Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2584–2588, October 1971

Cell Cycle-Dependent Immune Lysis of Moloney Virus-Transformed Lymphocytes: Presence of Viral Antigen, Accessibility to Antibody, and Complement Activation

(complement components/cytotoxicity/gammaglobulin/Fischer rats/rabbit)

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Communicated by Robert A. Good, July 23, 1971

ABSTRACT **The expression of Moloney leukemia virus on the surface of a viral-induced lymphoma cell, availability of the virus to anti-viral antibody, and the nature and extent of activation of the complement system during the cell cycle were studied *in vitro*. Viral antigen was present on the cell surface, accessible to antibody, and was able to activate complement in the presence of antibody throughout all cellular growth phases, while cytotoxicity was confined to the G_1 phase of cell growth. In addition, when cells were arrested in metaphase, viral antigen could be demonstrated on the cell surface by immunofluorescence, and budding virus was seen by electron microscopy. All nine components of complement were activated on the addition of antibody throughout the cell cycle. Additional experiments indicated that in the presence of antibody, C3 and/or C4 were immunospecifically bound to viral-induced lymphoma cells throughout the cell cycle as a result of complement activation. These results indicate that the inability to lyse the cells in the presence of specific anti-viral antibody and complement during the logarithmic phase of cell growth is not due to the lack of expression of Moloney virus antigen(s) on the cell surface, inaccessibility of this surface antigen(s) to antibody, or failure to activate the complement effector system.**

It is well established that after transformation with tumor viruses, cells develop new surface antigens which are, at least in some systems, coded for by the viral genome (1, 2). The fate of the tumor cell *in vivo* may depend in part on the ability of the host to respond to these antigens in a manner that is cytotoxic for the cells which bear them. Clearly, this is a complex process requiring recognition of the antigen by the host and synthesis of antibody which, on combination with antigen at the cell surface, is able to activate effector systems such as complement and/or other mediators in cellular reactions. Further complexity in this process is suggested by Cikes' report that the cytotoxic sensitivity of continuously growing, virus transformed, murine lymphoma cells to anti-viral antibody and complement varies with the phase of the growth curve, being maximal in the stationary phase and virtually absent in the logarithmic phase in culture (3–5). Cikes felt that within a single cell cycle, the limited period of cytotoxic sensitivity that was confined to the G_1 phase of cell growth was probably explained by the assumption of limited gene expression or covering of antigenic sites during phases other than G_1. There are, in addition to these possibilities, several alternative explanations for varying lytic susceptibility during phases of the cell cycle. Among these are: First, the combination of antigen with antibody may fail to activate an effector system because of the type of antibody or the distribution of the antigen on the cell surface. Linscott has recently proposed such a hypothesis (6). Second, cell damage may occur more readily in G_1 phase because of cell membrane conditions, which are unique to this phase of the cycle. Third, the complement system may be inhibited or rendered ineffectual during certain phases of the cycle. Fourth, the ability of the cell to repair damage to the plasma membrane may differ with the phase of the cell cycle. These suggestions imply that the fate of a tumor in the host may depend, in part, on the proportion of the cell cycle occupied by the G_1 phase, and the degree of synchrony of tumor cells *in vivo*.

In an attempt to differentiate among possible explanations for the differential lytic susceptibility, we have followed the presence of surface viral antigen, availability of antigen to antibody, and the nature and extent of activation of the complement system by anti-viral antibody during the cell cycle of Moloney virus-transformed lymphocytes.

The mouse lymphoma YCAB was obtained through the courtesy of Drs. George Klein and Matko Cikes of the Karolinska Institute, Stockholm, Sweden and maintained in our laboratory in continuous culture as described for other suspension cell lines (7).

Anti-Moloney virus antibody was obtained from Fischer rats with a transplanted Moloney sarcoma virus (MSV) tumor, which had been induced originally in newborn Fischer rats with plaque-purified Moloney virus. This antiserum was kindly provided by R. Wilsnack, Huntington Research Laboratories, Baltimore, Md. Fluorescent studies with antiserum to rat IgG showed that this antiserum combined only with virus-transformed lymphocytes and not with normal mouse cells. The presence of viral antigen was determined by an indirect immunofluorescent assay. 5×10^5 viable cells were initially incubated in suspension with rat antiserum to Moloney virus and then stained with fluoresceinated rabbit antiserum to rat IgG that was previously absorbed with YCAB cells. Details of preparation, purification, and conjugation of antibody to fluorescent isothiocyanate have been published (8). Additionally, the virus was visualized by electron microscopy during different phases of the cell cycle. Cytotoxic tests were performed on 5×10^1 cells that were incubated with 25 μl of neat antisera to Moloney virus for 20 min at 37°C, washed twice, and then incubated with 75 μl

Abbreviations: MLV, Moloney leukemia virus; YCAB, viral-induced lymphoma.

of guinea pig complement for 30 min at 37°C. Complement was previously absorbed with YCAB cells. The viability of target cells was assayed by the trypan blue dye-exclusion test as reported by Cikes (3). In addition, cytotoxic testing with ^{51}Cr-labeled target cells was used, as previously described (9, 10). Both assays of cytotoxicity gave similar results.

