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THE EXPRESSION AND LOCALIZATION OF SURFACE NEOANTIGENS IN TRANSFORMED AND UNTRANSFORMED CULTURED CELLS INFECTED WITH AVIAN TUMOR VIRUSES

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The presence and localization of neoantigens induced in cultured cells, infected or transformed with avian tumor viruses (ATV), were studied ultrastructurally on carbon platinum replicas of cell surfaces. The use of antibody, labeled with hemocyanin molecules, provided sensitive detection and analysis of cell surface antigen distribution. The subgroup-specific antigens of the viral envelope were found in considerable amount in the plasma membranes of ATV-infected chick embryo fibroblasts. The distribution of these antigens over the cell surface, evaluated on cells which were prefixed with glutaraldehyde, was found to be diffuse with a greater density on the cell processes in some cells. Reaction of antibody to viral envelope antigens with living ATV-infected cells resulted in a number of patterns of redistribution of membrane antigen-antibody complexes (AAC). Redistribution occurred in symmetrical or asymmetrical modes. The former consisted of randomly oriented aggregates (patches) of AAC over the cell surface. The latter included: (a) linear accumulation of AAC at cell margins; and (b) condensation of complexes into one or more centers of coalescence. These observations could be made on chick embryo cells infected (but not transformed) by avian leukosis virus, or on cells oncogenically transformed by avian sarcoma virus. The regions of coalescence were suggestive of the "capping" phenomenon seen in other systems, and their formation was temporally correlated with endocytosis of labeled AAC and the gradual loss of AAC from the surface.

The effects of several biologically perturbing substances on the processes of redistribution were investigated in ALV-infected fibroblasts. Sodium azide, puromycin, actinomycin D, and colchicine had no effect on either form of asymmetrical redistribution. Cytochalasin B (CB) and iodoacetic acid (IAA) appeared to have some effect on the marginal redistribution, and to completely prevent the condensation into foci of coalescence (FC). When treated with these compounds, reacted with antibody at low temperature, washed free of unbound antibody, and warmed at 37°C, cells rapidly cleared their surfaces of AAC. This was not accompanied by formation of FC or endocytosis. In some of these cells, a distribution was observed which suggested a possible centrifugal flow of antigenic sites – perhaps an alternate route for disposal of AAC. None of the drugs tested affected symmetrical redistribution.

Repeated attempts at detection and topographical analysis of a tumor-specific antigen on the surface of Rous sarcoma virus-transformed chicken and rat cells

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have provided no evidence for antibody to such an antigen in the serum of immunized animals. Autochthonous, homologous, and heterologous immunizations of chickens and rats did not produce a detectable antibody response to a virus-specific tumor surface antigen. Preliminary results, however, suggest the expression of an individual-specific (unique) tumor antigen on the surface of Rous sarcoma cells.

INTRODUCTION

The avian tumor viruses (ATV)[1] may be divided into two classes: the avian leukosis viruses (ALV) and the avian sarcoma viruses (ASV) (Table I). The former is leukemogenic in the susceptible host and the latter produces solid tumors (1). Both groups of viruses can infect cultured chick embryo cells in vitro. Infection of these cells with ASV results in the synthesis and production of virus and transformation to a malignant phenotype. This phenotype includes altered growth patterns (2), social behavior (3), morphology (4), and sugar transport (5). ALV infection, on the other hand, results only in virus production in an otherwise phenotypically normal cell.

Cultured chick embryo fibroblasts (CEF), infected with ATV, express new antigens at their surfaces. Such cells produce plasma membrane antigens identical to the subgroup-specific antigens of the viral envelope (6). These antigens appear to be the only new components in the plasma membrane of the ALV-infected cells. Cells infected with ASV, however, in addition to subgroup-specific viral envelope antigens have been reported to express a separate, group-specific, nonvirion antigen at the cell surface (6, 7). It has been suggested that this class of neoantigens consists of a complex of tumor-specific and retrogenically expressed embryonic antigens (8).

Early studies involving ultrastructural visualization of appropriately marked antibodies with specificities directed to cell surface antigens revealed clustering of antigenic sites into discrete sectors over the cell membrane (9–11). This finding raised the possibility that the cell surface could consist of a topographical mosaic of membrane components; and the heterogeneous distribution of sites suggested possible association between localization and function. Such an interpretation was not supported, however, by subsequent observations. The experiments of Frye and Edidin (12) demonstrated the fluid and mobile nature of the plasma membrane, and the later work of Taylor et al. (13), established that immunoglobulin molecules in the cell membranes of lymphocytes were readily redistributed from a diffuse orientation into "patched" aggregates, or into a condensed "cap" over one pole of the cell upon reaction with specific anti-IgG. Similar phenomena were also found to occur with a number of other receptor-ligand interactions (14–16).

[1] Abbreviations: AAC, antigen-antibody complex(es); ALV, avian leukosis virus; ASV, avian sarcoma virus; ATV, avian tumor virus; B77, Bratislava strain (subgroup C) of RSV; CB; cytochalasin B; CEF, chick embryo fibroblasts; FC, foci of coalescence; IAA, iodoacetic acids; ImS, immune serum; LI, labeling index; MR, marginal redistribution; PrS, preimmune serum; RAV, Rous-associated virus (subgroup A) of ALV; rD-O, REF transformed by SRV-D; rD-1, rat tumor induced in rat 1 by injections of rD-O; rD-19, rD-20, rat tumors induced in rats 19 and 20 by transplantation of rD-1 cells; REF, rat embryo fibroblasts; RSV, Rous sarcoma virus; TEM, Temin-modified Eagle's medium; TSSA, tumor-specific (cell) surface antigen; TATA, tumor associated transplantation antigen; SRV-A, Schmidt-Ruppin strain (subgroup A) of RSV; RSV-D, Schmidt-Ruppin strain (subgroup D) of RSV.

The less static concept of the spatial organization of components of the cell surface has not dampened interest in membrane topography. The preferential agglutination of oncogenically transformed cells by plant lectins, once thought a consequence of the greater number (17) or the discontinuous localization of lectin receptor sites (18) in the malignant state, has since been interpreted as occurring through a more facile ligand-induced redistribution of receptors in the fluid membrane (19–21). This phenomenon may be the result of a less viscous membrane matrix in transformed cells (22); or it may reflect an altered cellular control of surface topography. The latter prospect has stimulated enormous investigation and speculation. Results to date indicate that an energy-dependent mechanism, profoundly affected by the drugs colchicine and cytochalasin B, is responsible for specific topographical rearrangements stimulated by ligand-receptor interactions (23–25).

