially uniform distribution into patches and ultimately into caps. There was only a partial loss of labeled antibody from the cell surface into pinocytic vesicles. It appears that modulation precedes cap formation, but it is important to note that the fluorescence microscopy observations were made immediately following the preincubation, while the amount of antigenicity remaining on other cells from the same samples was determined during a subsequent cytotoxicity incubation lasting 45 min. Although it is likely that cytolysis is initiated shortly after the addition of fresh antiserum and C', further topographical displacement of TL antigen-antibody complexes may have occurred during this incubation which could influence the amount of TL antigenicity detectable on the cell surface. Therefore, on the basis of these observations, it is difficult to precisely correlate modulation of TL antigenicity with surface distribution of TL antigen-antibody complexes.

In order to clarify this relationship, we examined the fate of monovalent Fab' fragments of anti-ASL1 antibody used to modulate TL antigenicity from RADA1 cells, since these fragments have modulating capabilities comparable to bivalent antibody (16). Direct fluoresceination of Fab' fragments was not feasible, so indirect fluorescence staining was employed. Following modulation with Fab' fragments for a particular length of time, one aliquot of cells was fixed, incubated with rabbit antimouse IgG/FITC antibody, and then examined by fluorescence microscopy. The amount of TL antigenicity remaining on the cell surface was determined by cytotoxicity on another aliquot of cells from the same sample. Preparations of Fab' fragments of anti-ASL1 antiserum used in these experiments lacked the C'-binding Fc antibody fragment, as indicated by the complete lack of visible fluorescence staining when RADA1 cells incubated with Fab' fragments were subsequently incubated with fluorescent rabbit antimouse Fc fragments/FITC antibody.

After modulating RADA1 cells for 30 min with Fab' fragments, indirect labeling demonstrated a uniform distribution of these fragments over the entire cell surface (Fig. 7), while the TL antigenicity of these cells was reduced to about 50% of the initial level (Fig. 9). Uniform surface labeling was also observed on about 90% of the cells after 1 h of preincubation, with label on the remaining 10% of the cells appearing slightly aggregated. Less than 20% of the initial TL

FIG. 5.   Preincubated for 1 h. Label on the cell surface is completely aggregated into "patches," while internalized label has begun to coalesce into one region of the cell (arrows).

FIG. 6.   Preincubated for 2 h. Patches of label have concentrated into "caps" over one pole of the cell, with internalized label coalesced within the opposite pole of the cell in most cases (short arrows). The cap on one cell and internalized label within the opposite pole of the cell are visualized end-on (long arrow).

FIGS. 7-8.   Indirect immunofluorescence of modulation of TL antigenicity from RADA1 cells with Fab' fragments of anti-ASL1 antibody at 37°C. Antibody fragments were detected, after fixing the cells, by incubating with rabbit antimouse IgG/FITC antibody. × 1,200. Immunofluorescence and cytotoxicity results on the same samples are presented in Fig. 9.

FIG. 7.   Preincubated for 30 min. Label is distributed uniformly over the entire cell surface.

FIG. 8.   Preincubated for 2 h. Numerous aggregates of label are distributed over the entire cell surface.

antigenicity remained after 1 h. Only about 10% of the initial TL antigenicity remained on the surfaces of cells preincubated with Fab' fragments for 2 h. By this time, surface fluorescence on about 80% of the cells appeared as small aggregates of label distributed over the entire cell surface (Fig. 8), while the surfaces of the remainder of the cells were uniformly labeled. Only 2% of all labeled cells displayed cap formation (Fig. 9). Internalized label could not be visualized by this indirect staining procedure.