In order to determine if the complement was activated after antibody-virus union, purified human complement components and cellular intermediate complexes were prepared as previously described (11–14). Human serum, used as the source of complement in these studies, was absorbed with YCAB cells in the presence of EDTA for 60 min at 0°C, and then recalcified. In following YCAB cells through the cycle, 2×10^7 cells in duplicate were taken at each sampling interval and washed three times. Anti-Moloney virus antibody (heated at 56°C for 30 min) was added to one sample of cells and, after 10 min of incubation at 37°C, 50 μl of absorbed human serum was added to both cell samples. After an additional hour at 37°C, the cells were sedimented and the supernatant serum was analyzed for residual complement components by specific stoichiometric titrations with human complement intermediate complexes (12–16). The results were expressed as the percent of the number of effective molecules remaining in serum that are similarly incubated with cells in the absence of antibody. The sedimented YCAB cells were analyzed by immune adherence (17) with human type O erythrocytes as indicators for the presence of immunospecifically-bound complement components. The samples were also analyzed for activation of the complement bypass system, by immunoelectrophoresis, with antibody to the C3 proactivator (18).

Presence of virus antigen on the cell surface throughout all cellular growth phases

Initial experiments were designed to determine the presence of surface viral antigen, activation of complement, and cytotoxicity during different phases of cell growth in culture. Cytotoxicity was essentially confined to the stationary phase of growth, as described by Cikes. However, despite the absence of cytotoxicity during the logarithmic phase of growth, surface viral antigen was present on virtually every cell examined by fluorescent microscopy (Fig. 1). The distribution of viral antigen was not uniform, but seemed to occur in a crescent occupying only a portion of the cell surface (Fig. 1). A similar crescent distribution of viral antigen was noted by Cikes (4). The presence and distribution of viral antigen did not change throughout the various phases of growth. Electron microscopic studies confirmed the presence of virus in and on almost all cells during both the logarithmic and the stationary phases of growth. Furthermore, the cell population in the logarithmic phase of growth was able to activate the complement system in the presence of specific anti-viral antibody (see below). These results suggest that the inability to kill logarithmically growing cells was not due to the absence of viral antigen, inaccessibility of viral antigen to antibody, or inability of surface antigen and specific anti-viral antibody to activate complement.

Presence of viral antigen on the cell surface throughout a single cell cycle

Since logarithmically growing cells are random with respect to phases of the cell cycle, it was important to study the presence of viral antigen and cytotoxic sensitivity in a synchronized population of cells during a single cycle. Accordingly, cells were synchronized in the G_1 phase of the cell cycle and released from G_1-phase arrest as reported by Cikes and us (4, 7). Cells were susceptible to immune lysis only during the G_1 phase of the cell cycle, but as expected from the above studies, viral antigen, as determined by fluorescent and electron microscopy, was present on the cell surface during all phases.

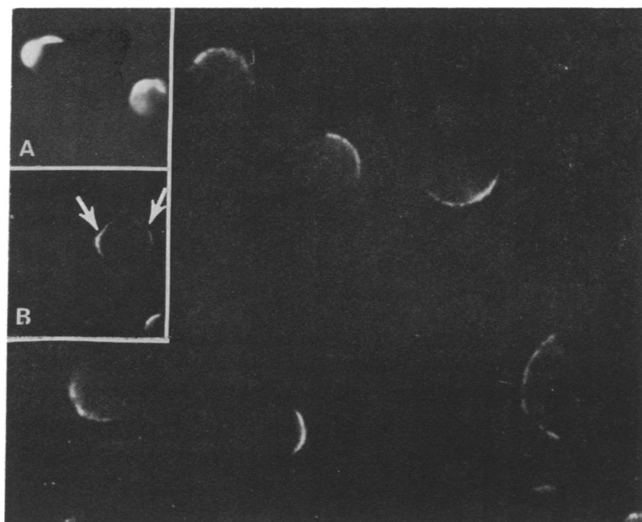

FIG. 1. Fluorescent photomicrographs of Moloney virus-induced YCAB culture cells in the logarithmic phase of cell growth. Unfixed cells were initially stained with rat anti-Moloney virus antibody and then with fluorescein-conjugated rabbit antiserum to rat 7S gamma globulin previously absorbed with YCAB cells. The presence of viral antigen, usually in a crescent distribution, is evident. Occasional cells showed antigen in a heavy polar distribution (*A*) or on multiple cell sites (*B*, *arrows*). Despite the presence of viral antigen and its accessibility to anti-viral antibody, cell lysis did not occur when complement was added. Magnification of crescent cells about ×487.