In this laboratory, we have investigated the topographical distribution of cell surface neoantigens associated with infection and transformation of cultured chick embryo cells by the Rous sarcoma virus (RSV). Both the unperturbed distribution and the rearrangement of these antigens induced on reaction with specific antibody were examined.

MATERIALS AND METHODS

Animals and Immunizations

Chickens of the line RPL-6 (Regional Poultry Laboratory, U.S. D.A., East Lansing, Mich.); or of SPAFAS origin (SPAFAS, Inc., Roanoke, Ill.) were immunized with subcutaneous injections of ATV-infected CEF, or autochthonous skin or muscle fibroblasts infected in vitro with ATV. Resulting tumors were surgically excised, cultured in vitro, and used for autochthonous hyperimmunization. One chicken was xenogeneically immunized with repeated injections of RSV-transformed rat embryo fibroblasts.

Rats of the W/Fu line were immunized with: repeated injections of rat embryo fibroblasts, transformed in vitro with the Schmidt-Ruppin strain – subgroup D – of RSV (rD-O); repeated injections of UV-treated, transformed rat embryo fibroblasts (rD-O [UV]); repeated injections of UV-treated, uninfected rat embryo fibroblasts (nREF [UV]); or transplantation of tumor homogenate from a tumor originating by inoculation of a separate rat with rD-O cells. Rats of W/Fu or W/Fu × BN hybrid strains were xenogeneically immunized with multiple inoculations of cultured RSV-induced tumors which had been explanted from tumor-bearing chickens.

Cell Labeling Procedure and Evaluation

Cultured target cells were grown on sterile glass squares. It was necessary to avoid detection of artefactual surface antigens caused by adherence to the cell surface of serum components in the culture medium. Thus, if heterologous (e.g., fetal calf) serum had been used to grow the immunizing cells, a different (e.g., chicken) serum was used for culture of target cells. Alternatively, in some cases, antisera were exhaustively adsorbed with insolubilized fetal calf serum. When target cells were ready for labeling, they were washed with Temin-modified Eagle's tissue culture medium (TEM) without serum, and covered with the serum preparation to be tested. The labeling procedure is outlined in Fig. 1. Cells were incubated at the specified temperature for a measured period of time, washed

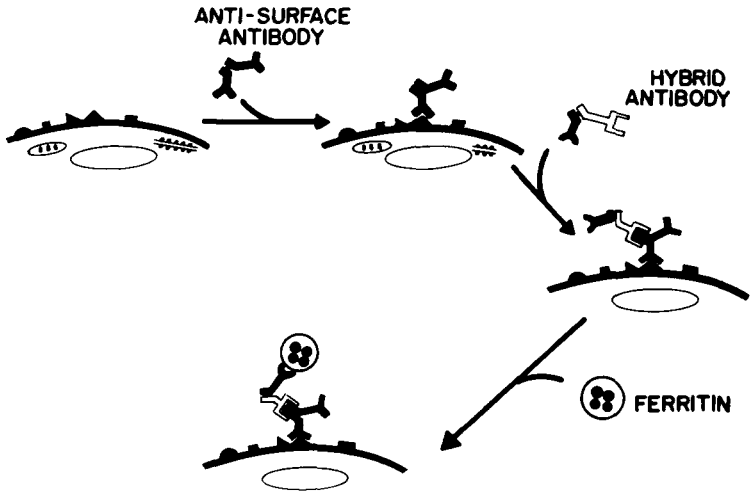

Fig. 1. Schematic illustration of process of labeling by the hybrid antibody technique. Bivalent antibody to cell surface antigen is reacted with the cell. Then hybrid antibody, with specificity against the attached immunoglobulin at one active site and against the marker (ferritin or hemocyanin) at the other, is applied. The hybrid serves as a bridge between the bound, specific, anti-surface IgG and the subsequently added marker.

thoroughly, and fixed for 10 min with 0.5% glutaraldehyde in phosphate-buffered saline. After washing with phosphate-buffered saline (PBS) and blocking of free aldehydes with lysine-HCl, the specimens were incubated for 25 min with hybrid antibody with mixed specificities for IgG (chicken or rat) and hemocyanin (anti-IgG/anti-Hcn). In some cases, hybrid anti-IgG/anti-ferritin was used. The samples were then washed and incubated for 20 min in 5 mg/ml of the marker molecule (hemocyanin or ferritin). After thorough washing, cells were again fixed in 1% glutaraldehyde, postfixed in 1% OsO_4, and processed for electron microscopy. Preparation techniques for immunoelectron microscopy have been described in detail elsewhere (26). Hemocyanin-labeled specimens were used in preparation of surface replicas after the method of Smith and Revel (27) and as described previously (26). Ferritin-labeled specimens were embedded and used for the preparation of thin sections of in situ-embedded materials (28). All specimens were examined by transmission electron microscopy.

RESULTS

Subgroup-Specific Antigens

Chicken antiserum prepared against UV-killed subgroup A avian leukosis virus (RAV-1)-infected cells, or antibody to RSV subgroup A envelope (immunospecifically purified by adsorption to and elution from a virus pellet), was used to examine the distribution of subgroup-specific viral antigens over the cell surface. The observed distribution patterns are summarized in Figs. 2 and 3. The native, unperturbed distribution was evaluated on cells fixed for 10 min with low concentrations of glutaraldehyde (0.1–0.5%) in

PBS before reaction with antiserum. Replicas of the surfaces of prefixed, nontransformed, leukosis virus-infected cells were examined ultrastructurally after labeling of antigenic sites with hemocyanin molecules. Arrangement of hemocyanin was diffuse over the dorsal cell surface; but some cells showed denser labeling of cell processes (Fig. 2A). Reaction of unfixed ALV-infected cells with antibody to components of the viral envelope at temperatures permitting redistribution (24° or 37°C) resulted in several patterns of rearrangement (Figs. 2, 3): (a) a diffusely labeled dorsal surface and pronounced accumulation of marker at the cell margins (marginal redistribution = MR); (b) randomly oriented aggregates of label (patches) over the cell surface; (c) randomly situated patches with intense linear aggregation at the cell margins. All patterns of antigen distribution could be observed in a single culture preparation – including the persistence of the pattern described with prefixed cells.