These results indicate that modulation of TL antigenicity precedes, and is not dependent upon, redistribution of cell surface-bound TL antibody into patches or caps, since such gross redistribution was not induced by Fab' antibody fragments. However, as in modulation with bivalent anti-ASL1/FTC antibody, residual TL antigenicity was measured during a 45-min cytotoxicity incubation
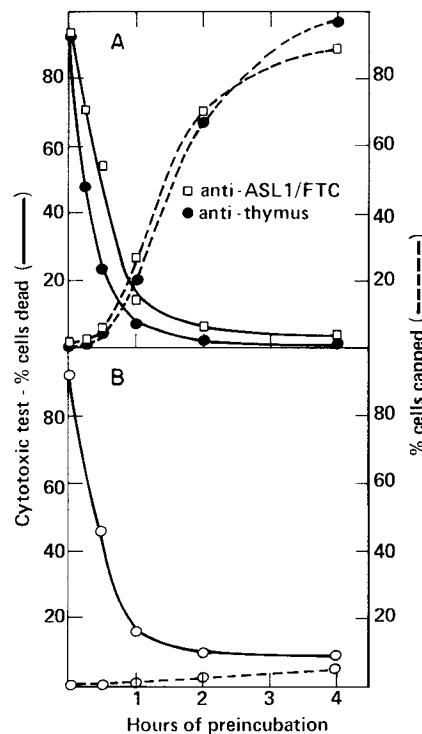

FIG. 9. Residual TL antigenicity and extent of cap formation on RADA1 cells modulated with bivalent and monovalent TL antibody. (A) Cells modulated with anti-ASL1/FTC antibody (1/10) or antithymus antiserum (1/10) at 37°C. Results for modulation with anti-ASL1/FTC antibody are from the same samples from which the immunofluorescence micrographs in Figs. 3–6 were obtained. Cytotoxicity was measured using anti-ASL1 antiserum (1/100) or antithymus antiserum (1/10) and toxic guinea pig C' (1/3). Cap formation on cells modulated with antithymus antiserum was determined by fixing the cells and incubating with rabbit antimouse IgG/FITC antibody. (B) Cells modulated with monovalent Fab' fragments of anti-ASL1 antibody (80 μg/ml) at 37°C. Data was obtained from the same samples from which the immunofluorescence micrographs in Figs. 7–8 were taken. Cytotoxicity was measured with anti-ASL1 antiserum (1/10) and toxic guinea pig C' (1/3). Cap formation was determined on cells fixed and then incubated with rabbit antimouse IgG/FITC antibody.

following the modulating incubation, although fluorescence microscopy observations were based on cells preincubated only. Therefore, since some further displacement of cell-bound Fab' antibody fragments might have occurred before cytolysis was initiated, a redistribution of TL antigen-antibody complexes into visible aggregates on the cell surface may be directly involved in modulation of TL antigenicity.

Immunoelectronmicroscopy of Modulation. To determine the site of cap formation on modulated cells, RADA1 cells were modulated for 2 h, and thymocytes for 4 h, with anti-ASL1/FTC antiserum as described above, and fixed. One aliquot of cells was examined by fluorescence microscopy, and label on the cell surface and within the cells appeared as in Fig. 6. Another aliquot of cells was incubated sequentially with hybrid antimouse IgG/anti-SBMV antibody and SBMV, and then prepared for electron microscopy. On both RADA1 cells and thymocytes, 70–80% of the caps of label observed in sections in which the Golgi region of the cell was evident were formed over the pole of the cell opposite from the Golgi region (Fig. 10), none were directly over the Golgi region, and the remainder occupied intermediate positions. Label observed to be internalized by cells modulated with anti-ASL1/FTC antibody must, therefore, become sequestered into the Golgi region of the cell, since it is usually apparent within the cell in a position directly opposite from the caps of external label (Fig. 6). Similar results were obtained using antithymus antiserum for modulation, although in this case cells were not prepared for fluorescence microscopy.