To extend these findings, cells were arrested in metaphase with colcemid (0.04 μg/ml) and were examined for the presence of viral antigen by fluorescent and electron microscopy. About 70% of the cells were found to be arrested in metaphase. This phase of the cell cycle is of most interest, because as a cell proceeds through the normal cell cycle, it gets further from the G_1 phase where immune lysis occurs. Viral antigen was demonstrated on the surface of virtually every metaphase cell by fluorescent microscopy and budding virus was seen by electron microscopy (Fig. 2). Despite the presence of viral antigen, no cytotoxicity was observed.

Activation of the complement system throughout the cell cycle

The ability of synchronized YCAB cells plus anti-viral antibody to activate the complement system was appraised in a synchronized population of YCAB cells during a single cycle. Although the consumption of complement by washed YCAB cells was observed in the absence of specific antibody at all phases of the cell cycle, there was a significant increase in complement utilization on addition of specific anti-viral antibody (Table 1). Furthermore, the extent of activation did not vary significantly during the cycle. Activation of the

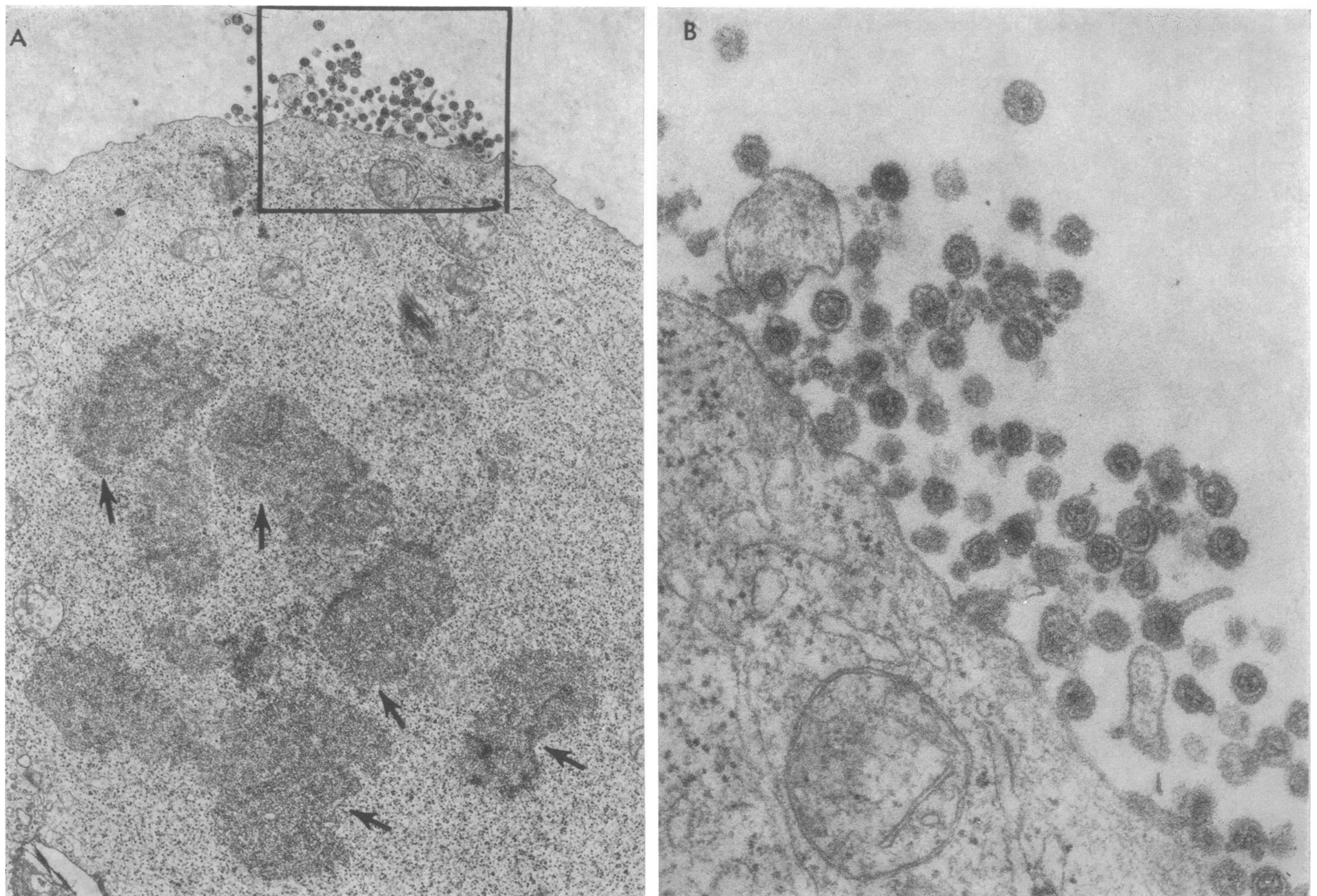

FIG. 2. Electron photomicrograph of a YCAB cell arrested in metaphase with colcemid. *A*. Absence of nucleus and the presence of chromosomes (*arrows*) are evident. Moloney virus at the surface of the cell is seen. Similarly, metaphase cells showed viral antigen on their surface when assayed by the indirect immunofluorescent technique. The addition of both specific anti-Moloney virus antibody and complement to these cells did not result in any detectable injury. Magnification about ×9,720 *B*. Enlargement of the cell area containing Moloney virus. Magnification about ×27,540.

complement system was not confined to the early-reacting components, but rather involved the entire reaction sequence, since the terminal complement components, C8 and C9, were also consumed (Table 1). In addition, immunoelectrophoretic analysis of the serum that was exposed to YCAB cells and antibody revealed the conversion of the C3 proactivator into the enzymatically active C3 activator (Fig. 3). This observation indicates that the viral–antibody reaction is able to initiate complement activation via the C3 activator system, an alternative pathway of complement activation that does not require participation of C1, C2, and C4 (18).

Despite activation of the entire complement sequence during all phases of the cell cycle, cytotoxicity was observed only during the G_1 phase (Table 1). In order to determine whether the complement reaction was occurring on the surface of YCAB cells, immune adherence was performed at 0(early G_1), 5(late G_1), 11(S), and 23 hr (S, G_2, and M phases) after release from the G_1 phase. The minimum number of cells giving a positive immune adherence (number of YCAB cells/ml giving +2 reaction with 8×10^7/ml of human type-O erythrocytes) was 1.2×10^6 from 0–23 hr after release from G_1 phase (5×10^6 YCAB cells were required for immunoadherence in the absence of specific antibody). The positive immune adherence observed indicates that C3 and/or C4 were immunospecifically fixed to YCAB cells throughout the cell cycle as a result of complement activation. In addition to documenting the presence of an immune reaction on the surface of the YCAB cells, the immune adherence reaction showed that the cells possessed a binding site for C3 and/or C4 during all phases of the growth cycle.