CEF transformed with the Schmidt-Ruppin strain of RSV, subgroup A (SRV-A), revealed the same "native" distribution of antigens when fixed prior to the antibody reaction as did ALV-infected cells. Transformed cells were heterogeneous in morphology – ranging in a continuum from flat, virtually normal-appearing cells, to rounded cells with multiple, prominent microvilli projecting from the surface. Cells with a flat, fusiform morphology displayed the same antigen redistribution patterns observed with ALV-infected cells. The round morphologically transformed cells, however, assumed a centripetal coalescence of sites – with the highest concentration located on a central, or nearly central, elevated area of the cell (Fig. 3).

Each of the observed redistribution patterns was accomplished under standard conditions, i.e. 20 min of incubation in antiserum or purified antibody at 24° or 37°C. To further investigate the sequence of events and the end result of redistribution of antigen antibody complexes (AAC), the time course of these phenomena was examined more closely in ALV-infected cells. Two different protocols were used: (a) cells were incubated in antiserum for varying periods of time at 37°C without removal of excess, unreacted antibody; (b) cells were incubated with antiserum at low temperature (ca. 0°C), washed free of unreacted antibody, and incubated for measured time periods at 37°C.

Both conditions allowed redistribution of AAC to occur as indicated above. In addition, beginning at about 20 min, and continuing thereafter, an increasing number of ALV-infected cells in a given preparation began to accumulate AAC into foci of coalescence (FC) (Fig. 4). These were frequently multiple (two or three) over the cell surface, and usually involved a concentration gradient – of either individual markers or small aggregates – diminishing away from the focus. The FC were not observed to be associated with any topographical cellular location or feature. Specimens incubated in the absence of excess unreacted antibody presented much more distinct FC, with clearing of peripheral membrane regions, than did those incubated continuously in antiserum during redistribution. The latter conditions obscured the foci by the abundance of peripheral label which persisted in either diffuse or "patchy" distribution.

In cells which had undergone redistribution after washing away of excess antibody, the proportion with FC increased with time up to about 1 hr. Thereafter, both the frequency of cells with FC and the size of individual FC, began to diminish. By 4 hr few cells were visually labeled and almost all those that displayed label had reduced FC.

Thin sections of cells marked with ferritin rather than hemocyanin which had been

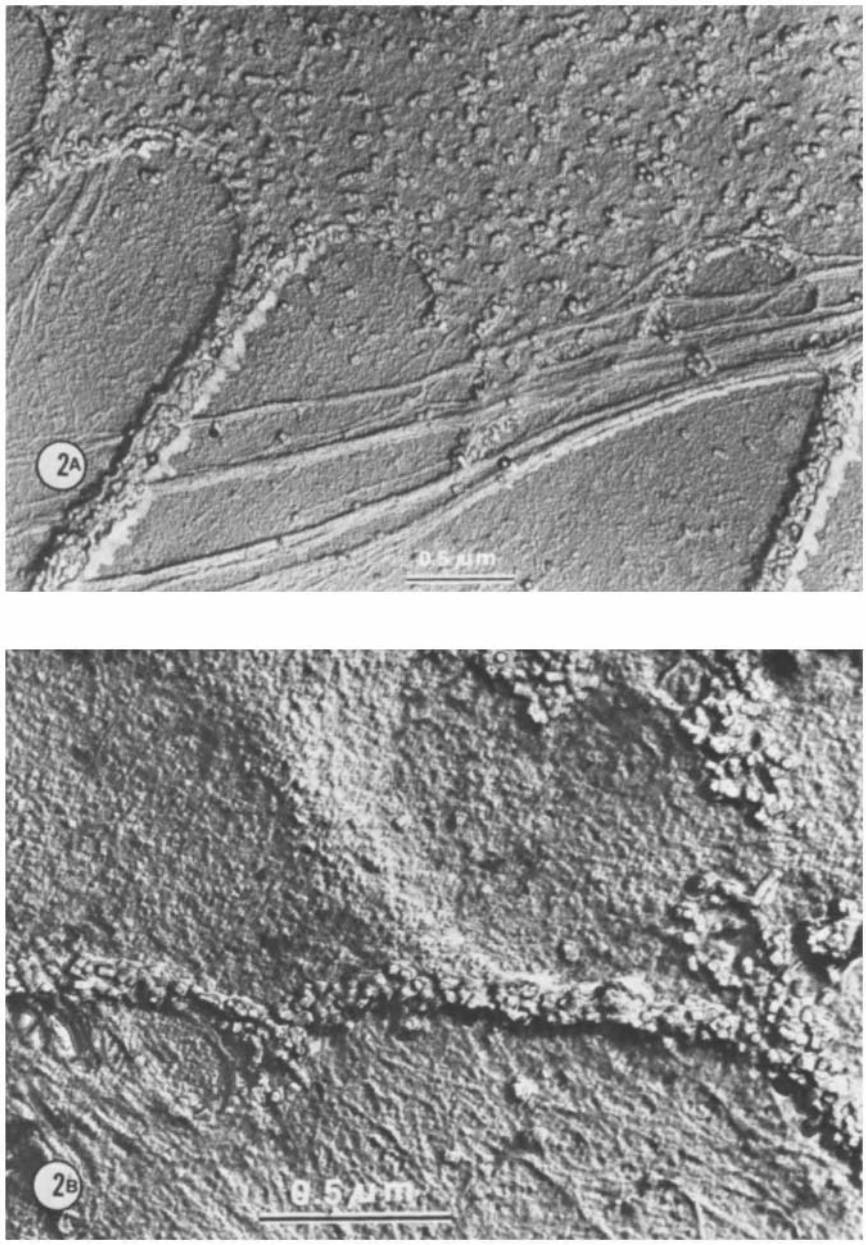

Fig. 2. (A) RAV-1-infected CEF treated with antibody to subgroup-specific viral envelope antigens. Sites of antigen-antibody reaction are marked with cylindrical hemocyanin molecules (350 Å). The cell was fixed with glutaraldehyde to immobilize membrane constituents before reaction with antibody. Label is diffusely distributed over the cell surface, but the cell processes are more intensely labeled. (B) RAV-1-infected CEF, similar to that in Fig. 2A but treated with antibody without prior fixation of the cell. The marked linear accumulation of the label at the cell edge represents marginal redistribution (MR). Note that label on the dorsal surface has accumulated into patches. Reproduced with permission from: Phillips, E. R., and Perdue, J. F., J. Cell Biol. 61:743 (1974).