Effect of Complement (C') on Modulation. The cytotoxic titer of anti-ASL1 antiserum on RADA1 cells was considerably higher (1/10,000) when absorbed rabbit serum was used as a source of lytic C' than when toxic guinea pig C' was used (1/5,000). Because of this greater sensitivity, we tested residual TL antigenicity using absorbed rabbit C' following modulation. When absorbed rabbit C' was substituted for toxic guinea pig C' in the cytotoxicity test, the loss of TL antigenicity from RADA1 cells during a modulating incubation with anti-ASL1 antiserum was considerably reduced (Fig. 11). After 4 h of incubation, only 21% of the initial TL antigenicity was lost when cytotoxicity was assessed using absorbed rabbit C', compared to a 95% loss when toxic guinea pig C' was used.

This would seem to indicate that following modulation nearly 80% of the initial quantity of TL antigens remains on the cell surface, unless absorbed rabbit C' operates by an unusual mechanism which does not require direct interaction with cell surface-bound antigen-antibody complexes. In order to test this possibility, we attempted to prevent the cytolytic activity of absorbed rabbit C' by covering cell-bound TL antibody with a secondary rabbit antimouse IgG antibody. Bivalent F(ab')$_2$ antibody fragments lacking the C'-binding Fc portion were used to avoid cytolytic interaction of absorbed rabbit C' with these antibody molecules. Incubating cells with these F(ab')$_2$ fragments for 1 h at 37°C following modulation for 1 h resulted in a complete loss of the residual TL antigenicity indicated by cytotoxicity with absorbed rabbit C' (Fig. 11). Cytolysis by absorbed rabbit C' must therefore depend upon direct interaction with cell surface-bound antigen-antibody complexes.

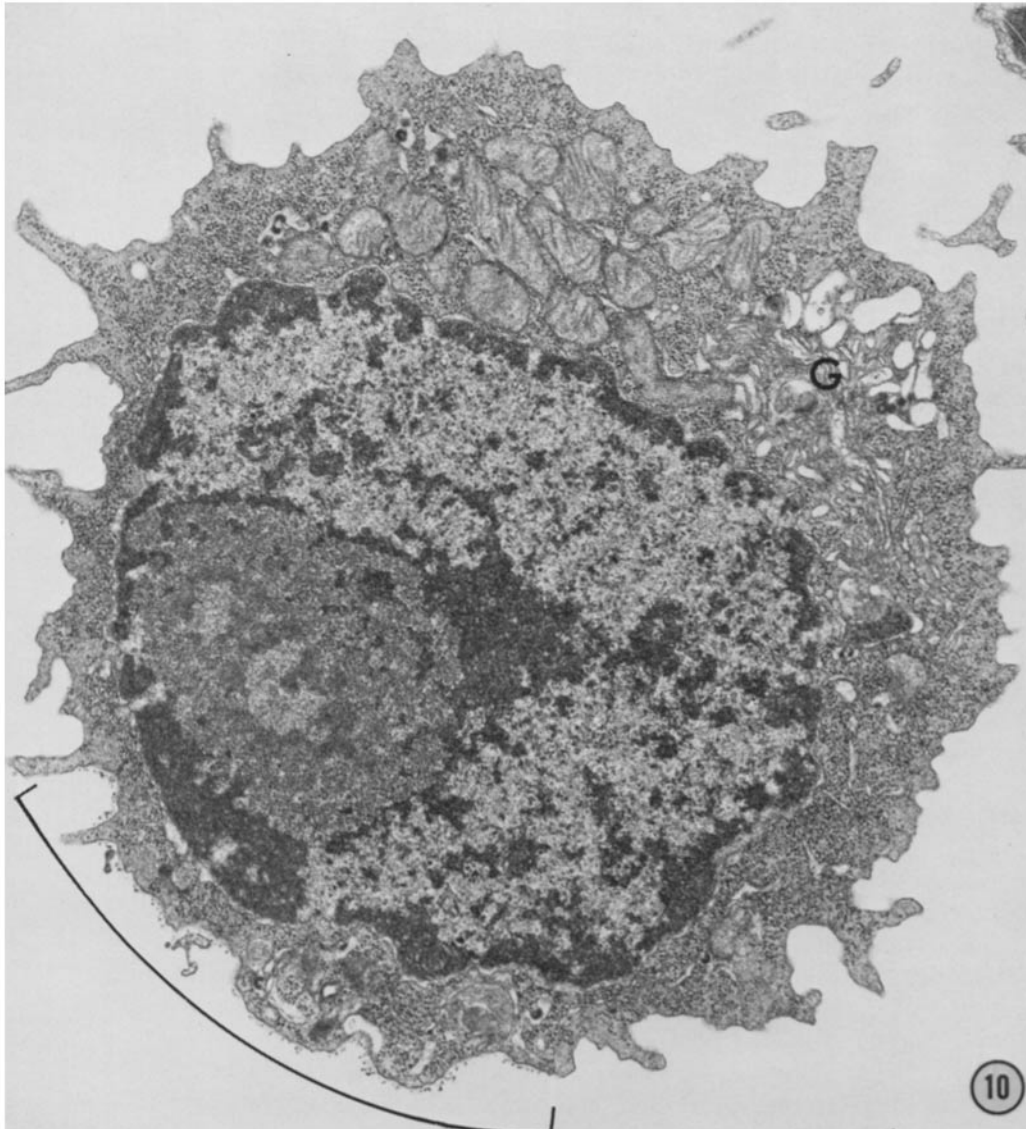In contrast, incubating RADA1 cells under modulating conditions with