DISCUSSION

Our studies confirm the observation of Cikes (3–5) and others (19) that nucleated cells are less susceptible to the cytotoxic effects of antibody to surface antigens and complement during the logarithmic phase of cell growth. However, it is clear that these differences are not due to the lack of antigen on the cell surface during different phases of the cell cycle, since comparable amounts of virus can be demonstrated by fluorescent and electron microscopy throughout the cycle. The fluorescent studies also show that the viral antigen is accessible to antibody at all times during the cycle. We also have shown that the combination of antigen with specific anti-viral antibody activates the effector system, complement, via two different mechanisms. Furthermore, this activation involves the entire complement sequence, including the terminal components, C8 and C9. The number

of molecules of the components consumed, i.e., the extent of activation of the complement system, was essentially constant through the cell cycle, although cell lysis occurred only during the G_1 phase. Since as much complement activation occurred in phases other than G_1, the lack of cytotoxicity could not be due to the inability of the virus-antibody union to activate the mediator system.

The resistance of cells to the cytotoxic effects of antibody and complement at phases of the cell cycle other than G_1 must, therefore, be explained by one of the following possibilities. First, the complement reaction may not occur on the cell surface, but rather may proceed in free solution at a distance from the target cell surface. Since immune adherence specifically detects the presence of C4 and/or C3 bound on a cell surface as a result of activation of the complement system, the finding of immune-adherence-positive YCAB cells throughout the cell cycle indicates that at least the initial portion of the complement reaction proceeds on the surface of the YCAB cells. It is not possible from the present data to determine whether the terminal portion of the complement reaction is also on the cell surface, a probable requirement for cytotoxicity. It is entirely possible that the binding sites for the later components, as for example C8, are present on the cell surface only during a limited period of the cell cycle. Second, there may be cell cycle-related changes in configuration, charge, or structure of the plasma membrane that render it resistant to cytotoxicity. Third, the ability of the cell to repair damage to the plasma membrane may differ during the cycle. We are unable to differentiate among these possibilities on the basis of the present data. Nevertheless, our results have implications for those studies attempting either to assay cytotoxicity or to explain the growth of tumor cells in the presence of circulating antibody and complement. One interesting point to consider is the relationship of these findings to the induction of immunological tolerance, where antigen–antibody union presumably also occurs at the surface of a cell. One might expect that resting (G_1) antigen-sensitive cells would be more susceptible to the injurious effects of large doses of antigen than cells entering the mitotic cycle. The known difficulty of inducing tolerance, once an immune response has been induced, might depend in part on such a phenomenon.