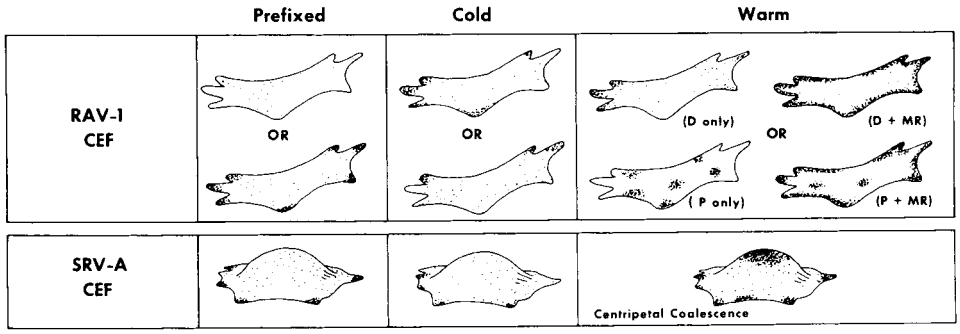

Fig. 3. Schematic representation of distribution and redistribution patterns encountered in leukosis virus (RAV-1)-infected nontransformed CEF, and in sarcoma virus (SRV-A)-infected transformed CEF. Cells were prefixed with glutaraldehyde before treatment with antibody; or were treated with antibody at ca. 0° –4°C (cold) or at 24° or 37°C (warm) for 20 min without prefixation. Redistribution of antigen-antibody complexes (AAC) in cells which had not been prefixed was stopped by cold washing and fixation with glutaraldehyde. The AAC were labeled with hybrid antibody and hemocyanin, and replicas were examined electron microscopically for patterns of distribution. D only = diffuse label only; D + MR = diffuse label with marginal redistribution; P only = patchy distribution of label; P + MR = patches of surface label plus marginal redistribution. (See also Fig. 2 and Table II.)

allowed to redistribute with the entire antigen-antibody hybrid-marker complex, revealed extensive cytoplasmic, ferritin-containing vesicles (Fig. 5). No distinctive association of these vesicles with other cellular organelles was noted.

To investigate the cellular controls for AAC redistribution, several biologically interfering drugs were evaluated for their effects on modes of redistribution. Studies included inhibition of oxidative phosphorylation (sodium azide, 30 mM); inhibition of protein synthesis (puromycin, 20μg/ml); inhibition of RNA synthesis (actinomycin D, 2 μg/ml); inhibition of glycolysis (iodoacetic acid (IAA), 1 mM); the use of antagonists of microfilaments (cytochalasin B [CB], 12.5 μg/ml); and microtubules (colchicine, 1 μM). Specimens preincubated in each compound for a time sufficient to allow full effect,[2] were reacted with antibody (in the continued presence of the drug) for 20 min at 37°C, and subsequently washed repeatedly with cold medium containing the compound. Replicas of hemocyanin-labeled cells were then examined for the following distribution patterns: diffuse label over the entire cell surface (D only); diffuse surface label plus marginal redistribution (D + MR); randomly distributed patches over the cell surface (P only); or patches plus marginal redistribution (P + MR). The percent of cells with each pattern in a typical experiment is given in Table II. None of the substances tested produced any definite effect on formation of patches. Only cytochalasin B resulted in a reproducible reduction of total MR. Both P + MR and D + MR were reduced on CB-treated cells, although the latter configuration was more drastically affected. IAA also produced an effect on D + MR, but none on P + MR, and total MR was changed very little.

Specimens pretreated with the test compounds, incubated with antibody (plus compound) for 30 min in the cold, washed to remove unbound antibody, and then incubated

[2] Preincubations were: CB (or DMSO), 15 min; NaN_3, 30 min; IAA, 1 hr; puromycin, 4 hr; actinomycin D, 4 hr; colchicine, 1 hr.

TABLE I. Summary of the Avian Tumor Virus (ATV) Group

Properties	Class ASV	Class ALV
Leukemogenic	-	+
Fibrosarcomagenic	+	-
Replicates in cultured fibroblasts	+	+
Transforms cultured fibroblasts	+	-

Subgroups[1]	ATV used in these studies: ASV	ATV used in these studies: ALV
A	Schmidt-Ruppin strain (SRV-A)	Rous-associated Virus-1 (RAV-1)
B	Not used	
C	Bratislava strain (B-77)	Rous-associated Virus-49 (RAV-49)
D	Schmidt-Ruppin strain D (SRV-D)	Rous-associated Virus-50 (RAV-50)

[1] Defined by envelope antigenicity (in chickens) and host range of infectivity.
Classes: Avian Rous sarcoma virus (ASV or RSV), Avian leukosis virus (ALV).

for 60 min at 37°C were examined for the formation of FC. Results are presented in Fig. 6. Control cells, and cells treated with any of the drugs except CB or IAA, displayed FC on more than an estimated 60% of the labeled population. Cells treated with IAA or CB did not form FC, but also showed nearly complete loss of surface labeling. The loss of label was not due to a loss of antigen sites as a result of treatment, since cells pretreated with CB or IAA and then fixed with glutaraldehyde before reaction with antibody were extensively labeled with a diffuse distribution. Nor did the drugs cause antigen-antibody dissociation since in both IAA and CB treatments free virus particles were well labeled. If cells were fixed after the antibody- and drug-induced loss of antigen, and treated with fresh antiserum, surfaces were again abundantly (and diffusely) labeled. To investigate these unexpected findings in CB-treated cells, samples were examined after 0, 5, 10, 25, and 60 min of incubation. The striking feature of the CB treatment was the rapidity with which surface AAC were lost. Even at 0 time (i.e. 30 min of incubation with antibody in the cold followed by washing) noticeably fewer cells had significant quantities of label compared to controls. This was present as diffuse or slightly patched marker with occasional MR. By 25 min, very few cells retained surface AAC, with the exception of characteristic small "tufts" of label at the tips of cell processes and projections. At 5 or 10 min, when more label was present, it was usually observed as patches with some brief areas of MR. In some of these cells a clearing of the central cellular area in favor of peripheral regions was noted (Figs. 6 and 7). Thin sections of ferritin-labeled CB-treated cells revealed very small amounts of endocytosis of AAC in contrast to untreated controls (see above). It should be noted that CB treatment produced dramatic morphological effects on these fibroblasts, as reported by others (24, 29), including contraction of the cell body,