Fig. 10. Electron micrograph of a RADA1 cell modulated with anti-ASL1/FTC antibody (1/10) for 2 h at 37°C, fixed, and then incubated sequentially with hybrid antimouse IgG/anti-SBMV antibody and SBMV at 0°C. Label is confined to a "cap" (bracket) over the pole of the cell opposite from the Golgi region (G). × 16,000.

anti-ASL1 antiserum which had been heated at 56°C for 1 h significantly reduced the usual loss of TL antigenicity as measured by cytotoxicity using toxic guinea pig C' (Table I). Modulating capacity was restored to this heated antiserum by adding either NMS or hyperimmune mouse antiserum directed against cell surface antigens (H-2^b antigens) not detectable on RADA1 cells. A heat-labile factor present in TL antiserum and mouse serum in general is therefore essential for the demonstration of modulation with guinea pig C'.
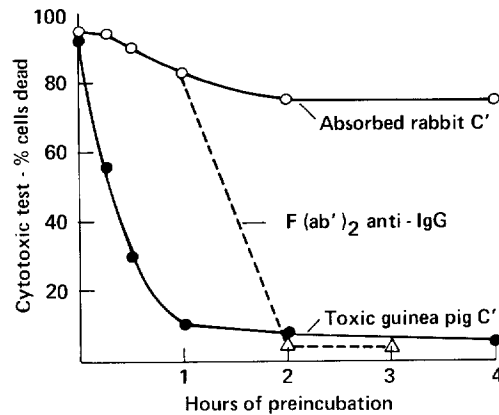
Fig. 11. Residual TL antigenicity on RADA1 cells modulated with anti-ASL1 antiserum (1/100), as measured by cytotoxicity using different sources of lytic C′. Following modulation, cell surface TL antigenicity was tested with anti-ASL1 antiserum (1/100) and either toxic guinea pig C′ (1/3) or absorbed rabbit C′ (1/4). After 1 h of modulation at 37°C, samples of cells were washed twice and incubated with 100 μg/ml of F(ab′)₂ fragments of rabbit antimouse IgG antibody [F(ab′)₂ anti-IgG] at 37°C for 1 and 2 h, after which cytotoxicity was measured using absorbed rabbit C′.