TABLE 1. *Consumption of complement components on addition of anti-viral antibody and serum to a synchronized population of YCAB cells*

Time after release from G_1 phase (hr)	Phase of cell cycle*	Percent of complement component consumed in presence of anti-viral antibody†						Cytotoxicity‡
		C2	C3	C4	C5	C8	C9	
0	early G_1	38	67	36	31	38	64	++
5	late G_1	38	59	32	17	30	53	+
11	S	38	67	36	31	38	64	0
23	S, G_2, M	38	67	40	35	38	67	0
	M§		70					0

* MSV antigen was present throughout all cellular growth phases (in $\geqslant 50\%$ of the cells).

† These percentages represent consumption by cells and antibody after excluding complement utilization by cells alone. Average complement consumption by cells was: C2 (47%), C3 (25%), C4 (56%), C5 (32%), C8 (18%), and C9 (24%). Effective molecules/ml in controls, in the absence of cells and antibody were: C2 (1.1×10^{11}), C3 (7.5×10^{10}), C4 (3.8×10^{12}), C5 (1.5×10^{12}), C8 (4.1×10^{11}), and C9 (1.3×10^{12}).

‡ ++ (>50%), + (<50%), 0 (not detectable).

§ In colcemid-arrested cells in a separate experiment.

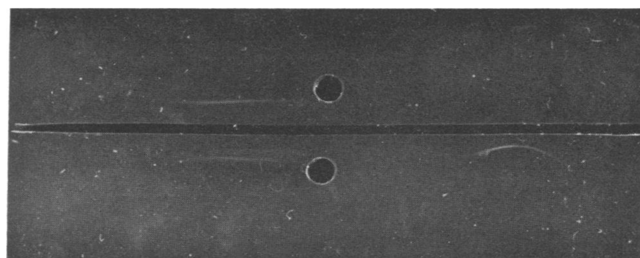

FIG. 3. Demonstration by immunoelectrophoresis of activation of the complement bypass system by YCAB cells and antiviral antibody. YCAB cells were incubated with antibody and serum (*upper panel*) or with serum alone (*lower panel*) for 1 hr at 37°C. Supernatant fluids were analyzed in 2% agar gel in a barbital buffer at pH 8.6 and ionic strength 0.05, containing 1×10^{-2} M EDTA. Anode is to the right. The trough was filled with rabbit antibody to human C3 proactivator.

NOTE ADDED IN PROOF

Later studies with heterologous antiserum to murine lymphocytes showed that lymphocyte-membrane antigens were present on the surface of YCAB cells, by both immunofluorescent and radioimmunochemical methods, throughout all cellular growth phases. The amount of antigen did not appear to vary significantly throughout the cell cycle. Furthermore, this antigen–antibody combination activated the entire complement sequence, and binding sites for late complement components were detected throughout the cycle. Although cytotoxicity was more marked during the stationary phase of cell growth, it was highly dependent on the number of antibody molecules offered. Structural proteins of the membrane might be expected to give different results in cell-cycle-dependent cytotoxicity assays than budding virus particles that are located at angstrom distances from the membrane. Of course, the interpretation of any of these results depends on whether one expresses the data as per cell or per cell surface. Since even suspension cells are not simple spheres (i.e., consider villi), the exact surface area cannot be determined.

We thank Dr. G. Tonietti for the electron photomicrographs. This research was supported by a grant from the Council for Tobacco Research, United States Public Health Service Grants CA-10596, AI-09484, and AI-07007, the Violet June Kertell Memorial Grant for Research on Multiple Sclerosis from the National Multiple Sclerosis Society, and United States Atomic Energy Commission Contract AT(04-3)-730. R. Lerner, M. Oldstone, and N. Cooper are recipients of Career Development Awards, KO4 AI-46372, KO4 AI-42580, and KO4 AI-33630 from the U.S. Public Health Service, respectively.

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