TABLE II. Influence of Various Metabolically Active Compounds on Redistribution of Virus Subgroup-Specific Cell Surface Antigens

		Percent of Cells in each pattern						
Cmpd.	No. Cells Counted	D only	D + MR	P only	P + MR	Total D	Total P	Total MR
None	128	22	18	34	27	40	61	45
NaN_3	70	13	18	46	23	31	69	41
DMSO	77	30	16	34	25	46	59	41
CB	82	32	1	54	12	33	66	13
IAA	62	34	0	32	34	34	66	34
Act D	85	21	33	20	26	54	46	59
Puro	79	8[1]	5[1]	51	37	13	88	42
Col	72	37	10	30	29	47	59	39

RAV-1-infected CEF were pretreated with various drugs in TEM at 37°C and then reacted with chicken antiserum to RAV-1 envelope antigens at 37°C for 20 min. Cells were then washed with cold medium and fixed with glutaraldehyde. All reagents up to the point of fixation contained the particular compound in the appropriate concentration (see Materials and Methods). Sites of antigen-antibody complexes were then labeled with hybrid antibody and hemocyanin, and the patterns of distribution (redistribution) were examined electron microscopically on carbon-platinum replicas. A number of cells on each specimen were examined and the percent showing the following were determined: diffuse label only (D only); diffuse surface label, but with linear packing of label at the cell edge as marginal redistribution (D + MR); randomly oriented patches of label (P only); randomly oriented patches of label with additional linear marginal redistribution (P + MR). Act D = actinomycin D; Puro = puromycin; Col = colchicine.

[1] The low numbers observed here for D only and D + MR modes of distribution are not typical for puromycin treatment as determined in other experiments in which the values were in keeping with those of untreated controls.

and extensive arborization of the processes and periphery.

A comparable study of cells treated at 37°C with CB but in the constant presence of excess antibody presented different results. Surface AAC labeling remained quite heavy and persisted in a diffuse distribution or a random dispersion of small patches with or without MR. Unlike control cells (without CB exposure), no FC were formed. Experiments with IAA yielded similar, although less clear, results.

Tumor-Specific Antigens

Detection of group-specific, tumor-specific cell surface antigen (TSSA) of RSV-transformed chick embryo cells, and evaluation of the distribution/redistribution properties was attempted. CEF, transformed with a given subgroup of RSV and treated with serum from a bird immunized with CEF transformed by a different subgroup (or with stock virus of a different subgroup), revealed no evidence for antibody to such antigen (26) in contrast to results reported by others (6, 30). Because of these conflicting results, the matter was re-examined in both avian and mammalian systems. Cell surface labeling of presumptive neoantigens was effected with specific antiserum, hybrid antibody, and hemocyanin as described above, and evaluation was done electron microscopically on sur-

Fig. 4. Two neighboring RAV-1-infected cells labeled with antiserum against viral envelope components for 20 min in the cold (0°–4°C), washed with cold medium, and then placed in warm (37°C) medium for 90 min. The substratum (S) is seen between the two cells, and multiple FC (arrows) are seen on one of the cells. Typical concentration gradients diminishing away from the FC can easily be appreciated.

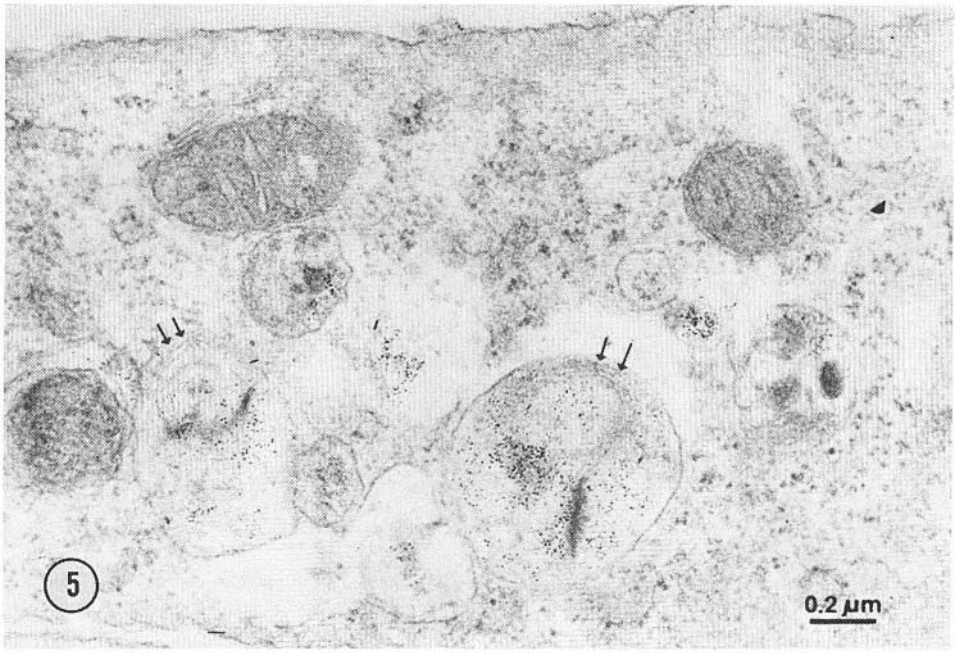

Fig. 5. Thin section of an RAV-1-infected CEF treated sequentially with chicken antiviral envelope antigen, hybrid anti-chicken IgG/antiferritin, and ferritin in the cold. Cells were then washed, and allowed to incubate for 2 hr at 37°C. Multiple cytoplasmic vesicles of endocytosed ferritin can be seen, frequently containing laminated, redundant membranous material (arrows).

face replicas. To quantitate the labeling, the number of labeled or unlabeled cells on a given specimen were counted – both using immune serum (ImS) and serum obtained from the same animal before immunization (preimmune serum = PrS). A labeling index (LI) was calculated as follows:

$$LI = \frac{\%\text{ unlabeled cells with PrS} - \%\text{ unlabeled cells with ImS}}{\%\text{ cells unlabeled with PrS}}$$

A value for LI of >0.3 was considered significant.