TABLE I

Effect of Heating TL Antiserum on Modulation of TL Antigenicity from RADA1 Leukemia Cells

Modulating serum*	Cytotoxicity test‡ (% cells killed)
None	92
Anti-ASL1 antiserum	8
Anti-ASL1 antiserum, heated§	68
NMS	94
Anti-ASL1 antiserum, heated + NMS	12
Anti-H-2^b antiserum‖	90
Anti-ASL1 antiserum, heated + anti-H-2^b antiserum	8

* Cells were incubated for 1 h at 37°C, with serum, when added, diluted 1/100.
‡ After the modulating incubation, cells were washed and incubated for 45 min at 37°C with fresh anti-ASL1 antiserum (1/100) and toxic guinea pig C′ (1/3); killed cells were distinguished by uptake of trypan blue dye.
§ 56°C for 1 h; after heating, this antiserum retained its full cytotoxic titer on RADA1 cells (1/5,000) when tested using toxic guinea pig C′.
‖ This antiserum (H-2^a vs. H-2^b) has no cytotoxic activity on RADA1 (H-2^a) cells.

Discussion

Our results indicate that in vitro modulation of TL antigenicity from the surfaces of RADA1 leukemia cells and normal thymocytes does not involve an extensive loss of TL antigen-antibody complexes from the cell surface, as has been

postulated (6, 7), and moreover is demonstrable by C'-mediated cytotoxicity only under certain conditions.

Modulation of TL antigenicity with bivalent antibody, indicated by a loss of cytotoxic sensitivity to lytic guinea pig C', was accompanied initially by a significant amount of pinocytosis, which decreased as antibody complexes remaining on the cell surface became progressively displaced into patches and caps. Cap formation usually occurred over the pole of the cell opposite from the Golgi region. Analysis of capping on mouse lymphoid cells has shown that surface receptors on splenic lymphocytes generally cap over the Golgi region, accompanied by extensive pinocytosis, while receptors on thymocytes most often cap opposite from the Golgi region, with little pinocytosis occurring (23). It is evident that capping is not directly related to modulation, but since the Golgi region is apparently the portion of the cell most active in pinocytosis, mobilization of TL antigen-antibody complexes away from this region of the cell during modulation may in part account for the retention of modulated complexes on the cell surface.

Modulation with monovalent Fab' antibody fragments was nearly completed before any aggregation of TL antigen-antibody complexes on the cell surface became apparent. Although the extent of pinocytosis occurring during modulation with Fab' fragments could not be determined directly, these antibody fragments remained readily detectable on the cell surface following modulation, indicating that pinocytosis could not have been extensive. Since pinocytosis may require ligand-induced aggregation of receptors on the cell surface (24), Fab' antibody fragments may not induce sufficient aggregation to stimulate pinocytosis, at least until after modulation is completed.

The continued cytotoxic sensitivity of RADA1 cells to (a) TL antiserum and absorbed rabbit C' after a standard modulation incubation with TL antiserum, and (b) TL antiserum and toxic guinea pig C' following preincubation with heated TL antiserum provides further evidence that a considerable number of TL antigen-antibody complexes remain bound to the cell surface following modulation in vitro. The observed loss of about 20% of the initial level of TL antigenicity on RADA1 cells after several hours of preincubation with TL antiserum may represent the approximate percentage of TL antigen-antibody complexes actually lost from the cell surface either by pinocytosis or exfoliation into the extracellular environment, or by a combination of these processes.

The fate of TL antigen-antibody complexes during modulation of TL antigenicity from the surfaces of ASL1 leukemia cells has recently been examined by Yu and Cohen (7) by isolating solubilized cell surface TL antigens by immunoprecipitation. Modulation of ASL1 cells, measured by the cytolytic effect of a subsequent exposure to fresh TL antiserum and guinea pig C', occurred very slowly by comparison with modulation rates we observed for RADA1 cells and thymocytes, modulation being completed only after 10–15 h of preincubation with TL antiserum. Although the rate of intracellular synthesis of new TL antigens remained essentially the same in cells undergoing modulation and in unmodulated cells, the recovery of TL antigens from the surfaces of modulating cells diminished gradually compared with unmodulated cells, loss of antigen becoming apparent initially after 12 h of incubation. Pinocytosis was implicated in this observed loss of TL antigens, since the extent of exfoliation into the

extracellular environment was no greater from modulating cells than from unmodulated cells, and there was no evidence for localized degradation of cell surface-bound antigens (7).