Sera of chickens immunized with autochthonous biopsy material, transformed in vitro with B77 virus (Bratislava strain, subgroup C of RSV), did not show antibody activity against the viral envelope or the cell surface of CEF transformed with Schmidt-Ruppin subgroup A or subgroup D RSV (LI <0.12). This was found to be the case for all sera whether obtained in the tumor-bearing state, shortly after complete excision, after regression of recurrent tumor, or after repeated hyperimmunization with cultured autochthonous tumor cells.

Cultured rat embryo fibroblasts (REF) were also transformed with the Schmidt-Ruppin subgroup D virus. When injected into adult W/Fu rats, these in vitro transformed cells (rD-O) gave rise to tumors after a latent period of 2 mo. Sera from rats bearing multiple tumors induced by rD-O and from rats with a single tumor (produced by transplanta-

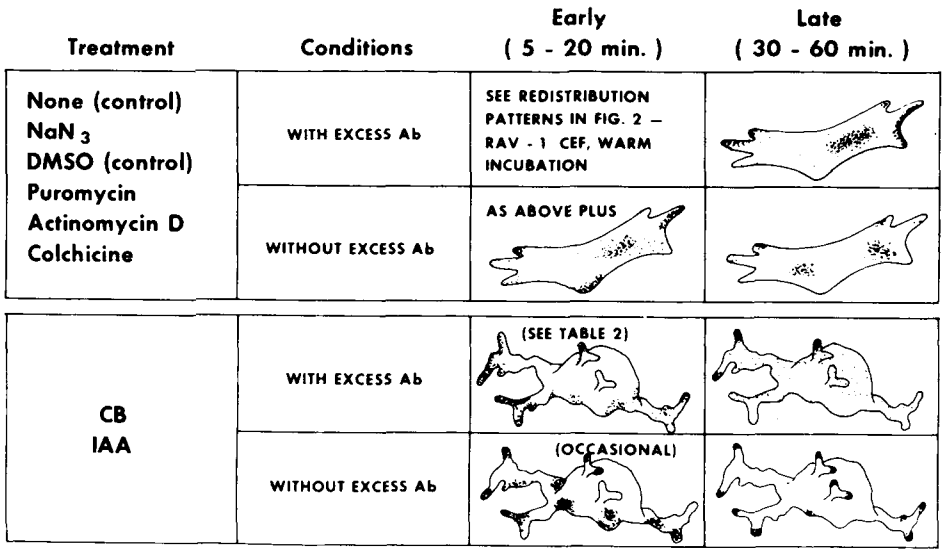

Fig. 6. Schematic demonstration of the redistribution of cell surface viral envelope antigens on RAV-1-infected CEF upon treatment with specific antisera in the presence of various biologically perturbing compounds. Two protocols were used: one in which cells were exposed to antibody at 0°–4°C to restrict redistribution, washed free of unbound antibody, and allowed to redistribute at 37°C for varying periods of time (without excess Ab); and one in which the cells were warmed to 37°C without removal of the excess antibody (with excess Ab).

tion of an rD-O-induced tumor and excised 1 wk prior to bleeding) were tested for the presence of antibody to neoantigens on cultured tumor cells explanted from tumor-bearing rats. The results of these analyses (Table III) indicate that none of the sera were reactive against the tumors tested – with the exception of serum from one rat (rat 1) on cultured autochthonous tumor, or on cultured tumor derived (in rat 20) by transplantation from rat 1. Serum from rat 20 showed activity of borderline significance against autochthonous tumor in one experiment but less in another. Rat 19 serum showed no significant activity against autochthonous tumor. Serum from rat 20 on rat 1 tumor cells was clearly without activity.

Sera from chickens or rats immunized as described, when adsorbed on normal fibroblasts of the opposite (heterologous) species and tested for the presence of a common, cross-reacting antigen (i.e. rat serum on RSV-transformed CEF (or cultured RSV-induced chicken tumor); or chicken serum on cultured rat RSV-induced tumor) gave negative results. In addition, the serum of rats immunized with cultured chicken tumors and the serum of a chicken immunized with RSV-transformed REF displayed no activity against tumor cells or transformed fibroblasts of the homologous species. In all cases of labeling, including that of the apparent autochthonous, individual-specific antigen, the distribution was quite sparse and evenly dispersed on the surface membrane – when incubated either with antiserum or at 0°C at room temperature for 30 min.

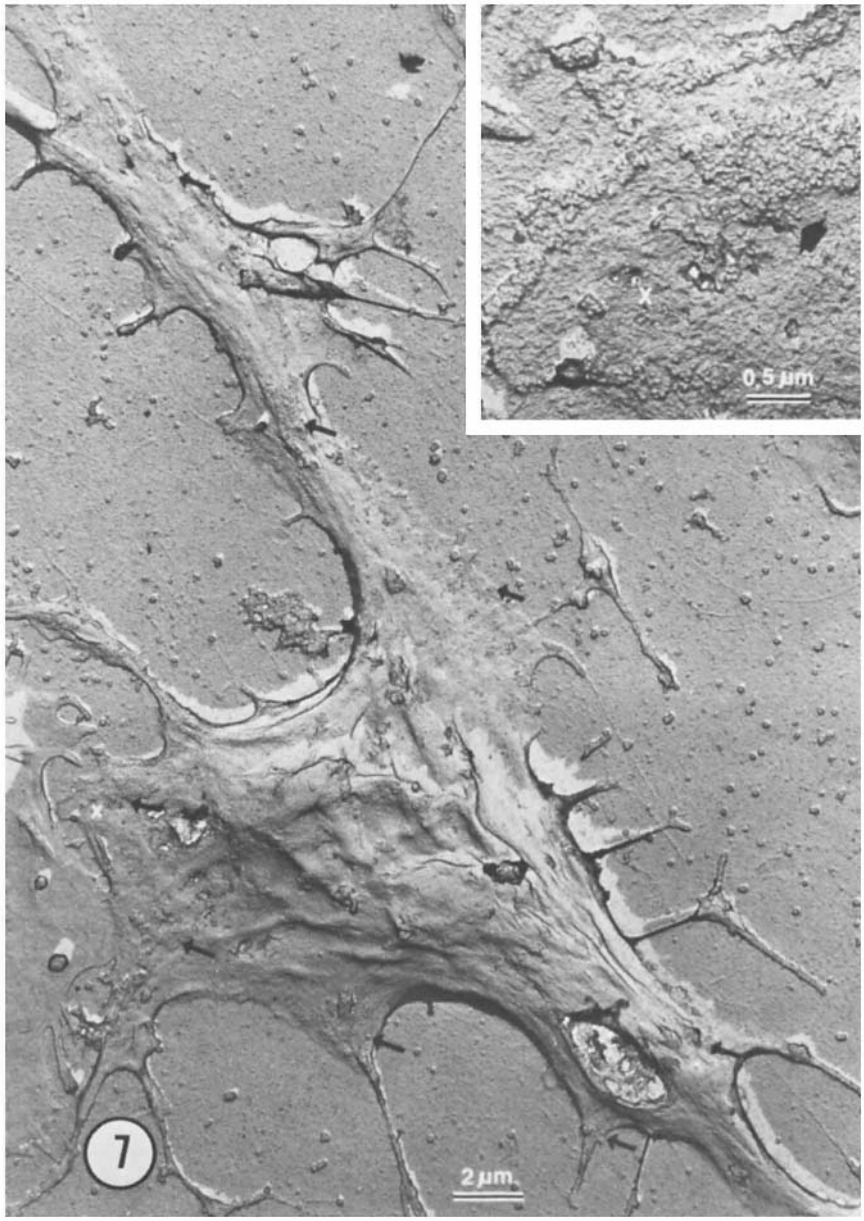

Fig. 7. RAV-1-infected CEF, treated with CB, reacted with antiviral envelope serum at 0° –4°C, washed free of excess antibody, and incubated at 37°C for 5 min before fixation and marking of sites with hemocyanin. Note the typical morphological effect (cell body contraction and arborization of periphery) of CB, and that the labeled antibody complexes (arrows) are largely located peripherally with clearing of the central area. This pattern was observed in a significant fraction of cells treated in this manner. Inset: high magnification to aid in identification of hemocyanin label on the less-magnified perspective of the whole cell (X marks the region of identity).

TABLE III. Antibody Activity of Homologously Immunized Rats Against Cultured Rat Tumors Induced By SRV-D

Rat no.	Immunogen	Serum	Target cell	[2] LI
1	rD-0	Tumor-bearing	rD-1	0.58
1	rD-0	Tumor-bearing	rD-20	0.66
1	rD-0	Tumor-bearing	nREF	0.08
2	rD-0	Tumor-bearing	rD-1	<0.1
4	rD-0 (UV)	Hyperimmunized	rD-1	0.16
5	nREF (UV)	Hyperimmunized	rD-1	<0.1
19	rD-1	Postexcision	rD-19	0.18
19	rD-1	Postexcision	rD-20	<0.2
20	rD-1	Postexcision	rD-1	-0.06
20	rD-1	Postexcision	rD-20 (A)[1]	0.31
20	rD-1	Postexcision	rD-20 (B)[1]	0.23

Rats were immunized by repeated injections with living SRV-D, in vitro transformed rat embryo fibroblasts (rD-0), UV-treated (killed) rD-0 (rD-0 [UV]), UV-treated uninfected rat embryo fibroblasts (nREF [UV]); or by a single injection of tumor homogenate from the excised SRV-D tumor induced in rat 1 (rD-1). Rats 1 and 2 developed multiple tumors and antiserum from the tumor-bearing animal was tested. Rat 4 and rat 5 had no detectable tumors. Rat 19 and rat 20 developed rapidly growing single tumors which were excised 4 wk after inoculation – serum was obtained 1 wk after excision. Target cells were cultured tumor cells from the indicated rat (e.g., rD-1 represents cultured cells from rat 1, etc.), or normal rat embryo fibroblasts (nREF). Evaluations were made by labeling cells by immunoelectron microscope techniques. Labeling index (LI) was determined as described in Table I.

[1] rD-20 (A) and rD-20 (B) represent two different experiments. Target cells in experiment A were grown in chicken serum-containing medium, and target cells in experiment B were grown in fetal calf serum-containing medium.

[2] Labeling indices of >0.3 were considered significant.

DISCUSSION

Our work has been directed toward the detection and mapping of new antigens appearing on the surfaces of avian tumor virus-infected or transformed cells. A large number of existing reports describe the presence of ATV-specific, tumor-specific surface antigen expression on avian and mammalian cells transformed by these viruses. Such an antigen has been reported on the basis of transplantation techniques (31–33), in vitro cell-mediated immunity tests (7, 34, 35), and a number of in vitro methods for evaluation of serum antibody. These serologic techniques have included complement-mediated cytotoxicity (30, 34, 36), mixed hemadsorption (37), immunofluorescence (30), and immunoelectron microscopy (6, 38). In addition, these tests have been applied to sera obtained from homologous and heterologous immunizations. It should be noted that not all studies in the avian species have revealed evidence for tumor-associated transplantation antigen (TATA) (39, 40), and evidence for interspecies cross-reactivity has been limited (41).

Since the discovery of common antigenicity of tumors induced by polyoma virus (42), the concept of virus specificity of tumor-specific antigens in all virus-induced tumors has become very nearly axiomatic. Recent studies with the DNA virus SV 40 have cast some doubt on the applicability of this rule – at least with respect to serologic detection by immunofluorescence. Tevethia et al. (43) demonstrated apparent nonidentity between the serologically detected "S antigen" and TATA, and Collins and Black (44), using careful autochthonous immunizations, were unable to demonstrate a virus-specific surface antigen. This report was contradicted, however, by the results of Ting and Herberman (45) employing the radiolabeled antibody technique. Similarly, in the case of mouse cells transformed by the nonproducer murine sarcoma virus (MSV), nonvirion, virus-specific tumor surface antigens could not be demonstrated by immunofluorescence or by lymphocyte cytotoxicity tests in vitro – and evidence for isograft resistance based on such surface antigens was not firm (46). This, too, was countered by serologic immunoferritin studies which indicated the presence of such an antigen in small amounts (47).

Repeated attempts on our part, using immunoelectron microscope serologic methods, have failed to detect the tumor-specific antigen on RSV-transformed avian or rat cells. Both homologous and heterologous hyperimmunization programs were used. However, our results do suggest the possibility of (an) individual-specific or unique antigen(s) on the surfaces of RSV-induced rat tumors. General applicability of this observation to other or all RSV tumor systems will be the subject of further investigation. The presence of such a unique antigen in RSV-induced chicken tumors has been indicated by the work of Wainberg et al. (48). Moreover, Collins and Black (44) have described the occurrence of unique individual-specific antigens in SV 40-transformed hamster cells. These findings parallel the classic description of individual-specific surface antigens in chemically induced neoplasms (49), and the recently reported demonstration of unique antigens on radiation-induced tumors (50).