It is difficult to correlate our results with those of Yu and Cohen (7) for several reasons: (a) since their modulating TL antiserum also contained non-TL antibodies, they were unable to determine by immunofluorescence whether TL antibody remained cell-bound following modulation; and (b) in their experiments cells were modulated with antiserum which had previously been heated at 56°C for 30 min, a procedure which we found to suppress modulation, and which may explain why modulation occurred so slowly.

While our results indicate that some TL antigens remain on the surfaces of cells modulated in vitro with TL antibody, modulation of other surface receptors, such as surface Ig on mouse lymphoid cells (8), may involve a more complete loss from the cell surface, since surface receptors cap in different locations on the cell surface and are thus differentially susceptible to pinocytosis (23). It will also be interesting to determine whether TL antigens remain on the surfaces of cells modulated in vivo (1).

The interaction of modulating TL antibody, or antibody fragments, with TL antigens on the cell surface resulted in a topographical displacement of these antigens, although modulation preceded extensive redistribution into patches and caps. A more subtle alteration in the organization of molecules bearing TL antigenic determinants within the cell surface membrane, or in the topographical interrelationships between these molecules, which would precede visible redistribution, may be more directly involved in "blocking" of guinea pig C'-mediated cytolysis. On thymocytes, TL antigens are much more readily capped than H-2 antigens (25). This difference in the tendency for these antigens to be displaced topographically as a result of specific antibody binding may be correlated with their tendency to modulate, since H-2 antigens are modulated very poorly with H-2 antibody alone, but much more readily when an antimouse Ig antibody is added (8, 9). Another indication that some type of redistribution may be involved in modulation is the difference in the rates at which TL antigenicity was modulated from RADA1 cells and thymocytes under the same conditions. By the criterion of cytotoxicity sensitivity, there appears to be a greater quantity of TL antigens on thymocytes than on RADA1 cells. An inverse relationship between the quantity of a particular receptor on the cell surface and the rate and extent of redistribution into caps has been observed with normal mouse lymphoid cells (25). The tendency for TL antigens to modulate may also be partially related to the amount of antigen on the cell surface.

Further elucidation of the mechanisms involved in in vitro modulation of TL antigenicity may explain why TL antigens appear to be unique among cell surface antigens in this regard, and may contribute to our understanding of the organization of the cell surface.

Summary

The modulation or loss of thymus-leukemia (TL) antigenicity from the surfaces of mouse RADA1 leukemia cells and normal thymocytes during incubation with

TL antibody in vitro at 37°C was investigated by cytotoxicity, immunofluorescence, and immunoelectronmicroscopy. The fate of bivalent and monovalent antibody during modulation was visualized by fluorescence microscopy. Considerable antibody remained bound to the cell surface after modulation, bivalent antibody being displaced topographically into "patches" and "caps" while monovalent antibody was only slightly aggregated on the cell surface. Some antibody was internalized, presumably by pinocytosis, and was sequestered into the Golgi region of the cell. Capping usually occurred over the pole of the cell opposite from the Golgi region, which may explain the lack of extensive pinocytosis of modulating bivalent antibody. Since modulation with monovalent antibody occurs without patch or cap formation, gross topographical redistribution of TL antigen-antibody complexes is not required for modulation, although more subtle displacement of these complexes may be involved.

Modulation was demonstrable by cytotoxicity with guinea pig C' but not with absorbed rabbit C', indicating that modulated TL antigens remain bound to the cell surface. A heat-labile factor in TL antiserum and in mouse serum in general is responsible for "blocking" the cytolytic interaction of guinea pig C' with modulated TL antigen-antibody complexes.

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