Although the indications for individual-specific antigens in our work is yet preliminary, it seems clear that a virus-specific tumor surface antigen is not expressed, is weakly or inconstantly expressed, or is pooriy immunogenic to the antibody-mediated limb of the immune system. We have little reason to challenge the existence of such an antigen in terms of recognition by the cell-mediated mechanisms of immunity, or by the intact host in isograft rejection. However, we now believe that antibody to a tumor-specific surface antigen is either lacking or present in extremely small quantities in the serum of the numerous animals we have tested under a variety of tumor-host conditions.

It is possible that the positive results of others may be attributed to artefactual detection of other antigenic materials, e.g., components of the serum in the culture medium adhering to the cell, antigens of contaminant viruses or of endogenous viruses, or heterophile or species antigen. It is also possible that only occasional, individual animals have the capacity for a humoral response to the tumor-specific antigen, or that only certain species can respond to heterologous immunizations. Whatever the explanations accounting for the differences, it seems likely that circulating antibody plays little if any role in the in vivo immunity to Rous sarcomas.

There is little dispute about the expression of subgroup-specific viral antigen on the surfaces of avian cells infected with and actively producing ATV. Our ultrastructural studies have confirmed the presence of the subgroup-specific surface antigen, and have

mapped the two-dimensional distribution at high resolution. The "native" distribution of these determinants over the surface of infected cells appears to be generally diffuse with enhanced density on the processes of some cells. Gelderblom et al. (6) have reported a patchy distribution in prefixed cells which is in direct conflict with our findings. Dougherty et al. (51), in examining living cells, found a patchy distribution in CEF infected with subgroup A RSV and a diffuse pattern in those infected with subgroup B. Our results indicate that "patchy" configurations are the result of antibody-induced redistribution of antigenic sites, and that within any population of cultured cells (infected with subgroup A ATV) the redistribution patterns are not uniform.

Patch formation as a mode of ligand-induced redistribution has been reported in almost all cell-receptor-ligand systems. The phenomenon of marginal redistribution (MR) described here and previously (26), however, appears unique to the viral envelope antigens on the surface of ATV-infected CEF. The higher density of antigen on the cell processes and the ease with which antibody relocates these sites toward the cell margins suggest that perhaps a very slight concentration gradient of surface antigen exists – increasing toward the periphery. It has been considered that such a condition may be related to either the sites of virus production (budding), or a favored marginal location of antigen synthesis and insertion. However, as an argument against these possibilities, actinomycin D in concentrations inhibiting 95% of RNA synthesis, and puromycin inhibiting more than 90% of protein synthesis, had no effect on MR. Inhibitors of oxidative phosphorylation and microtubule assembly also had no effect on MR. Only CB resulted in pronounced inhibition of total MR. The effect is difficult to interpret since the morphological alteration may significantly change the nature of cell "margins." Moreover, under conditions favoring distinct FC formation in control cells, CB-treated cell appeared to undergo a centrifugal displacement of AAC, but not to the point of the linear accumulation defined as MR. IAA treatment seemed to result in partial inhibition of MR. Since IAA reduced ATP content of CEF to 18% of normal (52), the reduction in MR (complete loss of the diffuse + MR configuration) may indicate an energy requirement for this process. This would be expected since the formation or intensification of any asymmetry (gradient) of antigenic sites should be a thermodynamically energy-consuming process. The negative effect of sodium azide is not surprising because, under the culture conditions used, CEF appear to grow and metabolize normally in the presence of this inhibitor (reference 53, Phillips and Perdue, unpublished observations).

Initial observations seemed to indicate that RSV-transformed cells redistributed viral antigen-antibody complexes into central "caps" while untransformed ALV-infected cells did not (26). A difference in redistribution properties between normal and malignant cells has been shown in a number of cases (20, 54, 21). However, more extensive examination of ALV-infected fibroblasts revealed that these, too, are able to form apparent centers of coalescence. These were often multiple on a single cell and randomly situated on the surface while the centers on transformed cells were usually single – located over the "roundest" portion of the cell. The finding of FC on phenotypically untransformed cells significantly reduced the probability of qualitative differences existing between the redistribution properties of untransformed and RSV-transformed CEF.

The influence of the various biologically interfering compounds mentioned above on FC formation was examined. Azide, puromycin, actinomycin D, and colchicine were

without effect. In experiments in which redistribution was allowed after removal of unbound antibody, cells treated with IAA or CB rapidly cleared their surfaces of AAC – but not concommitantly with FC formation or endocytosis. Indeed, the occasional centrifugal displacement of AAC on CB-treated cells, and the residual labeling of the tips of the cellular processes in both IAA- and CB-treated cells inspires speculation that antigens (with attached antibody) may move outward (toward the periphery) and be shed – either freely into the medium, or perhaps as the envelope of viral particles. One hypothesis we have entertained regarding the role of viral subgroup-specific cell surface antigens is that these may constitute the "pool" of envelope antigens which are concentrated and utilized during the assembly of viral particles at the plasma membrane.

60 min of redistribution in the continuous presence of antibody resulted in FC formation with persistent "background" peripheral label in control cells, but no FC were found on CB- or IAA-treated specimens in spite of abundant surface label. It is tempting to attribute these observations to specific actions commonly associated with CB or IAA, i.e. disruption of microfilaments, or interruption of glycolysis, respectively, but each of these compounds undoubtedly has multiple biological actions. In this regard, CB has been shown to be a potent inhibitor of glucose transport (55), but this property had no relevance to these experiments since absence of glucose from the medium did not mimic the CB effects. CB has been shown to have an inhibiting effect on the "capping" of receptor-ligand complexes in other experimental systems – either alone (13, 56, 57), or more dramatically, in combination with colchicine (58). Our results and the observations of others collectively suggest that the normal cellular means of handling surface AAC is by redistribution into regions of condensation (FC or "caps") and endocytosis. In this system, it appears that CB and IAA hinder this mechanism and/or stimulate (or permit) a different and very efficient mechanism of disposal. The particulars of this phenomenon remain to be elucidated.

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